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Research Paper

Comparative analysis of onychomycosis in Puerto Rico using molecular and conventional approaches

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ABSTRACT

Onychomycosis is the most prevalent nail ailment in adults, accounting for 50% of all nail infections. Dermatophyte fungi are the primary cause, but non-dermatophyte molds (NDM) and yeasts can also cause onychomycosis. It remains important to precisely determine the fungal cause of onychomycosis since the response to current treatments may vary between fungal classes. Real-time polymerase chain reaction (qPCR) has become a widespread tool for detecting fungal organisms for diagnosis due to its sensitivity and ability to detect down to the species level. This retrospective study aims to evaluate the qPCR Onycho+ test for dermatophyte detection using remnants of toenails from a cohort of patients from Puerto Rico. Two hundred forty-two toenail samples submitted for histological examination via Periodic acid Schiff (PAS) staining for suspected onychomycosis were analyzed by the Onycho+ test and Sanger sequencing of the internal transcribed spacer (ITS-2). Compared to the gold standard Sanger sequencing method, the Onycho+ test reported an agreement of 91.39%, a sensitivity of 100% and a specificity of 84.5% in detecting dermatophytes, superior to the histology method which had a 69.53% agreement, 85.1% sensitivity and 57.1% specificity. The distribution of fungal organisms detected in this cohort shows a dermatophyte majority but a higher-than-expected proportion of NDMs. Nails negative for the Onycho+ test and positive for histology were mostly NDMs. This study demonstrates that the clinical performance of the Onycho+ test is superior to histology in detecting dermatophytes and that a combination of Onycho+ and histology can result in a higher clinical accuracy.

Introduction

Onychomycosis is responsible for 50% of all nail infections [1–3]. It is the most frequent nail ailment in adults and is caused mainly by fungal organisms known as dermatophytes that recently have been categorized into seven main distinct genera: *Trichophyton, Epidermophyton, Microsporum, Nannizzia, Paraphyton, Arthroderma* and *Lophophyton* [4,5]. *Trichophyton rubrum* is the most prevalent dermatophyte associated with onychomycosis, but other dermatophytes are also associated [6–8]. Though usually considered commensal organisms, some non-dermatophyte molds (NDMs) including *Neoscytalidium, Scopulariopsis, Aspergillus* and yeast such as *Candida* sp. have been documented as causative agents of onychomycosis [3,6,9-11].

Onychomycosis has a global prevalence of 5.5% and a prevalence of 2–14% in the United States [6,12]. The main risk factors include age, tinea pedis, trauma, diabetes mellitus, peripheral artery disease, and genetic predisposition [3,6,7]. In Puerto Rico, studies are scarce, except for a panoramic view of dermatomycosis from 1930–1949. That study, which evaluated 205 infections of onychomycosis in Puerto Rico, found the most predominant organisms were *Trichophyton mentagrophytes* (68.8%), *Trichophyton rubrum* (30.20%), and *Epidermophyton floccosum* (2 cases) [13]. Similarly, in subtropical countries like Brazil, *T.rubrum,* and *T. mentagrophytes* were reported to be more prevalent in onychomycosis infections [14].

Several laboratory techniques are employed in the diagnosis of onychomycosis including fungal culture, direct microscopy following potassium hydroxide (KOH) treatment, and histopathologic microscopic examination following staining with the periodic acid-Schiff (PAS) procedure [12,15]. Despite their widespread and successful use, these techniques also have disadvantages. Fungal culture is time-consuming, requires qualified personnel, and has a high rate of false negative results [12,16,17]. Direct microscopy using KOH is highly dependent upon the skill of the practitioner [6,15,18]. And histological examination, along with KOH direct microscopy are both incapable of identifying the genus or species of the etiological agent (s) [6,18].

The molecular technique of real-time PCR (qPCR) is becoming more frequently used in diagnostic testing for onychomycosis

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[15,19]. Distinct advantages include high specificity and sensitivity, allowing the accurate identification of even small amounts of fungal DNA [8,18]. Additionally, its ability to identify the genus and species helps in determining anti-fungal therapy. For example, it is well documented that NDMs are often less responsive to mainline therapies [10,11]. Additionally, some therapies are labeled for the treatment of specific fungal species [20,21]. For these reasons, the benefits of molecular tests for the clinical diagnosis of onychomycosis are becoming more recognized [17,22].

Onychomycosis has been described as a nontrivial medical ailment, with different implications in the occupational, psychosocial, and physical aspects [23,24]. As the necessity to improve the patient's treatment increases, there is also a need to improve the current diagnostic methods. Given this, the present study was performed using clinical nail samples from a cohort of Puerto Rican patients to evaluate the Onycho+ PCR test designed to detect dermatophytes and compare it to histology and the gold standard reference of Sanger sequencing.

Methods

Study design and sample collection

This was a retrospective study approved by the Institutional Review Board from Ponce Health Sciences University (protocol # 2,212,124,490). The protocol scope was the validation of a laboratory developed qPCR test for dermatophyte detection. The tested nails were remnants of toenail samples that were received at the laboratory for histological examination via Periodic acid Schiff (PAS) staining for suspected onychomycosis between January 1, 2018, and August 31, 2022.

Histology staining procedure

The PAS staining technique was used to demonstrate the presence of hyphae, spores, pseudohyphae, and yeast in tissue samples [8,15]. The nail samples were deparaffinized with a xylene solution followed by two washes with 100% ethanol and a wash with tap water. The nails were then placed in a 5% periodic acid solution, washed with water and then Schiff's reagent was added. After this, the samples were washed with water and the counter stain, Light Green (Mercedes Scientific, Florida, USA), was added followed by a wash with water. Next, the samples were dehydrated with two washes of 100% ethanol followed by a wash with xylene. Finally, the samples were coverslipped and sent for microscopy examination**.** The PAS stain was considered positive if structures of fungi or yeast were observed.

Tissue lysis

The nail samples were grossed and cut to an approximate size of 0.3 × 0.3 × 0.4 cm (length, width, height) and placed inside 2 mL tubes containing 2.8 mm-ceramic beads (Omni International, Georgia, USA). The nails were added to a mixture containing MagNA Pure 96 DNA Tissue Lysis buffer (Roche Diagnostics, USA), proteinase K (20 mg/ml) (Fisher Scientific, New Hampshire, USA), and internal control. The mechanical lysis was performed in the Bead Ruptor Elite (Omni International, Georgia, USA) with the following parameters; 2 cycles of 4.2 m/s, for 30 seconds per cycle with a dwell time of 1 minute between cycles. Next, the samples were incubated for 15 minutes at 65^{0}C and 900 rpm in the Thermomixer C (Eppendorf, Germany), followed by centrifugation at 18,210 x g for 1 minute. The supernatant (lysate) was used in the purification process.

DNA purification

The lysates were placed in the source plate of the MagNA Pure 96 Instrument for DNA purification with the Viral NA small-volume kit and the universal pathogen protocol (Roche Diagnostics, USA). Reagent blanks served as negative extraction controls and were processed with each batch. The DNA was eluted in a volume of 100 μL and stored at -20°C for further analysis using the Onycho+ test.

Primer, probes and internal control

The primer and probe sequences (Table 1) for the *T. rubrum*, pan-dermatophytes and internal control were modified from Wisselink et al. [25] with the use of the National Center for Biotechnology Information (NCBI) database and Basic Local Alignment Search Tool (BLAST) "nucleotide blast" (nt/nr) query (See *blast.ncbi.nlm.nih.gov/Blast.cg*). The primers are localized within the region of the ribosomal RNA gene, in the intervening sequence regions, ITS -1 and ITS- 2, and the 5.8S rRNA (Fig. 1) [26]. The ITS region has been used as a DNA barcode for fungi, and it is the ideal tool for the development of amplicons capable of resolving to the level of genus and species [27–29]. Using these attributes, primers for *T. rubrum* and pan-dermatophytes were developed. The Geneious Prime software, Version 2022.2.2, (BioMatters Ltd, New Zealand) was utilized for primers and probes *in-silico* analysis.

Onycho+ test

The Onycho+ test is a multiplex qPCR capable of detecting *T. rubrum*, pan-dermatophytes, and an internal control. *T. rubrum* is speciated in the assay as it is responsible for most onychomycosis infections [6] and is specifically indicated in the FDA labels of the

Table 1
Sequences of the primers/probe sets used in the Onycho+ Test and primers for the ITS-2 Sanger sequencing.

Primer/Probe Name	Target Organism	Primers and Probes for Onycho+ Test Sequence (5′-3′)	Target Region	Dye
Trub-F	*Trichophyton rubrum*	CTG CCA GGG AGA GCC GT	ITS 1	N/A
Trub -R		GCT TGC TAA ACG CTC AGA CTG A		N/A
Trub-P7		/56-FAM/AC AGA CAC C/ZEN/A AGA AAA AAT TCT CTG AAG AGC TGT C/3IABkFQ/		FAM
PD-F	Pan-Dermatophytes	AGC GAA ATG CGA TAA GTA ATG TGA	5.8rRNA-ITS 2	N/A
PD-R		GAC GGT CGT CCA TCA CAC AAG		N/A
PD-P9		/5HEX/CA GAA TTC C/ZEN/G TGA ATC ATC GAA TCT TTG AAC G/3IABkFQ		HEX
PhHV-IC-F	Internal Control	GGG CGA ATC ACA GAT TGA ATC	PhHV DNA	N/A
PhHV-IC-R		GCG GTT CCA AAC GTA CCA A		N/A
PhHV-P		/5Cy5/TTT TTA TGT /TAO/GTC CGC CAC CAT CTG GAT C/3IAbRQSp/		Cy5
		ITS-2 Primers for Sanger Sequencing		
ITS2-F	–	**GTAAAACGACGGCCAG**GCATCGATGAAGAACGAGC*	ITS-2	–
ITS2-R	–	**CAGGAAACAGCTATGAC**TCCTCCGCTTATTGATATGC*	ITS-2	–

* M13 tails are depicted in bold.

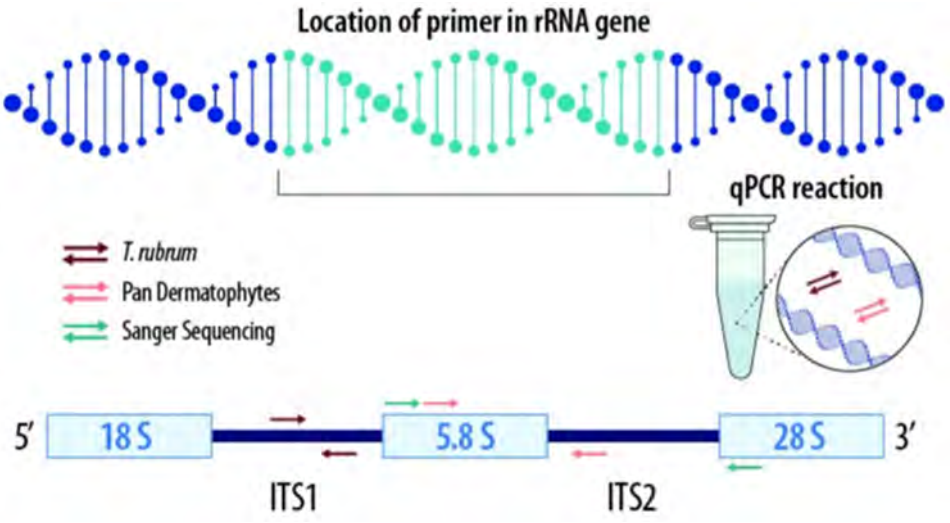

Fig. 1. Ribosomal RNA gene region and location of fungal PCR assay targets.
Ribosomal RNA gene depicting the 18S ribosomal RNA, 5.8S ribosomal RNA, and 28S ribosomal RNA regions, with the location of the different primers used for the Onycho+ Test. Primers in red color detected *T. rubrum*, and primers in orange color detected Pan Dermatophytes. Sanger Sequencing primers are also depicted in green in the image.

newest FDA-approved topical agents for treating onychomycosis, efinaconazole and tavaborole [20,21]. The internal control serves as both extraction and qPCR controls. This assay was designed and validated in our laboratory under CLIA regulations as a laboratory-developed test. The Onycho+ test was performed on the CFX Opus 96 from Bio-Rad. The reaction volume was 20 μl: 10 μl of the 2x LightCycler 480 Probes Master (Roche Diagnostics, USA), 1.2 μl of a primer mix (5 μM), 0.8 μl of the Probe Mix (FAM & HEX probes (5 μM)), Cy5 (2.5 μM), 6 μl of water and 2 μl of DNA. The thermal profile was: 10 min at 95°C; followed by 40 cycles of 10 s at 95°C, 1 min at 63°C; followed by 10 s at 72°C and a hold step at 4°C. A positive control and a non-template control (NTC) were included in each qPCR run. The results were analyzed using the Bio-Rad CFX Maestro Software, Version 2.3 (Bio-Rad, Hercules, California, USA).

ITS-2 sequencing

All samples were prepared for Sanger sequencing using the internal transcribed spacer 2 (ITS-2) region as described [26]. (i) An endpoint PCR was performed using M13-tailed primers targeting the ITS-2 region (Table 1 & Fig. 1). The total PCR reaction volume was 20 μl: 4 μl of DNA, 10 μl of the 2x LightCycler 480 Probes Master Mix (Roche Diagnostics, USA), and 1.2 μl of each primer for ITS2 (0.3 μM). The reaction thermal profile was: 10 min at 95°C; 40 cycles of 10 s at 95°C, 1 min at 65.5°C and 1 min at 72°C; a final extension for 15 min at 72°C and a hold step at 4°C. (ii) PCR amplicons were visualized through electrophoresis with 3%-agarose gel. (iii) Purified PCR products were obtained using the Monarch PCR & DNA Cleanup Kit (NEB, MA, USA) and sent for Sanger sequencing to Azenta (Burlington, MA, USA).

Fungal identification

In silico analysis was performed using the Geneious Prime software, Version 2022.2.2. Chromatograms were evaluated qualitatively; unreadable areas were manually trimmed, and undetermined bases were clarified. To establish fungal identity, sequences were queried using the Molecular ID tool from the MycoBank database (https://www.mycobank.org/, last accessed on September 23, 2022). The nucleotide blast tool (last accessed on September 23, 2022) from the Geneious Prime software was also used to evaluate the identity of the sequences, using the non-redundant nucleotide collection (nr/nt) and the Megablast Program. The criteria to perform the identification were the E-value score and the identity of the organism. The identification was retained when the best-match sequences from both databases (Mycobank and Blast tool) were concordant. Discrepancies were categorized as undetermined. An assessment tool was created by our team to categorize the quality of the chromatograms based on the E-value score and our qualitative observations (Table 2). Sequence identification was considered the gold standard reference method for comparison [30,31]. The sequence identification was considered definitive for sequencing results that had a quality score of 2 and 3.

Table 2
E-score correlation with chromatograms.

Sequence Quality	Quality Score	E-value	Description of Chromatograms
High Quality	3	$< 1e^{-140}$	Most of the sequence had discrete and evenly spaced peaks. Absence or low background noise. Absence of undetermined bases. A reliable sequence data should have less than 5% of bases identified as ambiguous after trimming [36].
Moderate Quality	2	$1e^{-139}$ to $1e^{-120}$	Some areas had discrete peaks and low background noise. However, most of the sequences had high background noise and overlapping peaks.
Low Quality	1	$1e^{-119}$ to $1e^{-50}$	The sequence had high background noise and overlapping peaks. Absence of evenly spaced peaks. Most areas with undetermined bases. The background noise was not acceptable when the peak height was more than 20% of the main peak height. When this panorama was observed the sequence was unreliable [36].
Unacceptable quality	0	$> 1e^{-50}$	The sequences consisted mostly of undetermined bases, high background noise, and the absence of discrete peaks. The sequence was not reliable for the analysis.

Statistical analysis

The CFX Maestro 2.3 version 5.3.022.1030 software from Bio-Rad was used for the post-qPCR data analysis. Microsoft Excel was used to assess sensitivity, specificity, and agreement. The Intellectus software (Florida, USA) was used for descriptive statistics, the Chi-Square test of independence, and the Pearson Correlation Analysis.

Results

PAS and Onycho+

Two hundred and forty-two dry nail samples submitted for fungal analysis through the standard clinical workflow were evaluated by the histological PAS staining procedure and compared with the Onycho+ test. As shown in Table 3, a total of 115 samples (115/237= 48.52%) resulted positive for fungal elements, and 122 (122/237= 51.48%) resulted negative through the PAS examination. The Onycho + test detected 99 (99/237= 41.77%) positive samples and 138 (138/237= 58.23%) negative samples. Five samples were excluded as they had an invalid result for the test, leaving 237 samples for the analysis.

Sequencing quality score and correlation to Onycho+ test result

Using the assessment tool as described in Table 2, the ITS-2 Sanger sequencing results were categorized. As shown in Table 4, of the 99 samples that were positive by the Onycho+ test, 80 (80.81%) were of high-quality (n=67) and moderate-quality (n=13) sequence. The 138 samples that were negative by the test, had a more even distribution, with 71 (51.45%) being high (n=44) and moderate quality (n=27) and 67 (48.55%) being low (n=50) and unacceptable quality (n=17) sequence. Additionally, a Chi-square Test of Independence was performed to examine whether the Onycho+ test and the sequencing quality were independent. The results were significant based on an alpha value of 0.05, df=3, $\chi^2 = 31.41$, and $p < 0.001$, suggesting that the PCR result and sequencing quality are related to one another. Finally, a Pearson correlation analysis was conducted (data not shown) between the Onycho+Test and the sequence quality. A moderate positive correlation of 0.35 was found, indicating that a qPCR-positive result was expected as the quality score increased. For the purpose of our analysis, only sequences with a high quality score (quality= 3) and moderate quality score (quality= 2) were considered definitive for identification. Therefore, as shown in Table 3, only 151 out of 237 had a quality score of 2 and 3. Of those, 80 samples were detected positive by Onycho+ test, and 67 were confirmed as dermatophytes; *T. rubrum* (n=46), *T. interdigitale* (n=18), *E. floccosum* (n=2) and one case of a *Trichophyton sp* (data not shown). The Onycho+ test was negative for 71 samples of score 2 or 3, and 46 (46/71= 65%) were confirmed as NDMs, as shown in Table 3.

Table 4
Chi-square Test of Independence assessment between the Onycho+ results and sequencing quality.

	Onycho+ Test	
Sequence Quality	Positive	Negative
High	67[46.37]	44[64.63]
Moderate	13[16.71]	27[23.29]
Low	16[27.57]	50[38.43]
Unacceptable	3[8.35]	17[11.65]
Total	99	138

The results of the Chi-square test were significant based on an alpha of 0.05, x2 (df=3), 31.41, p < 0.001. Note: Values formated as Observed [Expected].

Method comparison for dermatophyte detection

Using the gold standard of Sanger sequencing as a reference (Table 5), PAS had a 69.53% agreement, 85.1% sensitivity, and 57.1% specificity for detection of dermatophytes. Onycho+ compared more favorably with a 91.39% agreement, 100% sensitivity, and 84.5% specificity for dermatophytes.

Distribution of PAS positive results in the Onycho+ test

Samples that were positive by both PAS and Onycho+ were overwhelmingly dermatophytes (87.7%). However, samples that were positive by PAS and negative by Onycho+ were a majority NDMs (78.6%) (Table 6).

Discussion

The specific identification of dermatophyte infection in nails represents a step forward in personalized medicine as it enables a more accurate diagnosis and better treatment options in patients with onychomycosis. Dermatophytes are responsible for most onychomycosis cases [8,18], but half of all nail disorders are not onychomycosis [19,32]. Therefore, a dermatophyte assay will be the most

Table 3
Dry nail samples were evaluated through PAS compared with the Onycho+ test.

	PAS and PCR by ITS-2 Sanger sequencing results				
	PAS		Onycho+ Test		
ITS-2 Sanger Sequencing	Positive	Negative	Positive	Negative	Total[6]
Etiological Agent[1]					
Dermatophyte	57 (61.29%)	10 (17.24%)	67 (67.68%)	0 (0.00%)	67
NDM[2]	26 (27.96%)	24 (41.38%)	4 (4.04%)	46 (33.33%)	50
Yeast	7 (7.53%)	22 (37.93%)	8 (8.08%)	21 (15.22%)	29
Other[3]	3 (3.23%)	2 (3.45%)	1 (1.01%)	4 (2.90%)	5
Sub Total	**93 (80.87%)**	**58 (47.54%)**	**80 (80.81%)**	**71 (51.45%)**	**151**
Low and Unacceptable quality[4]	22 (19.13%)	64 (52.46%)	19 (19.19%)	67 (48.55%)	86
Total[5]	**115 (100.00%)**	**122 (100.00%)**	**99 (100.00%)**	**138 (100.00%)**	**237**

[1] The identity of the sample was established by high and moderate-quality sequencing results, as described in the Methods Section.
[2] NDMs, non-dermatophytes molds.
[3] The identity of these samples was other than Dermatophyte, NDMs, and yeast.
[4] The identity of these samples was established by low and unacceptable quality sequencing results.
[5] Percentages correspond to each column.
[6] The total in each row is the sum of positives and negatives for each method independently (Onycho+ Test and PAS Test).

Table 5
The overall agreement, sensitivity and specificity for dermatophyte detection using the Gold Standard[1] as reference.

Techniques	Agreement	Sensitivity	Specificity	NPV	PPV
PAS	69.53%	85.1%	57.1%	0.83	0.61
Onycho+	91.39%	100%	84.5%	1.00	0.84

[1] Gold Standard, GS, is defined as a high or moderate-quality ITS-2 Sanger sequencing result.

Table 6
Distribution of the PAS-positive results in the Onycho+ test.

	Identity from PAS Positive results[1]				
Technique Onycho+ test	Yeast	Other[3]	NDM[2]	Dermatophyte	Total
Positive	3 (4.6%)	1 (1.5%)	4 (6.1%)	57 (87.7%)	**65 (100%)**
Negative	4 (14.2%)	2 (7.1%)	22 (78.6%)	0 (0.00%)	**28 (100%)**
Total	**7**	**3**	**26**	**57**	**93 (100%)**

[1] The identity of the sample was established by high and moderate-quality sequencing results, as described in the Methods Section.
[2] NDMs, non-dermatophytes molds.
[3] The identity of these samples was other than Dermatophyte, NDMs, and yeast.

informative test in diagnosing and treating cases of suspected onychomycosis. Other than fungal culture, which has poor clinical sensitivity, PCR is the only routine method that can specifically identify dermatophytes.

Methodologies may have different capabilities, and discordant results are expected between the Onycho+ assay and PAS, as the former is specific for dermatophytes, and the latter can detect filamentous fungal elements, but cannot distinguish fungal types [6,8,22]. However, the Onycho+ assay accurately detected dermatophytes in nail samples compared to PAS using Sanger sequencing as the reference method (Table 5). Sequencing enabled the identification of the etiological agent in the nail samples and additionally provided clarity in interpreting the results. Only sequencing results with a quality score of 2 or 3 were considered definitive for identification. Sixty-four percent of all nails (151/237) (Table 3), met these criteria, very similar to that reported by Pospischil et al [32]. From this present study, a strong correlation between sequencing quality and a positive Onycho+ result was found (Table 4). It is expected that the etiologic cause of onychomycosis would be the predominant fungal organism present and thus the predominant fungal DNA leading to a high-quality sequence. On the contrary, in cases where there is a mixture of organisms in the nail sample, such as the presence of fungal environmental contaminants, a mixture of fungal DNA is to be expected [33–35] resulting in overlapping or competing peaks producing low-quality sequences [36]. A practical illustration of this is in Figure A1 (see supplementary file 1). The PCR reaction of the ITS-2 region that have a prominent amplicon present suggesting the presence of a predominant sequence or organism, produced high-quality sequences. PCR reactions that have several amplicons suggesting the presence of mixed sequence with no predominant organisms have low, or unacceptable-quality sequences (Table A1 see supplementary file 1). From the Pearson Correlation Analysis, it was found that as the quality of the sequence increased, a positive result was expected by the Onycho

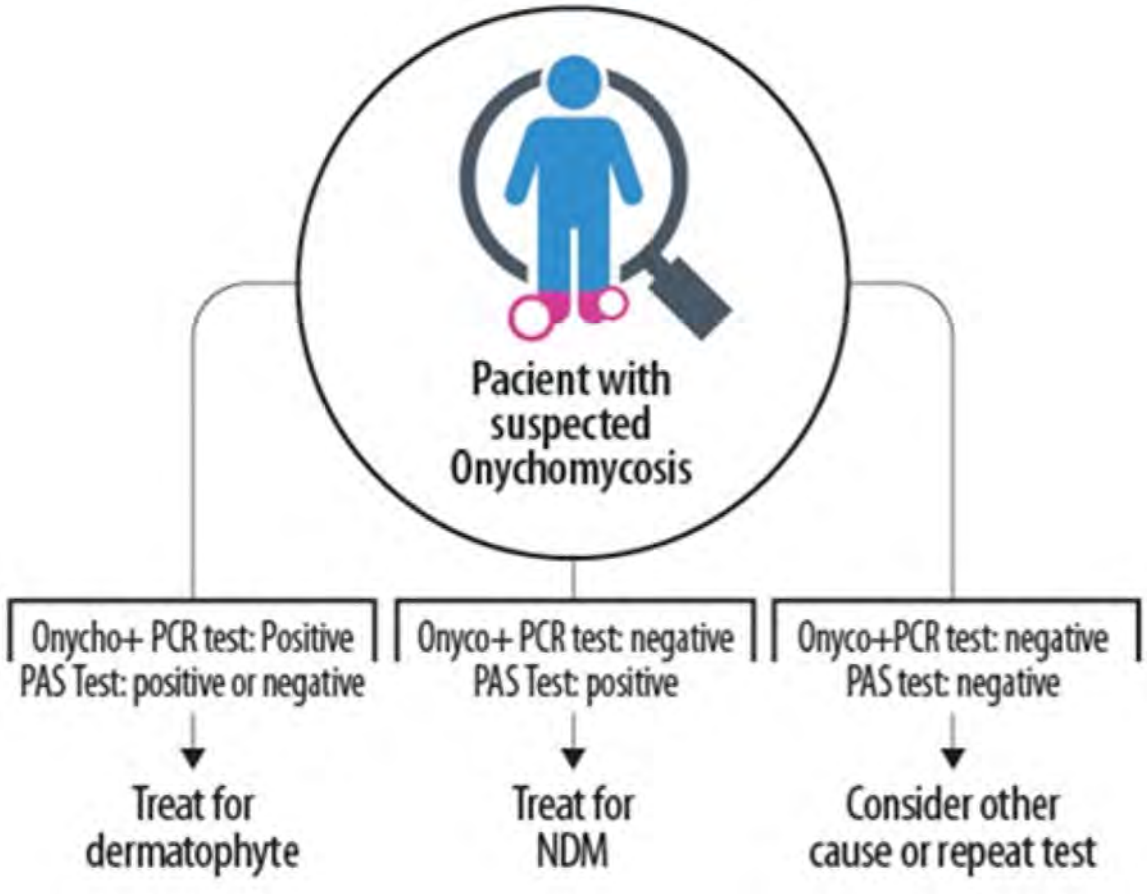

Fig. 2. Algorithm for onychomycosis diagnosis using Onycho+ PCR and PAS.

+ test. This is consistent with dermatophytes as the most prominent fungal organism causing onychomycosis.

Of the 151 samples with definitive sequence identification, dermatophytes represented the most numerous fungal organisms present in this study at 44.37% (67/151). However, NDMs represented 33.11% (50/151), a higher proportion than expected, as shown in Table 3. It has been suggested that warm, humid climates, such as the subtropical climate in Puerto Rico, may support a higher proportion of NDM in onychomycosis [9,35]. Recent studies performed in similar regions such as Brazil have documented the presence of mixed infection, with one or more NDM, in onychomycosis patients [37]. Our observation is important as many NDMs may require different treatment methodologies as they are reported to be less sensitive to standard therapies [32,38,39]. Still, NDMs can be problematic in onychomycosis diagnosis as they are almost ubiquitous in the environment and are present on body surfaces including nails [34]. As an example, environmental contamination with NDMs has long plagued dermatophyte culture leading to its poor sensitivity [39,40]. Additionally, most NDM lack keratinophilic properties of the dermatophytes [11] and are only present opportunistically as saprophytes on cells of the nail and skin. For these reasons, there have traditionally been stringent methods required for establishing an NDM as the etiologic agent in onychomycosis including: 1.) the isolation of the same organism on two separate occasions at least one week apart, 2.) demonstrating the absence of a dermatophyte and 3.) the presence of filamentous elements by microscopy [37]. These criteria rely on time-consuming and insensitive culture methods and the low specificity of direct microscopy from which many laboratories are moving away. However, combining methods to utilize the properties of each is one way to improve diagnostic certainty. Our data show that Onycho+ negative samples that are PAS positive are most likely NDMs (Table 6). This observation together with the high NPV of the Onycho+ test, suggests that the combination of histology with Onycho+ PCR can determine the presence of a dermatophyte, including the identification of *T. rubrum*, as well as indicate if a PAS positive nail is likely an NDM. This diagnostic algorithm is shown according to Fig. 2.

One limitation of this study is that it does not address mixed infections. We highlight that low sequencing quality is likely caused by the co-presence of ubiquitous, environmentally present NDMs which cause the poor-quality sequencing, especially in PAS and Onycho+ negative samples (Figure A1, Table A1 **see supplementary file 1**). Yet it is possible that some of the poor-quality sequencing results, especially in the PAS and Onycho+ positive samples, are mixed infections of a dermatophyte causing onychomycosis with an NDM, as described in [37]. The authors of that study estimate such mixed infections are present in up to 39% of cases and the proportion differs by geography. If correct, our positive results could be underestimated. A second limitation of this study is the lack of culture data for the clinical specimens. Fungal culture is highly specific and could resolve some issues such as the rate of mixed infections. However, fungal culture is problematic in both turnaround time and sensitivity. Other methods are supplanting the need for culture including PAS and PCR and using Sanger sequencing as a standard as in the current study.

Finally, from the totality of Onycho+ PCR, PAS and sequencing results, the organisms implicated in onychomycosis in this Puerto Rican cohort are the dermatophytes, including *T. rubrum, T. interdigitale* and *E. floccosum,* as expected (data not shown). In the case of NDMs and *Candida*, they appear mostly present as environmental mycobiota but can be etiologic agents of onychomycosis such as in some cases of *Aspergillus* sp., *Fusarium sp*. and *Neoscytalidium* sp. in this study (data not shown). Therefore, care must be taken to ensure accurate results. Though sequencing served to confirm the identity, these data show that the combination of PAS and Onycho+ PCR methods can be used to determine both a dermatophyte and an NDM etiology. This added value testing routine with the proposed diagnostic algorithm (Fig. 2) can be easily implemented in a clinical and pathology laboratory due to the wide availability of the standard techniques of tissue staining and PCR.

Conclusion

The Onycho+ PCR assay is a specific and sensitive test to determine the presence of dermatophyte fungi in human nail samples and is superior to histological staining with PAS in this Puerto Rican cohort with suspected onychomycosis. PCR and sequencing analysis indicate that the profile of the species causing onychomycosis is consistent with that found in the literature with a majority *T. rubrum* and *T. interdigitale*. However, the data also indicates that NDMs are present at a higher proportion than expected, and may also be an etiologic cause, especially from the genera *Aspergillus, Fusarium* and *Neoscytalidium*. In this study, samples that are negative by the highly specific dermatophyte qPCR test Onycho+ but also show filamentous fungal elements by PAS are sequenced as NDMs (Table 6). We conclude that a combination of qPCR by Onycho+ and PAS provides the information necessary to make an accurate onychomycosis diagnosis and point to the correct treatment.

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Declaration of Competing Interest

The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mycmed.2023.101412.

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