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Genetic variation of sequencing 18srDNA gene of *Trichophyton mentagrophytes* isolates

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ABSTRACT

Background. *Trichophyton* species are considered the most frequent causative and considerable agents in infection concerns.

Objective. The existing study was designed to conduct the sequencing analysis of nucleotides of ITS1-5.8S-ITS2 for *Trichophyton mentagrophytes.*

Methods. The isolation and identification of the pathogenic fungi, *Trichophyton mentagrophytes* from clinical specimens was done based on the morphological standard and molecular methods. The genomic DNA of fungal isolates was extracted and purified to amplify with primers of the 18S rRNA gene for detection and sequencing of the nitrogenous bases to define the genetic variation among clinical isolates of *Trichophyton mentagrophytes* compared with strains recorded in the NCBI GenBank.

Results. Eight isolates of *Trichophyton mentagrophytes* were isolated and identified from clinical specimen of dermatophytosis. The DNA sequencing analysis showed the presence of some genetic variation in nitrogenous bases between the local isolates and that recorded in locus of world NCBI GenBank. Multiple alignment sequence using NCBI BLAST revealed the recoding a new mutant isolate among the local isolates based on DNA homology percent.

Conclusion. The present results proved the successful use of molecular methods in diagnosis the fungal isolates of *Trichophyton mentagrophytes,* especially the sequencing analysis to find the genetic bases of these isolates.

Keywords: *Trichophyton mentagrophytes,* DNA sequencing, genetic variations

INTRODUCTION

Dermatophytoses are considered as one of the most common diseases in humans which promptly increased in last decades; although these diseases are not life threatening they have infected 10-20% of the world's population [1]. Dermatophytes are a group of related fungi with similar morphological and functional characteristics, capable of entering keratinized tissues of humans and animals, causing cutaneous infections or tinea [2]. Due to the fact that *Trichophyton* species show close similarity and confuse phenotypic features on culture media, these characteristics become a weak taxonomic tool in this state because it lacks or makes difficult the finding of teleomorph state for many clinical isolates complained with anamorphic polymorphism phenomena. Thus, these factors make the taxonomic classification of these dermatophytes so difficult especially the new species or strains that infect humans which are called anthropophilic dermatophytes. So, the diagnosis of these fungi comprises a taxonomic problem not only for species identification but for lacking the macroscopic and microscopic characteristics of *Trichophyton* species [3]. Due to this fact, the determination of genotypes of dermatophytes is very important to evaluate the levels of genetic variations among species that inhabit different ecosystems depending on the comparison of nucleotide sequences of target genes of fungal species, with the reference isolates sequencing recorded in the world GenBank. Due to the close correlation among dermatophytes that infect humans and animals, some isolates belonging to particular species of *Trichophyton* display different degrees of pheno-

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typic similarities and at the same time have different genotypes among one species of *Trichophyton*, named the *Trichophyton* species complex.

Liu et al. [4] used the method of random amplified polymorphic DNA (RAPD) to analyze the evolutionary relation among *Trichophyton* species complex. Also, the real-time PCR and restriction fragment length polymorphism (REFLP) were used to detect of *Trichophyton* species [5,6]. These species are unstable in taxonomy because of the emergence of new species or strains that differed in many morphologic and microscopic features from the wild type that it evolved from. So, the molecular methods began to solve this problem by using the sequencing analysis of nitrogen bases for the ITS region in rDNA gene [7]. So, the existing study was designed to conduct the sequencing analysis of nucleotides of ITS1-5.8S-ITS2 for *Trichophyton* mentagrophytes and determine the DNA homology between them in comparison with reference isolates in the GenBank.

MATERIALS AND METHODS

One hundreds three specimens including hair fragments, nail clippings, and skin scrapings were collected from patients from the Dermatology Department of AL-Diwaniya Teaching Hospital in Iraq during the period from the start of December 2021 till the end of April 2022. Then, all the samples were brought to the lab and diagnosed by direct examination and laboratory culture [8]. The typical medium for the isolation of pathogenic fungi from medical specimens is Sabouraud's dextrose agar (SDA) containing cycloheximide 0.5g/L to inhibit the growth of saprophytic fungi and chloramphenicol 0.05g/L to inhibit the growth of fast-growing bacteria. Cultures were incubated at 28+1ºC. Cultures were first examined after 4-7 days, and then twice weekly for a minimum of 3-4 weeks, formerly being deliberated negative. The distinctive characteristics of dermatophytes typically manifest within a span of 10-20 days. Many dermatophytes lose their unique cultural and microscopic traits when cultured for extended periods (Rippon, 1988). In this research, identification was primarily reliant on colonial and microscopic traits, employing specific media as outlined by Rippon [9,10]. A segment of the colonies was cultivated on SDA through a spot inoculation technique. These cultures were incubated at a temperature of 29 ± 2°C until visible fungal growth occurred, or until they reached 5-10 days of age, for early PCR analysis. The genomic DNA of fungal isolates (young fungal colonies) was extracted using a grinder in the presence of liquid nitrogen to initially break down the mycelia. The final DNA extraction was accomplished using a specialized purification kit. The resulting DNA solution, totaling 60 µl, was stored at 20°C until it was needed as a template in subsequent PCR experiments. The PCR primers, designed for detecting *Trichophyton sp.* and based on the 18S rRNA gene, were developed in this study using the NCBI Gene Bank database and the Primer 3 online design tool. These primers were obtained from the Macrogen company, Korea, as shown in Table 1.

TABLE 1. PCR Primers with their nucleotide sequence and amplicon size

Primer	Sequence (5'-3')		Product Size
Trichophyton mentagrophytes 18S rRNA gene	F	GACGTTCCATCAGGGGTGAG	584bp
	R	CTGAATTGGCTGCCCATTCG	

Fungal genomic DNA was extracted from fungal isolates using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, following the manufacturer's instructions. The quality and purity of the extracted genomic DNA were assessed using a Nanodrop spectrophotometer (THERMO, USA) by measuring absorbance at both 260 nm and 280 nm.

The primers were prepared as per the manufacturer's guidelines by reconstituting lyophilized primers with TE buffer to create a stock solution with a concentration of 100 pmole/ul. After centrifugation and an overnight incubation at 4°C, a working solution of the primers in TE buffer was prepared, achieving a final concentration of 20 pmole/ul for each primer, using the equation C1V1 = C2V2 (concentration versus volume).

The PCR master mix reaction was prepared using the AccuPower PCR PreMix Kit, following the manufacturer's instructions. Subsequently, these PCR master mix reaction components, as mentioned earlier, were placed in standard PCR tubes containing the lyophilized PCR PreMix, which includes all the necessary components for the PCR reaction, such as Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer, and tracking dye. The tubes were then centrifuged in an Exispin vortex centrifuge for 3 minutes before being transferred to a Mygene PCR thermocycler.

PCR thermocycler conditions for each gene were determined using a conventional PCR thermocycler system. The PCR products of each gene were subsequently analyzed using agarose gel electrophoresis, following the method outlined by Sambrook et al. [11].

The PCR products of ITS5-ITS4 primers were used to gain the nitrogen bases sequences of *Trichophyton* isolates which were sent to the Microgen company (USA). These sequences were compared with sequencing of reference isolates present in world the GenBank by using the NCBI blast nucleotide database program to get a high percent of compatibility for generic and specific name of anamorph or teleomorph state of each isolate.

Statistical analysis

The data underwent collection, summarization, analysis, and presentation through the utilization of SPSS version 25, along with Microsoft Office Excel 2010.

RESULTS AND DISCUSSION

The results indicated that the positive amplification of the ITS region of 18s rDNA gene for *Trichophyton* mentagrophytes had an amplicon size of 584bp PCR product (Figure 1).

The amplification of the ITS region of rDNA have been used by various workers [12-16].

Figures 2, 3, 4, 5, 6 show the Multiple Alignment sequence using NCBI BLAST of the partial 18S ribosomal RNA gene sequence of local isolates of *Trichophyton* mentagrophytes and NCBI Gene Bank. The results showed the close relation of local isolates of

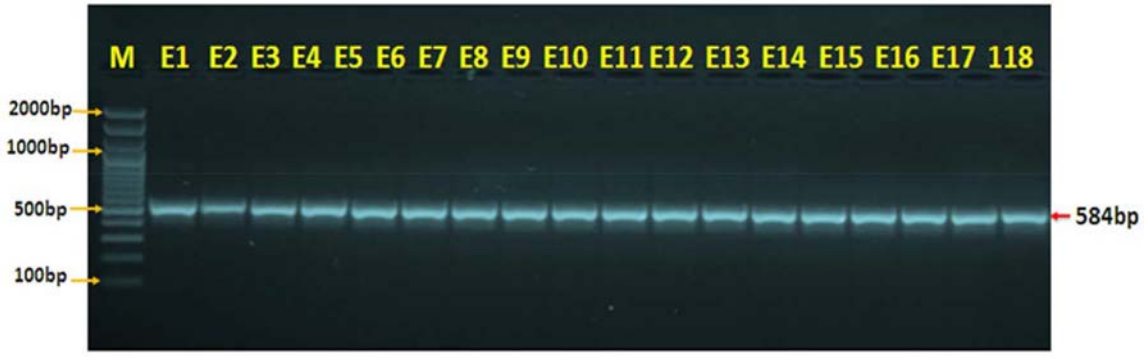

FIGURE 1. Image of electrophoresis Agarose gel of PCR product investigation of 16S ribosomal RNA gene of *Trichophyton mentagrophytes* isolates. (M=Marker ladder: 2000-100bp, lanes (1-18): positive (584bp) PCR product size

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REF. NCBI TCGATGAAGAAC-GCAGC-GAAATGCG-ATAAG-TAATGT-GAATTGCAGAATTCCGTGA
Tm1       ...........-.....-.....-.....-.....-.....
Tm2       ...........-.....-.....-.....-.....-.....-.....
Tm3       ..... -.....-.....-.....-.....-.....-.....
Tm4       .....- .....-.....-.....-.....-.....-.....
Tm5       ...A.....A.....-.....G....-.....-.....-.....-.....
Tm6       ...A.....TC.....C.....- A.....C....-.....-.....-.....-.....
Tm7       .....A..G.....-.....-.....-.....-.....-.....
Tm8       ..... A.....-.....-.....-.....-.....-.....
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FIGURE 2. Multiple Alignment sequence using NCBI BLAST of local isolates of *T. mentagrophyes* isolated from *Tinea capitis* (No. Tm1-Tm8) in compared with reference strainFJ746658.1

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REF. NCBI ATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTTCG
Tm1       .....
Tm2       .....
Tm3       .....
Tm4       .....
Tm5       ..... A.....T.....
Tm6       .....
Tm7       .....T.....
Tm8       G.....A.....G.....T.....G.....G.....T..... T.....
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FIGURE 3. Multiple Alignment sequence using NCBI BLAST of local isolate of *T. mentagrophyes* isolated from *Tinea pedis* (No. Tm1-Tm8) in compared with reference strain no. KJ606098.1

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REF.NCBI AGCGTCATTTC-AGCCCCT-CAAGCCCGGCTT-GTGTGATGGACGACCGTCCGGCGCCCC
Tm1.      .....-.....-.....-.....
Tm2       .....-.....- .....
Tm3       .....-.....-.....-.....
Tm4       .....-.....-.....-.....
Tm5       .....-.....T.....-..... .-.....
Tm6       … C.....T.....C-.....G.....-.....-.....
Tm7       ..... A..... -.....A… A-.....T....T.....-.....
Tm8       .....T-……..T.....-…. C...-C….-….A…..G.....G….-.. ..GA. A.....G....G……..G……
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FIGURE 4. Multiple Alignment sequence using NCBI BLAST of local isolate of T. mentagrophyes isolated fromTinea ungium (No. Tm1-Tm8) in compared with reference strain no. HQ014710.1

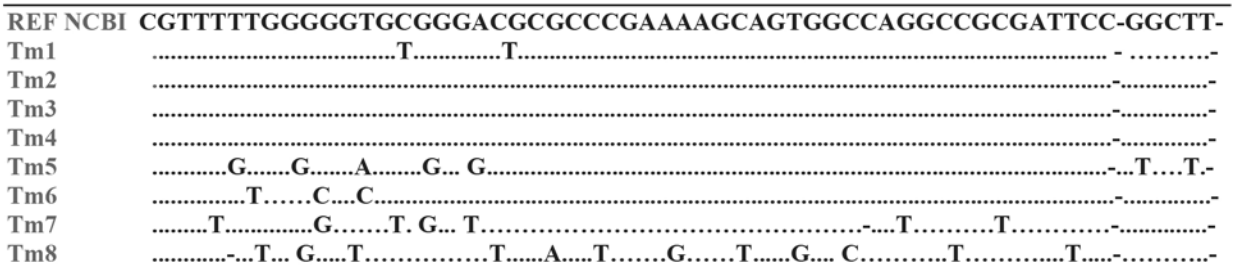

FIGURE 5. Multiple Alignment sequence using NCBI BLAST of local isolate of *T. mentagrophyes* isolated from *Tinea corporis* (No. Tm1-Tm8) in compared with reference strain no. HQ223449.1

FIGURE 6. Multiple Alignment sequence using NCBI BLAST of local isolate of *T. mentagrophyes* isolated from *Tinea barbae* (No. Tm1-Tm8) in compared with reference strain no. AF168125.1

T. mentagrophytes isolated from clinical specimens of dermatophytosis with reference strains NCBI Blast *Trichophyton mentagrophytes:* KX463658.1; FJ746658.1; KJ606098.1 HQ014710.1; AF168125.1).

The PCR amplicons generated were sequenced with the same primers to obtain partial 18SrRNA gene sequences. The incomplete 18S ITS1-5.8S-ITS2 incomplete 28 rRNA sequences examined by nucleotide blast and the percentage similarity were calculated by using method of alignments explorer Clustal X Microsoft Mega 6.0 package. The gene sequence of local isolates of *T. mentagrophytes* showed 97-100% homology with sequence of *T. mentagrophyes* standard strain. The DNA sequencing of the partial 18SITS1-5.8S-ITS2 partial 28S rRNA gene sequence data for each strain was compared to its nearest neighbor, and if the similarity score exceeded 97%, it indicated the presence of a new species [17].

In recent years, genotypic methods have demonstrated their utility in resolving identification issues related to dermatophytes. Genotypic variations have been deemed more stable and accurate compared to morphological characteristics [13]. The detection rate of dermatophytes by PCR assay showed a degree of variations due to the diagnosis of *Trichophyton spp.* by classic laboratory methods which gave phenotypic variability and pleomorphism, and the use of molecular methods gave high accuracy and specificity [14].

Comparing sequences within the ITS region is a common practice in taxonomy and molecular identification. It's favored because it can be easily amplified from small DNA samples, thanks to the high copy number of rRNA genes, and it exhibits significant variation even among closely related species. The ITS region stands out as the most frequently sequenced DNA region in the molecular diagnosis of fungi [15].

On the other hand, the results of analyzing the sequencing of ITS1-rRNAgene of local isolates *Trichophyton mentagrophytes* isolated from different tinea lesions showed a high genetic variation in nitrogen bases of nucleotides when compared with some strains recorded as reference in the NCBI GenBank. Table 2 shows the base sequences of some isolates under study and their percent of compatibility with reference isolates in the world GenBank isolates.

These results revealed the occurrence of point mutation either as mismatching or genetic gaps which differed in their number or types according to fungal species and sometimes based on isolate or strain of these species. This may be due to some stress of effect in their ecosystem or habitat of species which pay the fungus to form new strains and this is called eco-micro internal evolution [12].

CONCLUSION

The present results proved the successful use of molecular methods in diagnosing the fungal isolates of *Trichophyton mentagrophytes,* especially the sequencing analysis to find the genetic bases of these fungi.

TABLE 2. Type and position of mutation with genetic variation percentage in local isolates of *T. mentagrophytes* in comparison with reference NCBI world strain

No	Sample	Mutation	Genetic variation %
1	T. capitis Trichophyton_ITS1-rRNA_gene	A -> T	50%
2	T. capitis Trichophyton_ITS1rRNA_gene	A -> T	100%
3	T. capitis Trichophyton_ITS1-rRNA_gene	C -> T	50%
4	T. capitis Trichophyton_ITS1-rRNA_gene	C -> A	0%
5	T. capitis Trichophyton_ITS1-rRNA_gene	C -> A	50%
6	T. capitis Trichophyton_ITS1-rRNA_gene	A -> C	50%
7	T. capitis Trichophyton_ITS1-rRNA_gene	A -> C	100%
8	T. capitis Trichophyton_ITS1-rRNA_gene	T -> A	50%
9	T. pedis Trichophyton_ITS1-rRNA_gene	G -> T	0%
10	T. pedis Trichophyton_ITS1-rRNA_gene	G -> T	0.25%
11	T. pedis Trichophyton_ITS1-rRNA_gene	G -> A	0%
12	T. pedis Trichophyton_ITS1-rRNA_gene	G -> A	100%
13	T. pedis Trichophyton_ITS1-rRNA_gene	G -> A	50%
14	T. pedis Trichophyton_ITS1-rRNA_gene	C -> T	8.18%
15	T. pedis Trichophyton_ITS1-rRNA_gene	C -> T	50%
16	T. pedis Trichophyton_ITS1-rRNA_gene	C -> T	50.19%
17	T. corporis Trichophyton_ITS1-rRNA_gene	C -> T	24.40%
18	T. corporis Trichophyton_ITS1-rRNA_gene	G -> T	7.08%
19	T. corporis Trichophyton_ITS1-rRNA_gene	G -> T	49.97%
20	T. corporis Trichophyton_ITS1-rRNA_gene	G -> T	50%
21	T. corporis Trichophyton_ITS1-rRNA_gene	G -> T	0%
22	T. corporis Trichophyton_ITS1-rRNA_gene	G -> C	50%
23	T. corporis Trichophyton_ITS1-rRNA_gene	G -> C	0%
24	T. corporis Trichophyton_ITS1-rRNA_gene	C -> T	100%
25	T. ungium Trichophyton_ITS1-rRNA_gene	C -> T	100%
26	T. ungium Trichophyton_ITS1-rRNA_gene	C -> T	50%
27	T. ungium Trichophyton_ITS1-rRNA_gene	C -> T	100%
28	T. ungium Trichophyton_ITS1-rRNA_gene	A -> G	11.25%
29	T. ungium Trichophyton_ITS1-rRNA_gene	A -> G	63.67%
30	T. ungium Trichophyton_ITS1-rRNA_gene	A -> G	50.46%
31	T. ungiumTrichophyton_ITS1-rRNA_gene	A -> G	50%
32	T. ungiumTrichophyton_ITS1-rRNA_gene	G -> T	50%
33	T. barbae Trichophyton_ITS1-rRNA_gene	G -> T	56.31%
34	T. barbae Trichophyton_ITS1-rRNA_gene	G -> T	14.98%
35	T. barbae Trichophyton_ITS1-rRNA_gene	G -> C	50%
36	T. barbae Trichophyton_ITS1-rRNA_gene	G -> C	0%
37	T. barbae Trichophyton_ITS1-rRNA_gene	G -> C	50%
38	T. barbae Trichophyton_ITS1-rRNA_gene	C -> T	99.20%
39	T. barbae Trichophyton_ITS1-rRNA_gene	C -> T	0%
40	T. barbae Trichophyton_ITS1-rRNA_gene	C -> T	2.48%

Ethics Contemplation: This study adheres to the ethical guidelines set forth by the ethics committee of Al-Diwaniaya teaching hospital in Iraq. Prior to sample collection, verbal consent was obtained from the study participants or their relatives.

Conflict of interest: No identified engagement of attention linked with this publication.

Funding: This investigation did not get any funding from activities in the public, profit, or not-for-profit sectors

Availability of data and materials: The information used and/or examined during this study is accessible from the consistent authors on sensible invitation.

Competing interest: The authors professed that they take no challenging attention.

REFERENCES

1. Grumbt M, Monod M, Staib P. Genetic advances in dermatophytes. *FEMS Microbiol Lett.* 2011;320(2011):79-86. doi: 10.1111/j.1574-6968.2011.02276.x
2. Hainer BL. Dermatophyte infections. *Am Fam Physician.* 2003;67(1):101-108. PMID: 12537173
3. Gräser Y, De Hoog S, Summerbell RC. Dermatophytes: recognizing species of clonal fungi. *Med Mycol.* 2006;44(3):199-209. doi: 10.1080/13693780600606810
4. Liu D, Pearce L, Lilley G et al. PCR identification dermatophyte fungi Trichophyton spp. *J Med Microbiol.* 2002;51:117-122. doi: 10.1099/0022-1317-51-2-117
5. Wisselink GJ, van Zanten E, Kooistra-Smid AMD. Trapped in keratin; a comparison of dermatophyte detection in nail, skin and hair samples directly from clinical samples using culture and real-time PCR. *J Microbiol Methods.* 2011;85(1):62-66. doi: 10.1016/j.mimet.2011.01.023
6. Kadhim SK, Al-Hamadani AH, Al-Janabi JK. Genotyping and subgenotyping of Trichophyton rubrum isolated from dermatophytosis in Iraqi patients using RFLP-PCR. *IOSR J Pharm Biol Sci.* 2015;10(6):61-67. doi: 10.9790/3008-10616167
7. Cafarchia C, Iatta R, Latrofa MS et al. Molecular epidemiology, phylogeny and evolution of dermatophytes. *Infect Genet Evol.* 2013;20:336-351. doi: 10.1016/j.meegid.2013.09.005
8. McGinnis R. Infections caused by dematiaceous fungi: chromoblastomycosis and phaeohyphomycosis. *Infect Dis Clin North Am.* 1988;2(4):925-938. PMID: 3062095
9. Rippon JW. The Pathogenic Fungi and Pathogenic Actionmycetes. 3rd ed. Philadelphia, USA: WB Saunders; 1988.
10. Kwon-Chung KJ, Bennett JE. Medical mycology. *Rev Inst Med Trop Sao Paulo.* 1992;34(6):504-504. doi: 10.12691/ajmcr-8-3-7
11. Sambrook J, Fritsch ER, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
12. Rezaei-Matchkdaei A, Makimura K, De Hoog GS et al. Discrimination of Trichophyton tonsurans and T. equinum by PCR-RFLP and by β-tubulin and translation elongation factor 1-alpha sequencing. *Med Mycol.* 2012;50:760-764. doi: 10.3109/13693786.2012.661885
13. Grumbt M, Monod M, Staib P. Genetic advances in dermatophytes. FEMS Microbiol Lett. 2011;320:79-86. doi: 10.1111/j.1574-6968.2011.02276.x
14. Moskaluk AE, VandeWoude S. Current Topics in Dermatophyte Classification and Clinical Diagnosis. *Pathogens.* 2022;11(9):957. doi: 10.3390/pathogens11090957
15. Behzadi P, Behzadi E, Ranjbar R. Dermatophyte fungi: Infections, Diagnosis and Treatment. *SMU Med J.* 2014;1(2):53-61. Corpus ID: 85925460
16. Schoch CL, Seifert KA, Huhndorf S et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc Natl Acad Sci U S A.* 2012;109(16):6241-6246. doi: 10.1073/pnas.1117018109
17. Li HC, Bouchara J, Hsu MM et al. Identification of dermatophytes by sequence analysis of the rRNA gene internal transcribed spacer regions. *J Med Microbiol.* 2008;57:592-600. doi: 10.1099/jmm.0.47607-0
18. Reller L Barth, Weinstein MP, Petti CA. Detection and Identification of Microorganisms by Gene Amplification and Sequencing. *Clin Infect Dis.* 2007;44:1108-1114. https://doi.org/10.1086/512818