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Optimizing a real-time PCR assay for rapid detection of *Candida auris* in nasal and axillary/groin samples

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Abstract

Introduction. *Candida auris* is an emerging fungal pathogen. The organism can cause invasive infections associated with high mortality, has been implicated in outbreaks in healthcare settings and is frequently resistant to multiple antifungal agents, making it a significant challenge to infection prevention and patient treatment.

Aim. To implement a real-time PCR assay for detection of *C. auris* in patient surveillance samples collected with the Copan Liquid Amies elution swab (ESwab) collection and transport system.

Methodology. We optimized a real-time PCR testing procedure based on the sample collection device used in our institution.

Results. ESwab transport medium was strongly inhibitory to the real-time PCR. Removing the medium with centrifugation, followed by suspending the pellet in PBS-BSA buffer (concentration 1%), sufficiently eliminated the inhibition. The manual sample preparation method, freeze–thaw followed by mechanical disruption, allowed the detection of *C. auris* at the lowest cell concentration.

Conclusion. The optimized procedure was used to test 1414 patient surveillance samples. The real-time PCR detected all culture-positive samples with 100% sensitivity and 100% specificity.

INTRODUCTION

Candida auris is an emerging fungal pathogen. Since it was first recovered from an external ear canal drainage specimen from a Japanese patient in 2009, it has spread to many countries on five continents. The organism can cause invasive infections associated with high mortality, has been implicated in outbreaks in healthcare settings and is frequently resistant to multiple antifungal agents, making it a significant challenge to infection prevention and patient treatment [1].

Following the release of a *Clinical Alert to U.S. Healthcare Facilities* in 2016 asking laboratories and healthcare workers to monitor and report any cases of *C. auris* to the Centers for Disease Control and Prevention (CDC), *C. auris* guidance for clinicians and infection control personnel was issued [2]. Investigation of the first *C. auris* outbreak in a hospital showed that implementation of enhanced infection control measures, including isolation of all *C. auris* patients, creating patient cohorts, cohorting of healthcare personnel and ceasing new admissions to affected rooms, was necessary to limit the transmission [3]. Subsequently, guidance was released in the UK, the USA, Europe and South Africa recommending contact precaution isolation of patients, and thorough cleaning of shared equipment and the environments of affected patients. Accurately and quickly identifying patients with *C. auris* colonization and infection is critical to contain and prevent the spread of the organism.

Risk factors for a patient acquiring *C. auris* include contact with *C. auris*-positive patients or their environment [4]. The minimum contact period of 4h with a positive case or a contaminated environment may be sufficient for the acquisition of *C. auris* [3]. Investigation of *C. auris* hospital outbreaks revealed that patients infected with *C. auris* harboured the organism at multiple body sites, including nares, groin, axilla and rectum 1 to 3 months after initial detection of *C. auris*,

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Abbreviations: CDC, Centers for Disease Control and Prevention; FDA, Food and Drug Adminstration; ITS, internal transcribed spacer.

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and persistence of the organism in the patient room could be detected 3 months after patient discharge [5]. Nares, axilla and groin were identified as high-yield body sites for identifying colonized patients [6]. Assays for *C. auris* surveillance need to work with a common sample transportation medium for patient and environmental sampling.

Leach *et al*. developed a real-time PCR assay for the rapid detection of *C. auris* from surveillance samples [7]. During the process of implementing the assay for surveillance swabs collected with the Copan Liquid Amies elution swab (ESwab) collection and transport system, we found that the real-time PCR was strongly inhibited by the ESwab Liquid Amies. As the ESwab is the most commonly used specimen collection device, optimizing the real-time PCR assay for ESwab use will allow laboratories to adopt the real-time PCR assay. In this study, we tested several sample processing procedures and identified the optimal testing conditions.

METHODS

Strains

Fifty-two *Candida* control strains were obtained from the CDC and U.S. Food and Drug Adminstration (FDA) Antibiotic Resistance Isolate Bank. The strains included Antibiotic Resistance Isolate Bank numbers 0314 to 0345 and 0381 to 0400. Strain 0381 was used for assay optimization and testing for the lowest detection level.

Samples used for assay optimization

A 0.5 McFarland cell suspension was prepared from *C. auris* growth on an inhibitory mould agar plate. Serial dilutions of the cell suspension with the concentrations of 10, 10^2, 10^3, 10^4 and 10^5 c.f.u. ml^{-1} were prepared in ESwab Liquid Amies. One aliquot of 200 µl of each dilution was used for nucleic acid preparation, while another 100 µl aliquot was plated on an inhibitory mould agar plate and incubated for 14 days for colony counting. The colony counts were compared to the C_t value generated by real-time PCR in order to evaluate the DNA isolation yield. The procedure that resulted in the detection of the lowest organism concentration was selected for further evaluation. The C_t value cut-off for a positive result was determined based on the C_t value of the PCR that produced the lowest level of detection. To confirm the specificity of the *C. auris* real-time PCR, 52 yeast strains from the CDC and FDA Antibiotic Resistance Isolate Bank were cultured on inhibitory mould agar plates. Cell suspension with a concentration of 10^4 c.f.u. ml^{-1} was prepared with ESwab Liquid Amies and tested with real-time PCR.

Sample processing optimization

Nucleic acid samples were prepared using Qiagen QIAamp kit, Qiagen QIAsymphony tissue kit and manual freeze/thaw/homogenization. DNA isolation with kits followed the package instructions. Manual wash/freeze/thaw/homogenization included the following steps: (i) 200 µl cell suspension was transferred into a 1.5 microcentrifuge tube and then centrifuged for 10 min at 15000 r.p.m.; (ii) the entire 200 µl supernatant was removed and the pellet was re-suspended in 100 µl PBS-BSA buffer (concentration 1 %) (Millipore Sigma), then the suspension was transferred into to a 2 ml screw cap tube containing glass beads; (iii) the suspension was frozen at −80 °C for 30 min, and then incubated at 70 °C in a water bath for 30 min; (iv) the suspension was homogenized for 20 s twice with 10 s intervals using a Precellys 24 homogenizer (Bertin Instruments); (v) the homogenized cell suspension was centrifuged for 10 min at 15000 r.p.m.; (vi) the supernatant was carefully transferred to a fresh tube. Manual freeze/thaw/homogenization without the step (ii) washing step was also tested for comparison.

C. auris real-time PCR assay

The real-time PCR assay was developed based on the method reported by Leach *et al*., with the following modifications: (i) primers and probe targeting the human β-globin gene were used for the internal control; (ii) a QuantStudio 6 Flex instrument (Thermo Fisher Scientific) was used for PCR with 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A 5 µl aliquot of 100 µl nucleic acid extraction was used for the amplification reaction [7]. Quantabio PerfeCTa qPCR ToughMix low ROX was used for the PCR amplification. When testing patient surveillance samples, primers and probe that target *Homo sapiens* β-globin chain (GenBank accession no. AY260740.1) 5′-GGTTGGGA TAAGGCTGGATTATT-3′, 5′-CAGGAGCTGTGGGAGGAAGA-3′ and 5′-JOE/ZEN-CAAGCTAGGCCCTTTT GCTAATCATGTTCA-Iowa Black FQ-3′ were used as the internal control [8].

Testing of patient samples

The performance characteristics of the finalized assay procedure were established by testing patient samples collected with ESwabs (Copan) prospectively from October 2019 and December 2019. Two ESwabs, a nares swab and a combined axilla/groin swab, were collected from each patient. The samples were tested with the real-time PCR and culture simultaneously. Fungal culture was performed by plating on an inhibitory mould agar plate and incubating for 14 days at 30 °C. The accuracy, sensitivity and specificity of the assay were calculated using the culture result as the gold standard. Yeast isolates recovered by culture were identified with a Vitek 2 instrument with software v8.0. *C. auris* identification was confirmed with internal transcribed spacer (ITS) sequencing.

ITS sequencing

Nucleic acid extraction from cell suspension was performed with the QIAamp DNA mini kit (Qiagen), following the manufacturer’s instructions. PCR amplification of the ITS region was performed with primers ITS1 5′-TCCGTAGGTGAACCTGCGG- 3′ and ITS4 5′-TCCTCCGCTTATGATATGC- 3′, as described previously [9]. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced using a BigDye kit (Thermo Fisher Scientific) and an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific). Identification was obtained by BLAST search against GenBank.

Table 1. Comparison of three nucleic acid extraction methods.

Cell count per PCR (c.f.u.)†	*C. auris* real-time PCR with different extraction methods*			
	Manual freeze/thaw/homogenization (C_t)	Manual wash /freeze/thaw/homogenization (C_t)‡	QIAamp kit (C_t)	QIAsymphony tissue kit (C_t)
587	–	29.1	38.0	39.8
61	–	33.5	–	–
6	–	39.8	–	–
1	–	40.5	–	–
<1	–	–	–	–
NP				

NP, Negative control.
*– indicates that the real-time PCR failed.
†The mean colony count from culture of each dilution in triplicate.
‡Wash refers to the step to remove inhibitory Amies medium with centrifugation followed by suspending the pellet in PBS-BSA buffer with glass beads.

RESULTS

Method optimization

C. auris cells suspended in ESwab Liquid Amies were used to test different sample preparation methods. The intent of the procedure was to break *C. auris* cells and release nucleic acid. Using the samples prepared with the procedure for the PCR failed to produce any amplification signal, indicating that the PCR was strongly inhibited by the ESwab Liquid Amies (Table 1). To remove the inhibition, cells were pelleted by centrifugation and re-suspended in PBS-BSA buffer. After using this washing step, the manual method allowed the real-time PCR to detect as low as one cell per reaction (Table 1).

The lowest PCR detection level was determined with samples prepared using three sample preparation methods, the manual method, the Qiagen QIAamp kit and the QIAsymphony tissue kit. While the PCR detected 578 cells in the samples prepared with the QIAamp kit or with the QIAsymphony tissue kit, the samples prepared with the manual method allowed the PCR to detect as low as 1 cell per reaction (Table 1).

The C_t value of the PCR that produced the lowest level of detection was 39.8. The sample with a C_t value of 40 failed to produce a positive culture (Table 1). Based on the results, a C_t <38 was classified as a positive reaction, a C_t of 39 required repeat testing and a C_t >40 was classified as a negative reaction. If repeat PCR produced a C_t value less or equal to 39, the sample was classified as *C. auris* positive. If the C_t value of the repeat PCR was greater or equal to 40, the sample was classified as *C. auris* negative.

The optimal sample preparation procedure was used to test 52 yeast strains from the CDC and FDA Antibiotic Resistance Isolate Bank. All non-*C. auris* isolates tested negative by the *C. auris* real-time PCR. Ten *C. auris* strains tested positive by the *C. auris* PCR. Two strains, classified as *C. auris* (CDC reference numbers 0391 and 0392) in the Antibiotic Resistance Isolate Bank were tested negative by *C. auris* real-time PCR (Table 2). Sequencing of the nuclear ribosomal ITS region identified the two strains as *Candida duobushaemulonii*.

Real-time PCR testing of patient surveillance samples

Nasal swab and combined axilla/groin swab were collected to identify patients with *C. auris* colonization. Real-time PCR and fungal cultures were performed simultaneously using the same sample. A total of 1414 samples were tested. Of these, 38 samples tested positive by PCR, while 1376 samples were negative by PCR. Of the 38 PCR-positive samples, 30 samples (15 nasal swabs and 15 combined axilla/groin swabs) were from 15 patients. Among the remaining PCR-positive samples, three were nasal swabs and five were axilla/groin swabs (Fig. 1). Six patients had both nares and axilla/groin

Table 2. *C. auris* PCR of control strains

Two strains were identified as *C. duobushaemulonii* with ITS sequencing.

Organism	CDC reference no.	*C. auris* PCR
Candida glabrata	314–334	Negative
Candida parapsilosis	335–344	Negative
Candida tropicalis	345	Negative
Candida auris	381–390	Positive
Candida auris	391–392	Negative
Candida haemulonii	393, 395	Negative
Candida duobushaemulonii	394	Negative
Kodamaea ohmeri	396	Negative
Candida krusei	397	Negative
Candida lusitaniae	398	Negative
Saccharomyces cerevisiae	399, 400	Negative

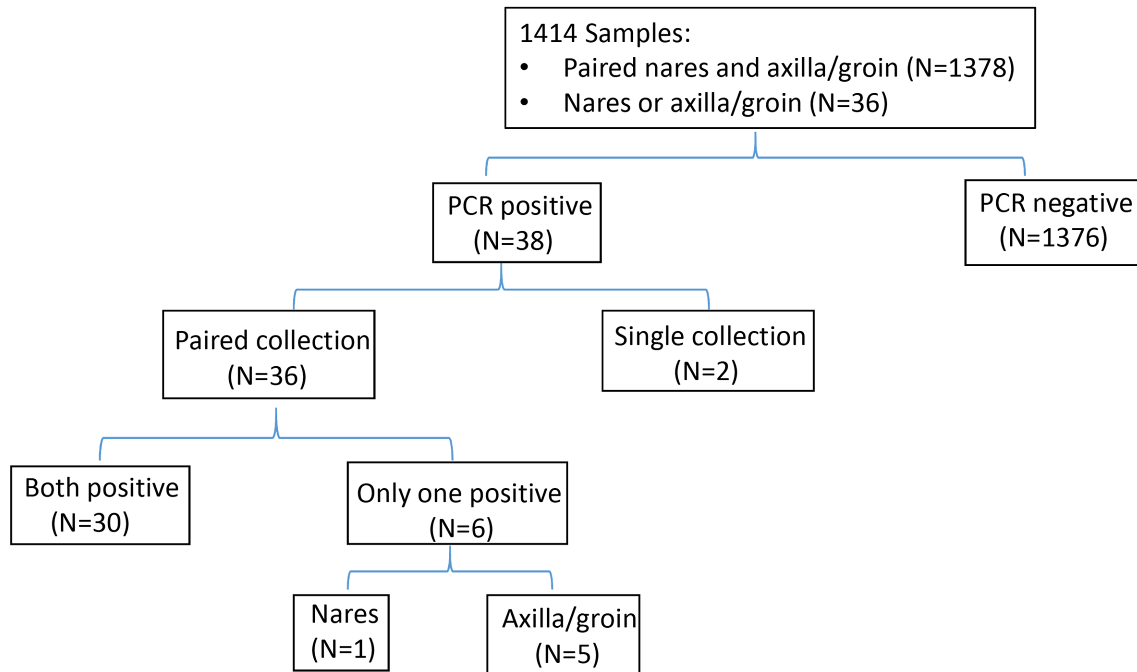

Fig. 1. Real-time PCR testing of patient surveillance samples.

swabs collected, but only one of the samples tested positive (Fig. 1).

Comparison of *C. auris* detection by real-time PCR and culture

C. auris was recovered from all the real-time PCR-positive samples. Cultures of PCR-negative samples were negative for *C. auris*. Using culture as the gold standard, the *C. auris* real-time PCR had 100% accuracy, and 100% sensitivity and specificity (Table 3).

Initial culture of six samples where *C. auris* PCR was positive, one nasal swab and five axilla/groin swabs, failed to recover *C. auris* (Table 3). Of the six cultures, one was negative for growth and five grew *Candida* species other than *C. auris* (Table 4). Repeat cultures were performed for the six samples, and identification with Vitek 2 analysis was performed on multiple colonies from each plate. *C. auris* was detected in the repeat cultures of all six samples (Table 4). A study limitation is that repeat culture was not performed to confirm the other PCR-negative samples.

DISCUSSION

The emergence of *C. auris* raises serious concerns for public health. Rapidly identifying patients with *C. auris* colonization in healthcare facilities is critical for implementation of proper infection-control measures to contain the spread. Swabs of nares, axilla and groin were identified as the high-yield body sites for identifying *C. auris*-colonized patients [6]. A real-time PCR assay testing for *C. auris* in patient surveillance samples has been reported. To implement the assay in our institution, we optimized the procedure based on the sample collection device used in our institution. Our study showed

Table 3. Comparison of *C. auris* detection by PCR and culture for surveillance samples

Sample	PCR positive		PCR negative	
	Culture positive for *C. auris**	Culture negative for *C. auris*	Culture positive for *C. auris*	Culture negative for *C. auris*
Nares	18 (1)	0	0	695
Axilla/groin	20 (5)	0	0	674
Total	38 (6)	0	0	1369

*The number in brackets indicates the number of samples that tested positive by repeat testing.

Table 4. Investigation of the samples with discrepant PCR and culture results

Sample	PCR	Initial culture result	Repeat culture result
Nares	+	*C. albicans*	*C. albicans, C. auris*
Axilla/groin	+	*C. albicans*	*C. albicans, C. auris*
Axilla/groin	+	No growth	*C. auris*
Axilla/groin	+	*C. glabrata*	*C. glabrata, C. auris*
Axilla/groin	+	*C. glabrata*	*C. glabrata, C. auris*
Axilla/groin	+	*C. tropicalis*	*C. tropicalis, C. auris*

that ESwab transport medium posed strong inhibition to the real-time PCR . Removing the medium with centrifugation, followed by suspending the pellet in PBS-BSA buffer, sufficiently eliminated the inhibition. We further compared three sample preparation methods and showed that the manual method allowed for the detection of *C. auris* at the lowest cell concentration. Using the established procedure to test patient surveillance samples and comparing the PCR results with culture results demonstrated the procedure had 100% sensitivity and specificity.

Rapid detection of micro-organisms with PCR relies on effective DNA extraction methods [10]. As yeast have cell walls composed of chitin, mannan and glucan, enzymatic or mechanical pre-treatments are necessary for nucleic acid preparation [11, 12]. We attempted to use proteinase K to break up yeast cells, followed by a silica-based purification of genomic DNA procedure. Compared to heat and mechanical disruption, proteinase K appeared to be less effective for DNA recovery. The lowest level of real-time PCR detection with samples prepared with heating and mechanical treatment was 600 times lower than that with proteinase K-treated samples. Methods based on the use of enzymes other than proteinase K, such as lysozyme, zymolyase and lyticase, for cell-wall destruction were reported [13–15]. Better DNA recovery after lyticase treatment was reported by Karakousis *et al.* [16]. Results from the study by Lech *et al.* showed that double-digestion with two enzymes, lyticase and proteinase K, improved the DNA isolation procedure and shortened the time of incubation with various enzymes [17]. Therefore, for enzymatic cell disruption, the use of two enzymes may be required to achieve an optimal result. Physical methods of cell lysis, such as boiling a cell suspension in a water bath for 15 min followed by mechanical disruption with glass beads, have been successfully used for sample preparation for PCR-based detection of *Candida* spp. in clinical sample [18]. Employing freeze–thaw as a physical method for cell disruption was later shown to be more effect than boiling in releasing DNA from the cell interior [19]. Our results showed that freeze–thaw followed by mechanical disruption was effective to release DNA from *C. auris* cells.

The ESwab is a widely used liquid-based, multipurpose collection and preservation system that maintains viability of aerobic, anaerobic and fastidious bacteria as well as *Candida* species for up to 48 h at refrigerator and room temperature. The ESwab system consists of a flocked swab and a screw-capped transport tube containing 1 ml Liquid Amies medium. The design ensures that the sample rapidly and completely elutes from the flocked swab as soon as it is placed in the transport medium, providing the lab aliquots of liquid sample suspension that can be used to run multiple tests from a single specimen collected. Samples collected in ESwabs are suitable for automation, Gram staining, traditional culture and molecular assays.

Amies transport medium contains sodium chloride at a concentration of 0.3% for optimal preservation of *Neisseria gonorrhoeae*. Potassium, calcium and magnesium salts serve to maintain osmotic equilibrium by controlling the permeability of cells. Phosphate buffer maintains the pH of the medium. Sodium thioglycolate and agar provide a reduced environment favourable to a variety of bacterial and fungal species. An inhibitory effect of Amies transport medium was noted by Gibb and Wong, who observed inhibition of PCR in throat swabs submitted in routine bacteriological transport medium. Further investigation showed that agar was the inhibitory agent [20]. Our result was consistent with their discovery. PCR amplification was completely inhibited when nucleic acid was released in Amies medium by heating and mechanical disruption. We showed that removing the Amies medium, with centrifugation followed by suspending the pellet in PBS-BSA buffer, was critical to avoid the inhibition problem.

Two isolates classified as *C. auris* in the CDC and FDA Antibiotic Resistance Isolate Bank, CDC reference numbers 0391 and 0392, tested negative by the *C. auris* PCR. Sequencing of the ITS region of the rRNA gene identified the isolates as *C. duobushaemulonii*, confirming the PCR results. *C. duobushaemulonii* belongs to the *Candida haemulonii* species complex. Members of the complex consist of two genotypically distinguishable species, *C. haemulonii* and *C. duobushaemulonii*, and a variety, *C. haemulonii* var. *vulnera* [21]. These species and other phylogenetically close relatives of *C. haemulonii*, *C. auris* and *C. pseudohaemulonii* cannot be reliably differentiated by the commercial yeast identification methods used in microbiology laboratories. Misidentifying *C. auris* as *C. duobushaemulonii* when using traditional biochemical methods has been reported [22]. Our results raise concerns of possible misidentification of the strains in the collection in the CDC and FDA Antibiotic Resistance Isolate Bank, and suggested that confirmation of identification may be necessary when using those strains.

Nares, axilla and groin are sites that are frequently colonized by *C. auris* [3], with the axilla found to be the most-frequently colonized site [23]. Our results demonstrated that *C. auris* could be detected in multiple body sites of some colonized patients; however, for some patients the organism was detected at only one site. Whether this was caused by a sampling error or was a result of colonization preference requires further investigation. Our results indicated that sampling one body site would miss some *C. auris*-colonized patients.

In summary, our study has shown that ESwab transport medium was strongly inhibitory to real-time PCR. However, the inhibition was eliminated with a single centrifugation. Compared to nucleic acid extraction with Qiagen kits, the manual freeze/thaw/homogenization method allowed the detection of *C. auris* at the lowest cell concentration. The optimized procedure was used to test 1414 patient surveillance samples. The real-time PCR detected all culture-positive samples with 100% sensitivity and specificity.

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Author contributions

M.M. and C.Q.: conceptualization, methodology, investigation, resources, data curation and writing – original draft preparation. N.D., S.R., J.R., L.D. and J.A.Z.: formal analysis. A.Z.: writing – review and editing.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Samples used for this study were residual clinical specimens not specifically collected for study purposes. Exemption was granted for patient consent. The human study protocol was approved by the institution review board of Northwestern University. Consent was obtained from participants for publication.

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