Research Article

Novel multiplex real-time quantitative PCR detecting system approach for direct detection of *Candida auris* and its relatives in spiked serum samples

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The multidrug-resistant opportunistic yeast species of *Candida auris*, *Candida haemulonii*, *Candida duobushaemulonii* and *Candida pseudohaemulonii* continue to endanger the healthcare settings around the globe. Due to the lack of a specific qPCR assay for detection of these species from clinical samples, we developed a multiplex qPCR assay. Analytical specificity and sensitivity showed 100% specificity and the sensitivity of up to ten genomes of target species with a high value of reproducibility (R^2 >0.99). Subsequently, from spiked serum samples, our qPCR specifically could detect up to ten genomes of *C. auris* and one genome of *C. haemulonii*, *C. duobushaemulonii* and *C. pseudohaemulonii* (R^2 >0.98). Lack of cross reaction with the human DNA, a high degree of specificity and sensitivity, showed the potential of our multiplex PCR for direct detection of *C. auris* and closely related species from serum samples of suspected patients. Future studies are warranted to assure its applicability in clinical settings.

Nowadays, controlling infections caused by opportunistic yeast species is challenged owing to increasing of immunocompromised population, emerging pathogenic species and increasing prevalence of antifungal-resistant isolates, which potentially lead to diagnostic and therapeutic failures and increased healthcare costs [1–4]. The globally fast-spreading *Candida auris* currently is regarded as one of the most important opportunistic emerging yeast species, which caused a huge concern for healthcare settings [5–7]. Using routine typing protocols, clinical isolates of *C. auris* are clustered in four distinctive clades namely, east Asian, south Asian, South American and African [8]. From 2012 to 2017, >361 isolates of *C. auris* were obtained from blood cultures, accounting for 67.48% of the all recovered isolates from clinical samples, worldwide [9]. On the other hand, the other closely related species of *C. auris* namely, *Candida haemulonii*, *Candida duobushaemuloni* and *Candida pseudohaemulonii* have been reported to possess a multidrug-resistant profile and all have been isolated from blood samples in various countries [10–12]. *Candida haemulonii,* which was first known as a human pathogen in 1984, is being increasingly reported worldwide [6,10]. Additionally, recent surveillance studies of candidemia conducted in Panama, revealed

Table 1. Primers and targets used in the qPCR assay.				
Target species	**Primer name**	**Primer sequence**	**Target loci**	**Product size (bps)**
Candida auris	Auris-F	CGGTGGCGTTGCATTCA	Internal Transcribed Spacer 2	244
	Auris-R	CTGCTGCTAGAACACCTCGT	28S rDNA	
Candida haemulonii	Haemu-F	CCGAGGACCGCAGCAAT	28S rDNA	181
	Haemu-R	CCCGTCCGAGCACTTCCA	28S rDNA	
Candida duobushaemulonii	Duobu-F	GAACGCACATTGCGCCTTGG	5.8S rDNA	115
	Duobu-R	GTAGACTTCGCTGCGGATATGTTA	Internal Transcribed Spacer 2	
Candida pseudohaemulonii	Pseud-F	TAACCTGCGGAAGGATCATTAAG	18S rDNA	197
	Pseud-R	AATGTGCGTTCAAAGATTCGATG	5.8S rDNA	

that almost half of 36 isolates primarily identified as *C. auris* were *C. duobushaemulonii* [13], which highlights the importance of correct identification of other closely related species of *C. auris. Candida pseudohaemulonii* that for the first time in 2006 was detected in the blood sample of a Thai patient showing resistance to amphotericin B and azoles [14], was accountable for approximately a third of clinical isolates identified as *C. haemulonii* in a Korean surveillance study and surprisingly all of the isolates were from blood samples [10].

For these multidrug-resistant pathogens, rapid and precise diagnosis is important to initiate appropriate antifungal treatment, prognosis improvement and outbreak control [15–17]. Up to now, >20 identification methods have been published for *C. auris* [9,18–20]. Biochemical assays as one of the most widely used phenotypic approaches, besides of being time consuming and expensive, cannot properly identify these species [21]. Recently, several species-specific PCRs have been established to identify *C. auris* from DNA samples derived from pure colonies or environmental swab samples [18,19]. Although the need for a culture-independent molecular method is urgent, unfortunately, this is still lingering as an unsolved issue.

Herein, we developed the first multiplex qPCR assay for *C. auris,* and its relatives, that is, *C. haemulonii, C. duobushaemulonii* and *C. pseudohaemulonii*, which can detect these opportunistic yeast species directly from spiked serum samples with a clinically relevant limit of detection (LOD).

Materials & methods

Fungal strains

Two hundred and eighty-two reference strains and clinical isolates were used in two different analytical validation test sets. The first test set included 177 reference strains consisted of 33 *C. auris*, 25 *C. haemulonii*, five *C. duobushaemulonii*, three *C. pseudohaemulonii* and 111 fungal strains representing 68 other closely or distantly related species, which were included for testing the analytical sensitivity, reproducibility, specificity and proficiency testing (Supplementary Tables 1 & 2). Reference strains were obtained from Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands. Human genomic DNA (male, 10 ng/μl, Thermo Fisher Scientific, MA, USA) was also used for specificity testing. The second test set included 105 clinical isolates obtained from Faculty of Medicine, Kuwait University, Kuwait, which were previously identified as *C. auris* by matrix-assisted laser desorption/ionization TOF mass spectrometry (MALDI-TOF MS; Bruker Corporation, MA, USA) [22,23].

Primer design

Sequences of rDNA for *C. auris* (including the isolates of the four clades analyzed by Lockhart *et al.* in 2016 [8] and more isolates after 2016) and its relatives were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/) for primer design. We analyzed the rDNA region of 233 global isolates for the reverse primer of *C. auris*, of which 138 of the sequences were from our own database and the rest were from Genbank. Only primers meeting the following criteria were chosen for further optimization: they should cover all the clades of target species; no cross reaction with other clinically and environmentally important fungi and human DNA; production of a single discriminative melting peak for each locus, which is at least 1°C different from the other locus; and being compatible with the rest of primers in the same reaction in terms of primer Tm values and primer homo/heterodimer formations. uMELT (www.dna.utah.edu/umelt/umelt.html) was used to predict PCR products melting temperature. IDT OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) was used to predict primer Tm values and homo/heterodimers. Subsequently, oligonucleotides were synthesized by Integrated DNA Technology Company (IDT, Leuven, Belgium). Table 1 shows the primers information used in this study.
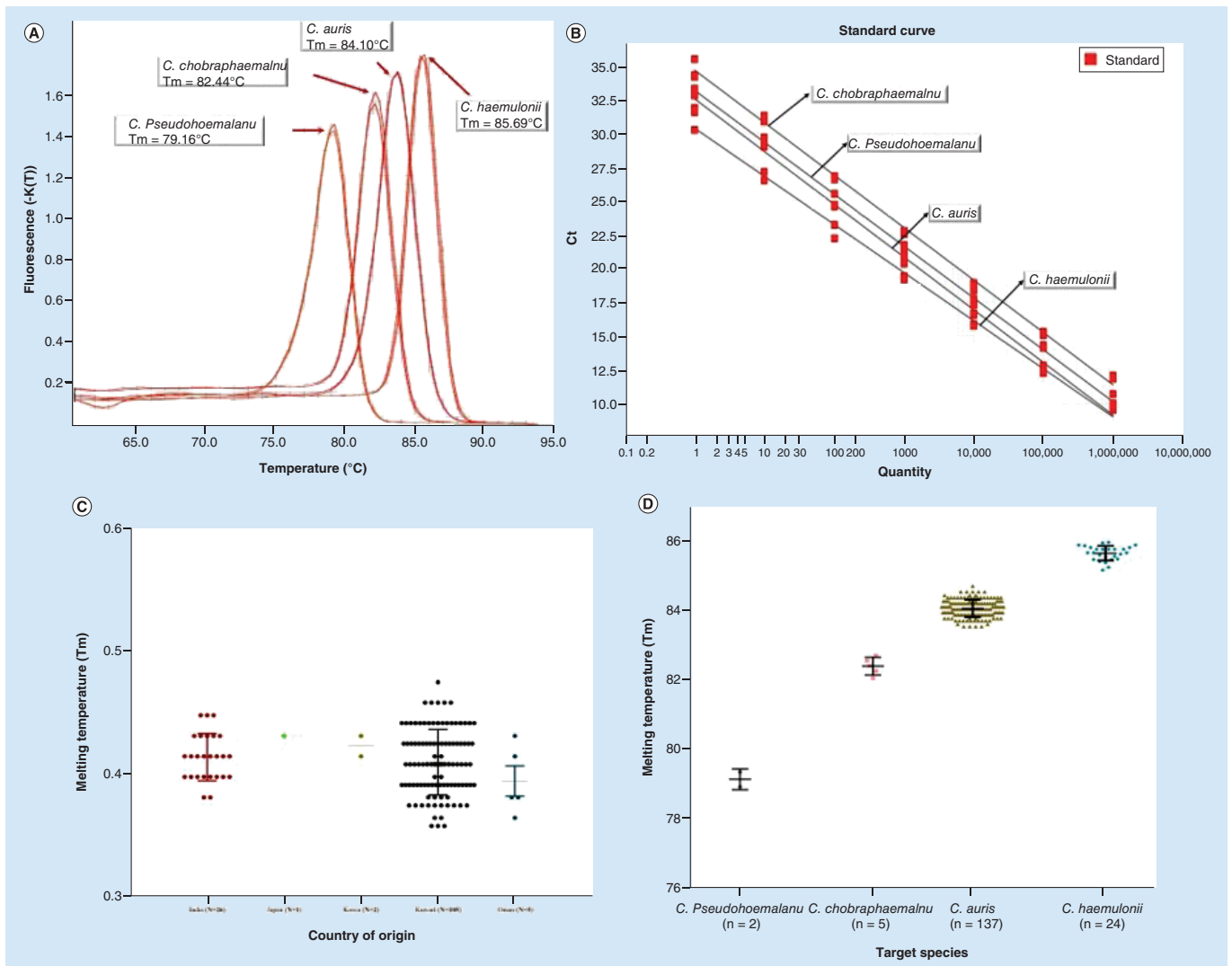
Figure 1.   Shows the analytical features obtained for our qPCR assay. (A) Melting profile of target species including *Candida pseudohaemulonii*, *Candida duobushaemulonii*, *Candida auris* and *Candida haemulonii*. **(B)** Standard curves of four target species over seven orders of magnitude. **(C)** Melting temperature distribution of *C. auris* in five countries. **(D)** Shows the Tm distribution of four target species.

Analytical validation

Log-10 serial dilutions (starting from 1 ng/µl) of DNA of all four target species were prepared to find the LOD and the reproducibility of our assay. The analytical specificity was evaluated using 1 ng/µl DNA of diverse range of other yeast species, filamentous fungi and human.

Two technicians ran the proficiency testing in a blind fashion using DNA of eight target strains of *C. auris*, *C. pseudohaemulonii*, *C. duobushaemulonii* and *C. haemulonii* dispersed among 111 nontarget strains representing 68 fungal species (Supplementary Table 3). One technician, who was aware of the identity of each DNA samples, randomly distributed them and provided the second technician with the blinded test set. Besides, single pure colonies of *C. auris*, *C. pseudohaemulonii*, *C. duobushaemulonii* and *C. haemulonii* were subjected to our multiplex PCR.

Institutional ethical committee approval

As clinical validation phase of our study required serum samples, the procedure of serum sampling was reviewed and approved by the ethical committee members of Medical University of Innsbruck.
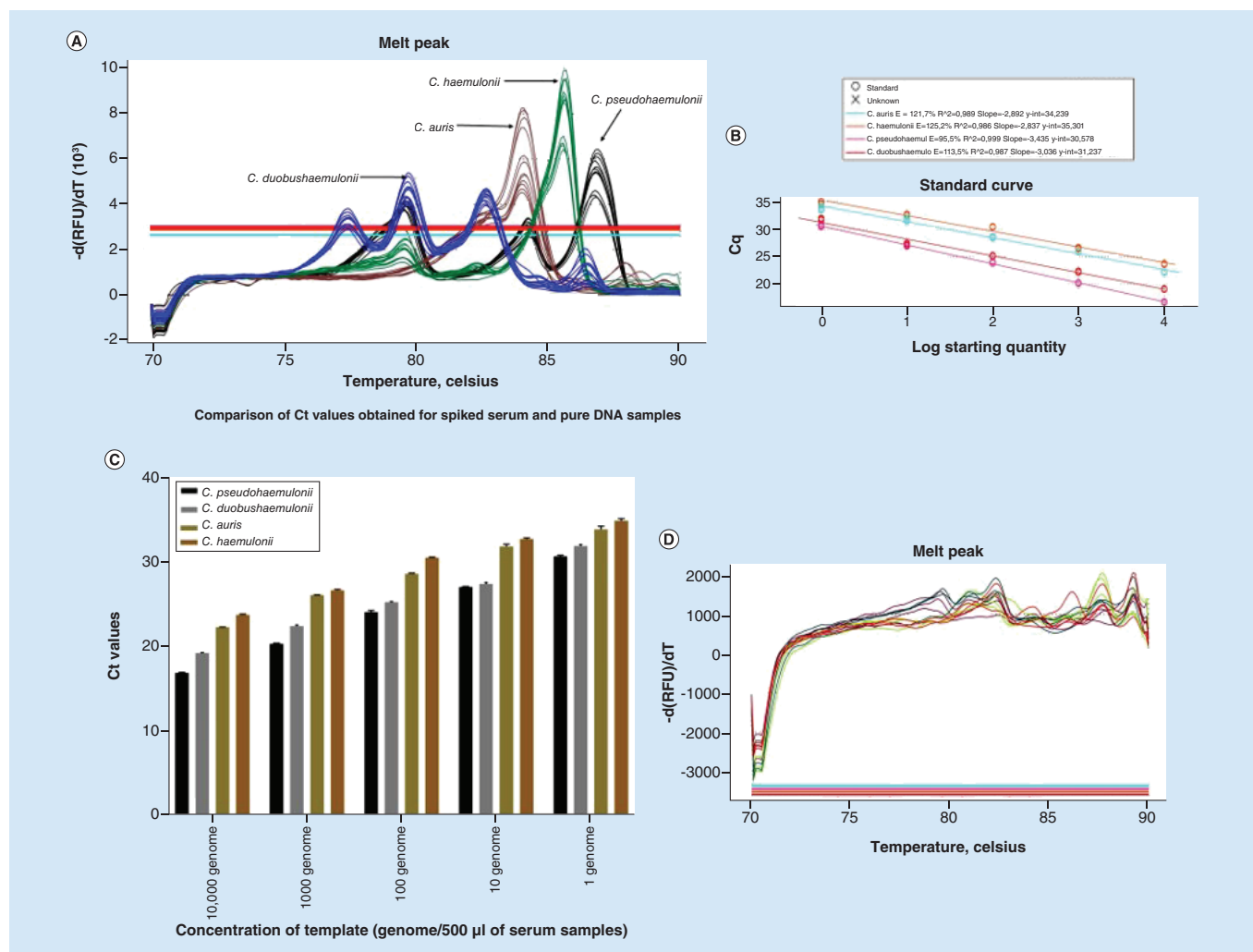
Figure 2.   Analytical information derived from spiked serum samples. (A) Serum samples spiked with five orders of magnitude (10^4–10^0) of the DNA of target species. **(B)** The efficiency of target species derived from spiked serum samples (R^2 >0.98). **(C)** Ct values of spiked serum samples with five order of magnitude of DNA (10^4–10^0) of four target species. **(D)** And, the irregular-shaped melting curves obtained from negative serum samples. The data obtained from the standard curves including efficiency, limit of detection and R2 values are automatically calculated by the software installed on the real-time PCR machine and they were not measured manually.

Spiked serum samples

Due to the lack of serum samples of suspected or proven cases, we proceeded with spiking serum samples with the DNA samples obtained from *C. auris* (CBS 10913), *C. haemulonii* (CBS 5149), *C. duobushaemulonii* (CBS 7798) and *C. pseudohaemulonii* (CBS 10004).

For serum preparation, blood clots were subjected to centrifugation with 3000 × *g* for 15 min. Serum samples were distributed in 500 μl aliquots in DNase/RNase sterile 2 ml tubes and stored at -20°C for future uses. Serum samples were collected from two donors.

Five hundred microliters of serum samples were spiked with serially diluted DNA samples (from 10^4 genome to 10^0 genome) obtained from *C. auris* (CBS10913), *C. haemulonii* (CBS5149), *C. duobushaemulonii* (CBS7798) and *C. pseudohaemulonii* (CBS10004). As, 100 pg of DNA of *C. albicans* estimated to be 10^4 genomes [24] and due to the similarity of genome size of the *C. albicans* (14.6 Mb) with those of *C. auris* (12.36 Mb), *C. haemulonii* (13.6 Mb), *C. duobushaemulonii* (12.58 Mb) and *C. pseudohaemulonii* (12.64 Mb), we considered 1 ng DNA of target species as 10^5 genomes.
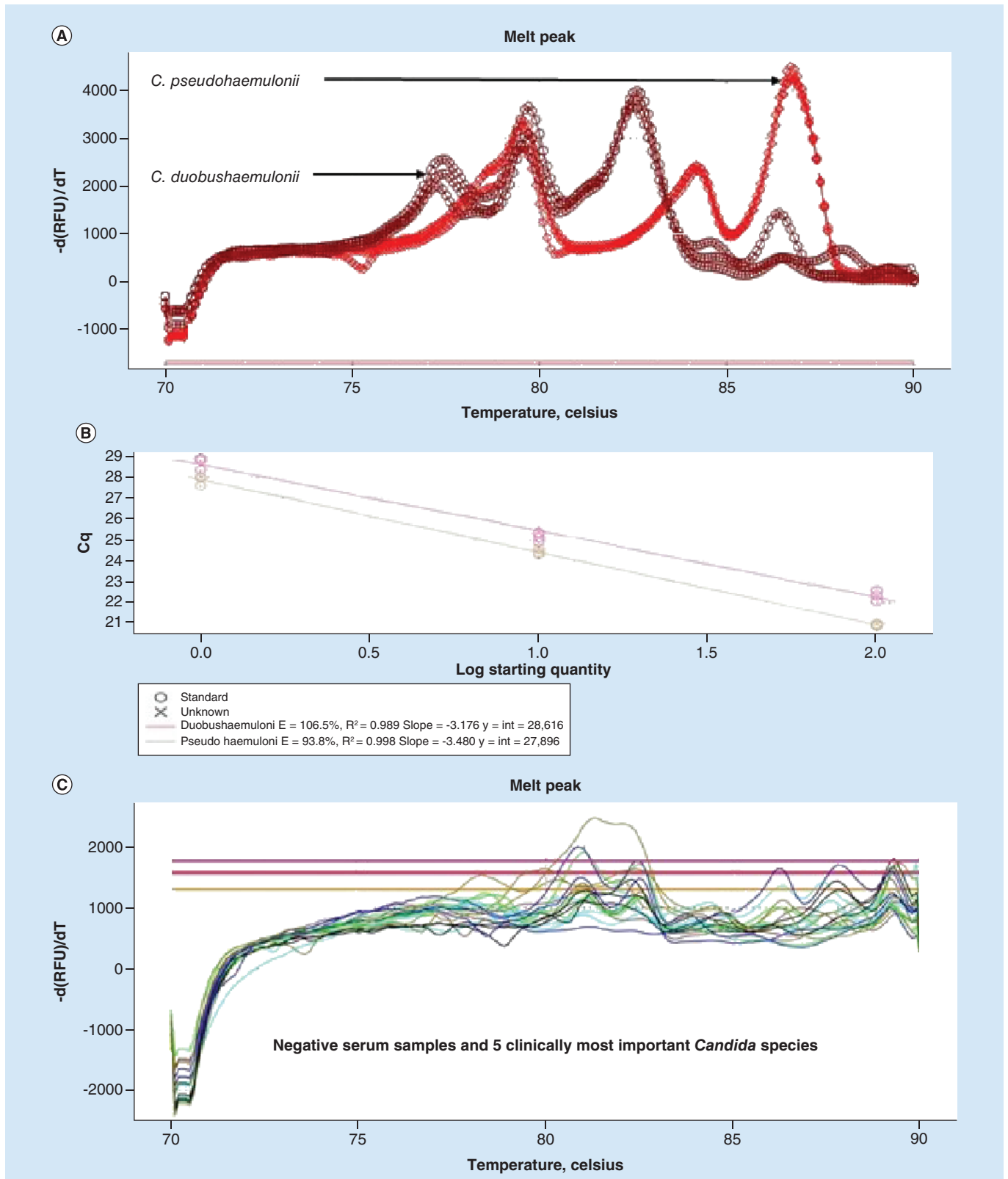
Figure 3.   Melting curve analysis for *Candida duobushaemulonii* and *Candida pseudohaemulonii* (102–100 genomes) and negative samples. Shows the melting curve obtained from spiked serum samples with DNA (10^2–10^0 genomes) of *Candida duobushaemulonii* and *Candida pseudohaemulonii* **(A & B)**, and *Candida albicans, Candida parapsilosis, Candida glabrata, Candida tropicalis, Candida krusei* and negative serum samples **(C)**. As it is evident from the figure, nontarget *Candida* species and negative serum samples **(C)** generated unusual melt curves that are readily distinguishable from those of target species **(A)**. Although, normal standard curves include at least four concentration points, due to the clinical concentration relevance, only three concentration points (1, 10 and 100 genomes) were used.
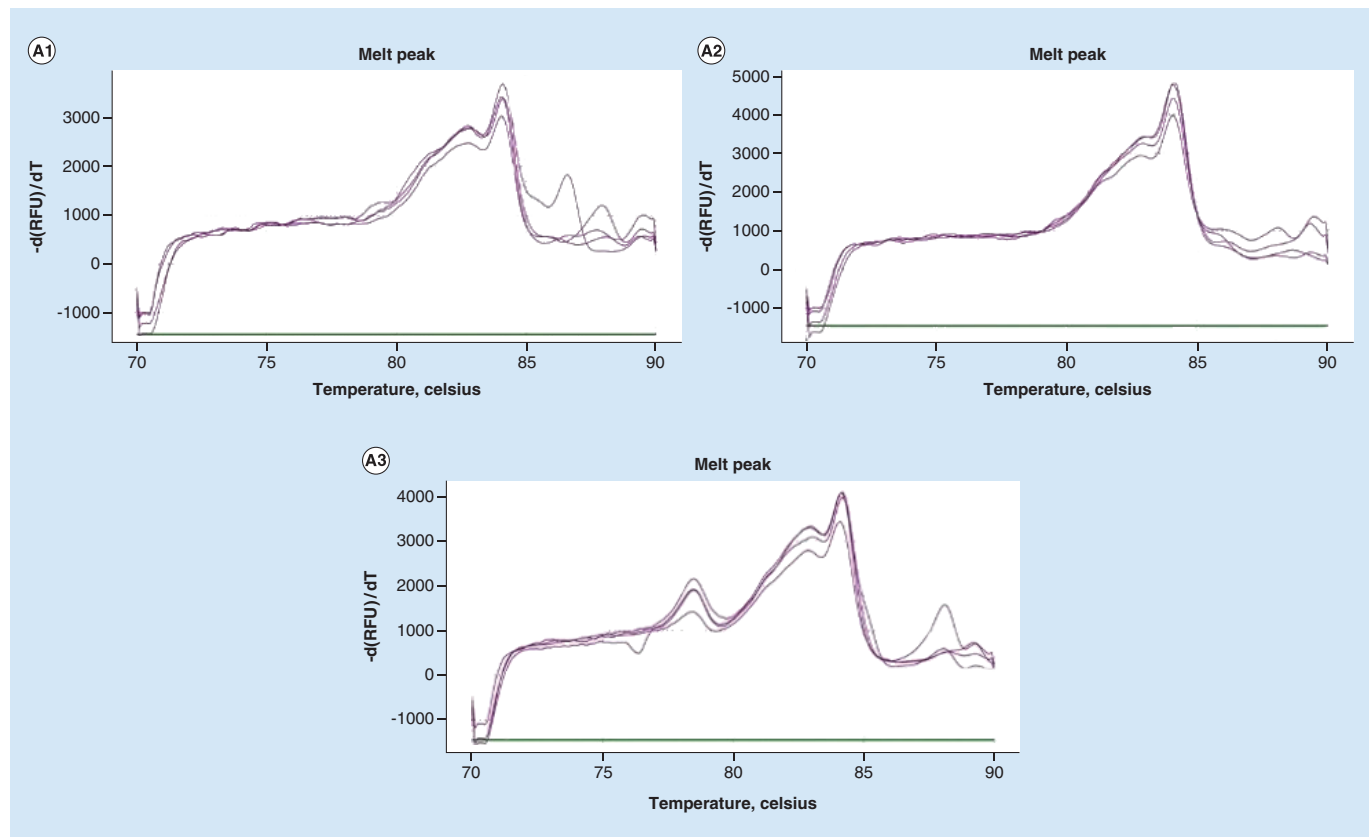
Figure 4.   Melt curves obtained from serum samples spiked with mixture of *Candida albicans* and each target species, individually. (A1) A total of 100 genome (Ge) of *Candida albicans*–10 Ge of *Candida auris*; **(A2)** 100 Ge of *C. auris*–10 Ge of *C. albicans*; **(A3)** 10 Ge of *C. auris*–10 Ge of *C. albi cans*; **(B1)** 100 Ge of *C. albicans*–10 Ge of *Candida duobushaemulonii*; **(B2)** 100 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(B3)** 10 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(C1)** 100 Ge of *C. albicans*–10 Ge of *Candida haemulonii*; **(C2)** 100 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(C3)** 10 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(D1)** 100 Ge of *C. albicans*–10 Ge of *Candida pseudohaemulonii*; **(D2)** 100 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*; **(D3)** 10 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*.

Simulating mixed infection in spiked serum samples containing the DNA of *C. albicans* & target species

As *C. albicans* shown to be the most predominant isolated species in coinfection cases [25], hence DNA sample of each of target species (100 and 10 genomes) were individually mixed with DNA samples of *C. albicans* (100 and 10 genomes as follows, 100 genome of *C. albicans*–10 genome of target species, 100 genome of target species–10 genome of *C. albicans*, 10 genome of *C. albicans*–10 genome of target species). Twelve serum samples (500 µl) were spiked with the DNA mixtures and the serum samples were subjected to DNA extraction as mentioned in the following.

DNA extraction from fungal cells & simulated serum samples

DNA extraction from pure colonies was performed by cetyltrimethyl ammonium bromide (CTAB) method [26]. The obtained DNA samples were checked for purity by NanoDrop™ 2000 (Thermo Fisher Scientific) and for quantity by QuBit dsDNA BR Assay Kit (Thermo Fisher Scientific Corporation). All the DNA samples were standardized to 1 ng/µl for further checking of sensitivity and specificity.

Serum samples were extracted by QIAamp® UltraSens® Virus kit (QIAGEN, Hilden, Germany) with a slight modification of the manufacturer's protocol. In the last step of elution of DNA samples, instead of two continuous elution stages with 30 µl, the DNA samples were eluted by two steps of 25 and 20 µl. In order to monitor the phenomenon of cross contamination during DNA extraction steps, negative serum samples (without DNA of target species) were included. Eight microliters of each extracted DNA samples were run in the qPCR assays in duplicate.
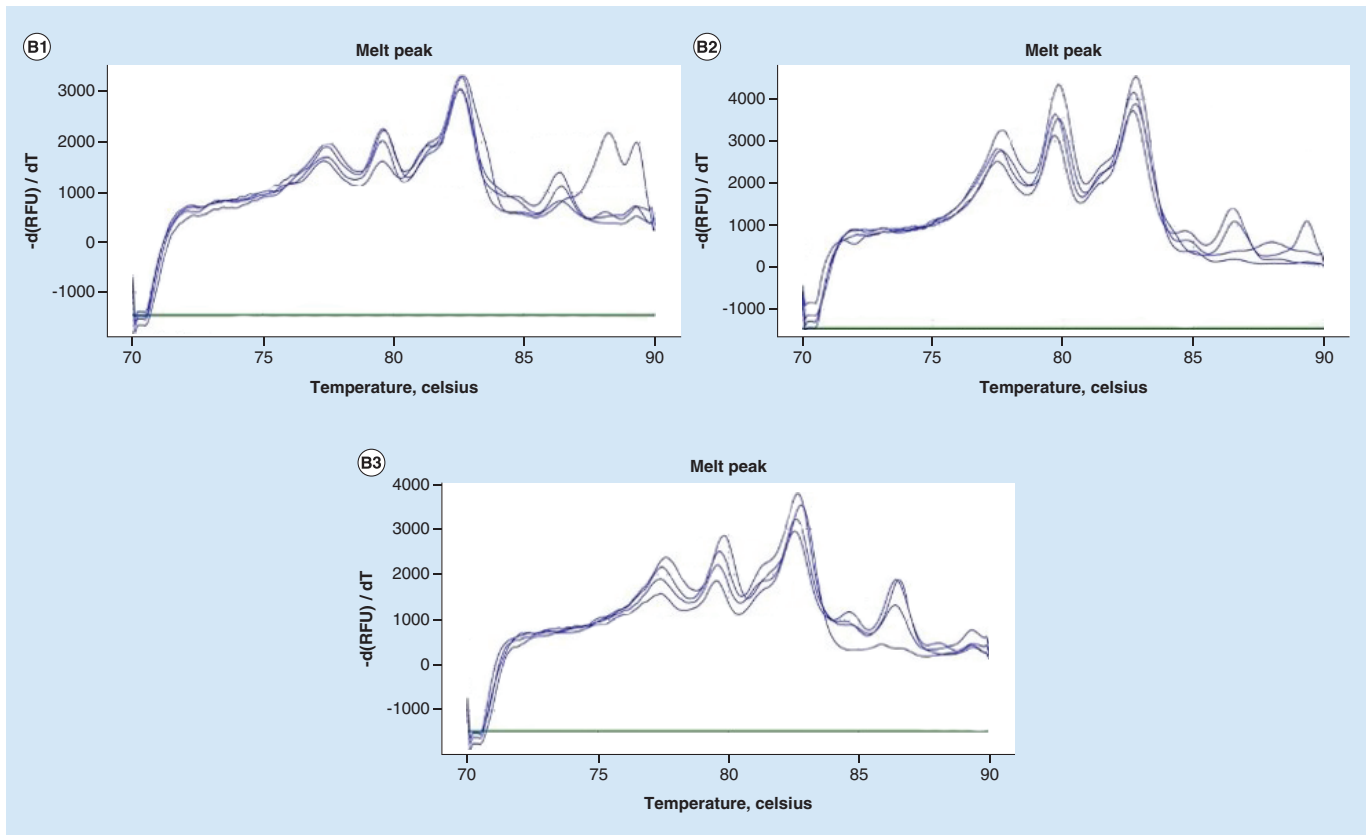
Figure 4.   Melt curves obtained from serum samples spiked with mixture of *Candida albicans* and each target species, individually (cont.). (A1) A total of 100 genome (Ge) of *Candida albicans*–10 Ge of *Candida auris*; **(A2)** 100 Ge of *C. auris*–10 Ge of *C. albicans*; **(A3)** 10 Ge of *C. auris*–10 Ge of *C. albi cans*; **(B1)** 100 Ge of *C. albicans*–10 Ge of *Candida duobushaemulonii*; **(B2)** 100 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(B3)** 10 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(C1)** 100 Ge of *C. albicans*–10 Ge of *Candida haemulonii*; **(C2)** 100 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(C3)** 10 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(D1)** 100 Ge of *C. albicans*–10 Ge of *Candida pseudohaemulonii*; **(D2)** 100 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*; **(D3)** 10 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*.

PCR conditions

As our study had two phases of optimization (Westerdijk Fungal Biodiversity Institute, Netherlands) and assessment of performance with spiked serum samples (Division of Hygiene and Medical Microbiology/Reference Centre for *Aspergillus* and *Aspergillus* Infections, Austria), hence, two kinds of master mixes and qPCR machines were used.

In the optimization phase, both DNA samples and pure colonies were subjected to qPCR assay (Applied Biosystems® 7500 fast, Thermo Fisher Scientific Corporation). The latter was to check whether our assay is able to detect the signal from pure colonies and, hence, skip the tedious DNA extraction procedures. The following PCR conditions were used for the optimization phase: 10 μl of Power Up SYBR Green Master Mix (A25742 Thermo Fisher Scientific Corporation), 10 pM of Auris-F, Auris-R, Pseud-F and Pseud-R, 8 pM for Haemu-F and Haemu-R, and 5 pM for Duobu-F and Duobu-R, 1 μl of DNA template (or 1 mm^3 of pure colony) and Milli-Q water (Merck Millipore, Darmstadt, Germany) to reach the final volume of 20 μl. The PCR program was as following: 50°C for 2 min, 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 64°C for 30 s. Melting curves were obtained from 65 to 95°C with the increment of 0.5°C/s, and data analysis was carried out by 7500 software version 2.3 (Thermo Fisher Scientific Corporation).

As for the DNA of the spiked serum samples, qPCR ingredients included 10 μl of SsoFast™ EvaGreen® Supermix with Low ROX (BioRad, CA, USA), 10 pM of Auris-F, Auris-R, Pseud-F and Pseud-R, 8 pM for Haemu-F and Haemu-R, and 5 pM for Duobu-F and Duobu-R, and 8 μl of DNA samples obtained from spiked serum samples in duplicate. The same PCR program (CFX96, Bio-Rad) used as it was optimized, with the exception of limiting melt curve analysis from 70 to 90°C, and the increment of 0.1°C/s. Obtained data were analyzed by Bio-Rad CFX
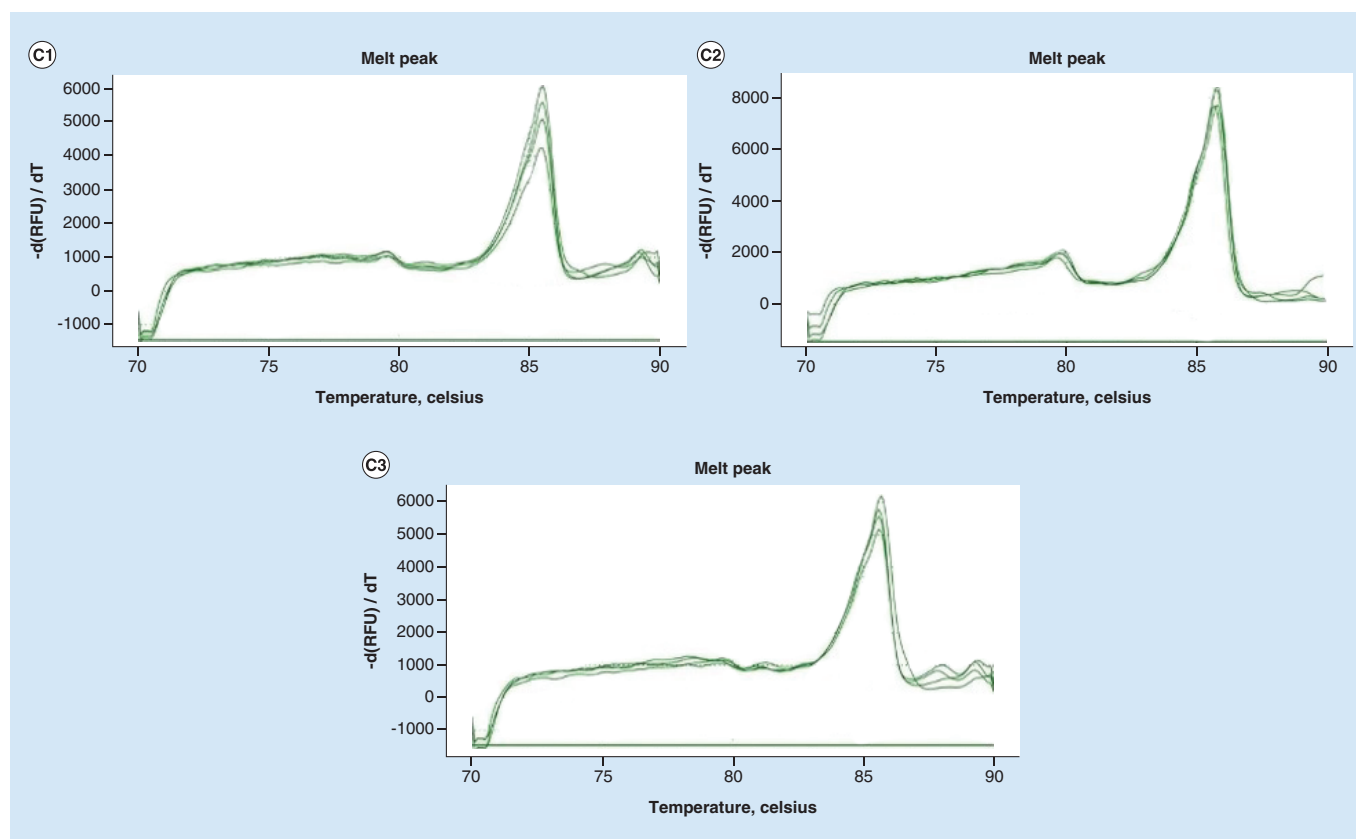
Figure 4.   Melt curves obtained from serum samples spiked with mixture of *Candida albicans* and each target species, individually (cont.). (A1) A total of 100 genome (Ge) of *Candida albicans*–10 Ge of *Candida auris*; **(A2)** 100 Ge of *C. auris*–10 Ge of *C. albicans*; **(A3)** 10 Ge of *C. auris*–10 Ge of *C. albi cans*; **(B1)** 100 Ge of *C. albicans*–10 Ge of *Candida duobushaemulonii*; **(B2)** 100 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(B3)** 10 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(C1)** 100 Ge of *C. albicans*–10 Ge of *Candida haemulonii*; **(C2)** 100 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(C3)** 10 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(D1)** 100 Ge of *C. albicans*–10 Ge of *Candida pseudohaemulonii*; **(D2)** 100 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*; **(D3)** 10 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*.

Manager Version 3.1 (Bio-Rad). Efficiency of utilized primers, costs and avoiding cross reaction were the most important factors ruling the concentration of primers. *Candida auris* as the main target species had the highest primer concentration (10 pM), while the rest of target species due to possessing higher efficiency (8 and 5 pM) required less amount of respective primers.

Sequencing

Sequencing of D1/D2 domain of large subunit of rDNA was performed for all the 283 strains to explicitly approve the identity of each strain. In order to report the identity of each species, obtained sequences were subjected to BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

SPSS (version 25, International Business Machines Corporation, NY, USA) was used for statistical analysis. Results were presented as mean ± standard deviation for normal data or median with interquartile ranges for non-normal data.

Results

Analytical sensitivity, reproducibility & specificity

Based on the melting temperature of the target species, the designed primers unequivocally distinguished *C. auris*, *C. haemulonii*, *C. duobushaemulonii* and *C. pseudohaemulonii*. Melting temperatures for *C. auris*, *C. haemulonii*, *C. duobushaemulonii* and *C. pseudohaemulonii* were 84.10 ± 0.25°C, 85.69 ± 0.21°C, 82.44 ± 0.25°C and
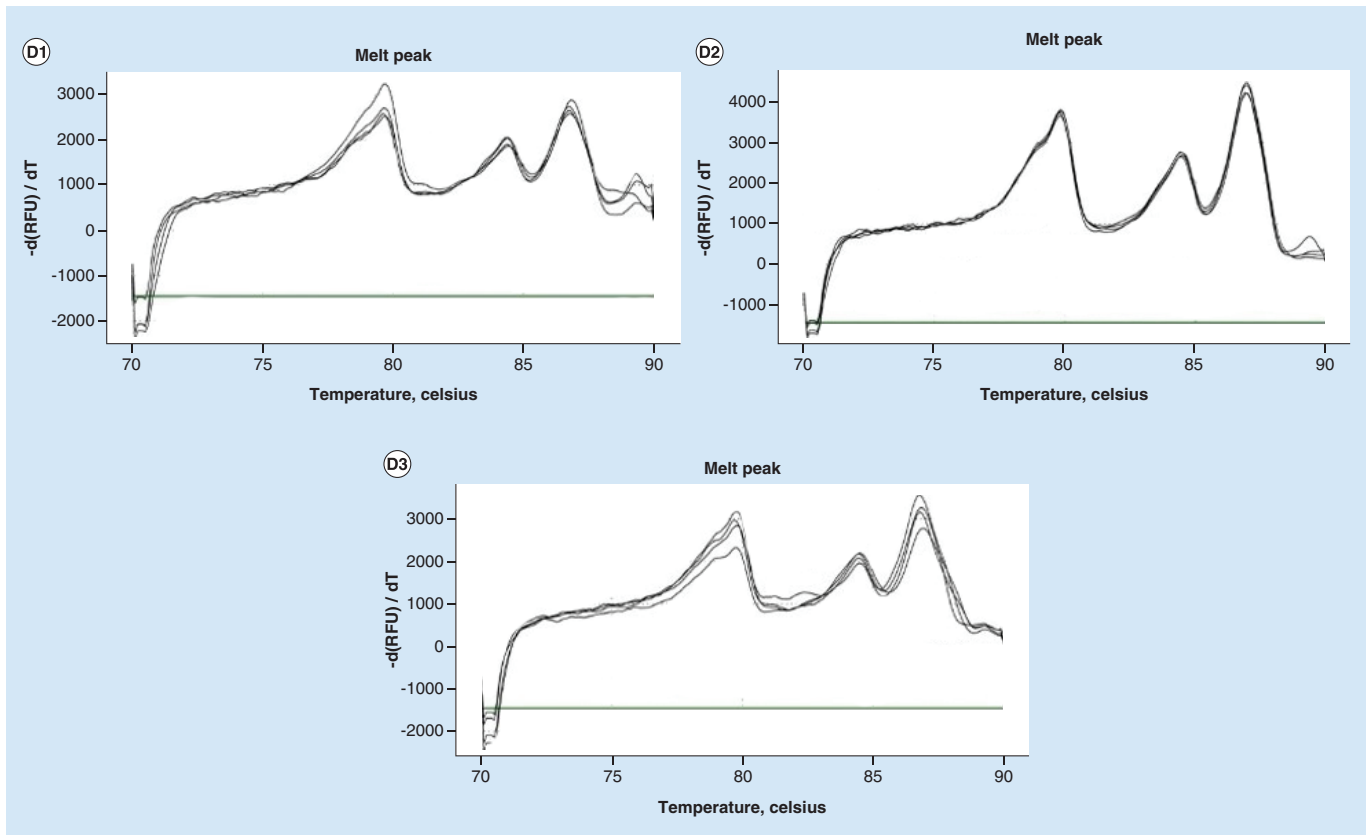
Figure 4. Melt curves obtained from serum samples spiked with mixture of *Candida albicans* and each target species, individually (cont.). (A1) A total of 100 genome (Ge) of *Candida albicans*–10 Ge of *Candida auris*; **(A2)** 100 Ge of *C. auris*–10 Ge of *C. albicans*; **(A3)** 10 Ge of *C. auris*–10 Ge of *C. albi cans*; **(B1)** 100 Ge of *C. albicans*–10 Ge of *Candida duobushaemulonii*; **(B2)** 100 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(B3)** 10 Ge of *C. duobushaemulonii*–10 Ge of *C. albicans*; **(C1)** 100 Ge of *C. albicans*–10 Ge of *Candida haemulonii*; **(C2)** 100 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(C3)** 10 Ge of *C. haemulonii*–10 Ge of *C. albicans*; **(D1)** 100 Ge of *C. albicans*–10 Ge of *Candida pseudohaemulonii*; **(D2)** 100 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*; **(D3)** 10 Ge of *C. pseudohaemulonii*–10 Ge of *C. albicans*.

79.16 ± 0.18°C, respectively (Figure 1A & D, Supplementary Table 4). No difference in melting temperature (p > 0.05) was shown among *C. auris* strains from different countries (Figure 1C). All the target strains were correctly identified except for CBS 12371, which was wrongly designated as *C. pseudohaemulonii*, while our assay in accordance to D1/D2 sequencing and MALDI-TOF MS identified this strain as *C. haemulonii*. When the DNA samples obtained from pure cultures, the LOD for *C. auris*, *C. pseudohaemulonii* and *C. duobushaemulonii* was 10 CFU/PCR with a Cq value of 32, and the LOD for *C. haemulonii* was 10 CFU/PCR with a Cq value of 29. The average of R^2 value for six consecutive runs of qPCR was 0.99, indicating a high degree of reproducibility of this assay (Figure 1B). Specificity tests showed no cross reactivity with the other fungal species and genomic human DNA. Subjecting 105 clinical isolates of *C. auris* from Kuwait to our qPCR gave the same results as those obtained by MALDI-TOF MS and D1/D2 domain of ribosomal large subunit (LSU) rDNA sequencing, resulting in 100% agreement with these methods. Using blinded test set, our qPCR showed 100% accuracy and pure colonies as the pure DNA samples successfully identified target species.

Performance assessment with spiked serum samples

Results obtained from spiked serum samples revealed that our qPCR assay can reliably identify and detect ten genomes of *C. auris* (Cq = 31.75) and one genome of *C. haemulonii* (Cq = 34.82), *C. pseudohaemulonii* (Cq = 34.82) and *C. duobushaemulonii* (Cq = 30.61) (Figure 2A & C), and showed no cross reaction with negative serum samples (Figure 2D). Spiked serum samples with *C. auris* and *C. haemulonii* showed an identical melt curve as what was obtained for the pure DNA of both species (Figure 2A). Unexpectedly *C. duobushaemulonii* and *C. pseudohaemulonii* generated unique triple peaks specific for each species (Figure 2A). Repeated experimentation of

spiking serum samples with *C. pseudohaemulonii* and *C. duobushaemulonii* provided the same results. However, in order to prove that these frequent melting peaks are unique for *C. pseudohaemulonii* and *C. duobushaemulonii*, 500 μl of serum samples were spiked with three orders of magnitude (10^2 genomes to 10^0 genome) DNA of *C. albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis* and *Candida krusei* (Figure 3A & C). As it is shown in the Figure 3, serum samples spiked with the DNA of aforementioned *Candida* species and negative serum samples generated irregular-shaped melt curves, while melt peaks for *C. pseudohaemulonii* and *C. duobushaemulonii* consistently and efficiently resulted in the same melt curve patterns and turned out that those patterns are specific and distinctive fingerprints of these two species (Figure 3A & B). In order to prove that frequent melting peaks were not resulted from serum background, we utilized serum samples from two healthy individuals and both serums resulted in identical and irregular melting peaks. Spiked serum samples with *C. auris*, *C. haemulonii*, *C. pseudohaemulonii* and *C. duobushaemulonii* showed acceptable reproducibility value ($R^2 \geq 0.98$). These observations indicated that serum samples could be a suitable blood fraction for detection and identification of target species when using our multiplex qPCR assay.

In simulated co-infection experiment, serum samples were spiked with DNA mixture of *C. albicans* and each target species individually. Results from the qPCR of simulated co-infection test revealed that regardless of concentration of DNA of *C. albicans* (ten-times more, ten-times less and equal to target species) target species were detected and distinguished unequivocally (Figure 4).

Discussion

The lack of reliable clinical diagnostic tools potentially led to underestimation and neglection of *C. auris* cases [17,27–29]. When facing *C. auris* outbreaks, microbiologists mainly handle cultures, environmental swab or clinical samples (blood, sputum, urine). Using biochemical assays such as VITEK, API20C-AUX, AuxaColor 2, BD Phoenix, MicroScan etc. 90% of the *C. auris* isolates are misidentified as *C. haemulonii* (most commonly), *Candida famata*, *Candida sake*, *Candida lusitaniae*, *Candida catenulata*, *Candida guilliermondii*, *C. parapsilosis*, *C. tropicalis*, *C. albicans*, *Rhodotorula glutinis* and *Saccharomyces cerevisiae* [5].

Detecting *C. auris* directly from clinical or environmental swab samples in a culture-independent manner is essential for outbreak control. For environmental screening purposes, Leach *et al.* (2017) reported the ability of single-plex probe-based qPCR for detection of *C. auris* from environmental swabs [18]. Although, in outbreaks environmental screening is an essential part of infection control, identification of patients suspected with blood-borne infections by *C. auris* and its relatives is of a great importance. Epidemiological studies revealed that *C. auris* can represent up to 5.2% of candidemia cases in India [30], although with a lower prevalence, fatal bloodstream infection due to this pathogen is reported from other countries [9]. Moreover, it is estimated that delay of 1 h in starting immediate appropriate antimicrobial therapy for ICU patients will increase the mortality rate [31]. Thus, the urge for a rapid, specific, reliable and economic molecular means of identification from clinical samples is highly relevant. Herein for the first time, we developed a multiplex PCR assay that can detect *C. auris* and its relatives directly from serum samples in a culture-independent manner. Testing our qPCR with the serum samples spiked with serially diluted DNA of target species, showed an exceptional LOD of one genome for *C. haemulonii*, *C. pseudohaemulonii* and *C. duobushaemulonii*, and ten genomes for *C. auris*. Moreover, serum due to its applicability for downstream applications such as serology tests and its ease of DNA extraction procedure, has earned reputation for being a suitable blood fraction for identification of fungemia [32]. It has been shown that for identification of invasive candidiasis, serum samples showed the same performance as whole blood samples when subjected to real-time PCR [33]. Additionally, serum has been regarded as a suitable matrix for validation of PCR-based assays meant to detect *Candida* species from infected patients [34–36]. The diagnostic strategy using our assay can dramatically shorten the clinical turn-around time (Supplementary Figure 1). Moreover, our qPCR assay utilizes the most basic and inexpensive qPCR chemistry, that is, SYBR GreenI and EVA Green, and offers the first qPCR assay with the potential clinical utility for identification of *C. auris* and its relatives. Moreover, despite the fact that *C. albicans* accounts for the majority of mixed infections, which could affect the specificity and sensitivity of PCR assays [25], our assay regardless of the DNA concentration of *C. albicans* (ten-times more, ten-times less and equal to target species) readily identified *C. auris*, *C. haemulonii*, *C. pseudohaemulonii* and *C. duobushaemulonii* in serum samples spiked the mixture DNA of target species and *C. albicans*. This observation reinforced the specificity of our qPCR assay in mixed infections.

For specificity testing, our assay was challenged with 111 reference strains representing 68 nontarget yeast and filamentous fungal species, while in the published melting curve-based qPCR, only 19 nontarget species were

tested [37]. Large difference in melting temperature between *C. auris* and its relatives is another advantage of our qPCR, as PCR inhibitors, such as humic acid, melanin, hematin and collagen, are commonly found in the environmental and clinical samples and they can change the melting curve temperature causing melt temperature shift followed by misidentification [38]. In our assay, the difference in average melting temperature between *C. auris* and *C. pseudohaemulonii*, *C. duobushaemulonii*, *C. haemulonii* were 5, 1.7 and 1.6°C, respectively. Additionally, the minimum difference in melting temperature between any two species was 1.1°C. While in the published melting curve qPCR, small differences in melting temperature between *C. auris* and *C. duobushaemulonii* and *C. haemulonii* were observed (0.4 and 1°C, respectively) [37]. On the other hand, despite the presence of heterogeneity in the submitted sequences of *C. haemulonii* complex, that is, *C. haemulonii* var. *vulnera* and *C. haemulonii* (*C. haemulonii* Group I), our assay successfully amplified various strains within this complex [39].

Although, a given developed diagnostic assays should be assessed with the relevant clinical materials obtained from suspected patients, in our situation due to the lack of such clinical sources we had to spike serum samples with the DNA of target species. Hence, clinical performance and utility of our qPCR assay warrants further experimentations in settings where suspected patients exist.

In summary, we developed a novel multiplex qPCR, which can discriminate *C. auris*, from *C. haemulonii*, *C. duobushaemulonii* and *C. pseudohaemulonii* in one multiplex qPCR reaction. The fact is that our multiplex assay has been validated by using a comprehensive set of reference strains and clinical isolates, and proved to be rapid, specific and sensitive with spiked serum samples, indicating that it might have the applications for environmental screening and diagnostic purposes from clinical samples.

Conclusion

Culture-independent and species-level identification of the multidrug-resistant opportunistic yeast species *Candida auris, C. haemulonii, C. pseudohaemulonii* and *C. duobushaemulonii* is an important clinical practice. Involved healthcare settings of various countries can evaluate the specificity and sensitivity our multiplex qPCR assay in the light of culture as the gold standard technique. If proved to be sensitive and specific, it will show implications in early diagnosis followed by adminstartion of appropriate antifungal drugs. Moreover, its inexpensiveness, rapidity and sensitivity might highlight its applicability for environmental screening.

Summary points

- Based on the simple melt curve technique, our multiplex qPCR can distinguish and identify *Candida auris*, and its relatives, *Candida haemulonii*, *Candida duobushaemulonii* and *Candida pseudohaemulonii*.
- When DNAs obtained from pure colonies were used, the limit of detection for *C. auris, C. haemulonii, C. pseudohaemulonii* and *C. duobushaemulonii* was 10 CFU/PCR, with a high degree of reproducibility (R^2 = 0.99).
- Specificity tests showed no cross reactivity with the other fungal species and genomic human DNA.
- Subjecting pure colonies of target strains showed the expected melting temperature, which indicated good compatibility of our assay when using pure colonies without DNA extraction.
- Results obtained from spiked serum samples revealed that our qPCR assay reliably identifies and detects ten genomes of *C. auris* (Ct = 31.75) and one genome of *C. haemulonii* (Ct = 34.82), *C. pseudohaemulonii* (Ct = 34.82) and *C. duobushaemulonii* (Ct = 30.61).
- Spiked serum samples with both *C. auris* and *C. haemulonii* showed acceptable reproducibility value ($R^2 \geq 0.98\%$).
- Simulated co-infection test revealed that regardless of concentration of DNA of *C. albicans*, target species are identified unequivocally.
- Having a high degree of sensitivity and reasonable difference in the melt profile of target species reveals the potential utility of our qPCR assay for both serum samples derived from suspected patients and environmental swab samples for infection control purposes.

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