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New MSPQC-PLS method for the early clinic identification of commonly encountered *Candida* species

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ABSTRACT

The early clinic identification of commonly encountered *Candida* species became more important with the increasing human candidiasis. In this paper, a new MSPQC-PLS (multi-channel series piezoelectric quartz crystal biosensor combined with partial least square) method was proposed for early identification of the most frequent *Candida* species encountered in human pathology. This method was based on these fact that (1) MSPQC method is a real-time monitoring method based on the sensitive frequency response to the change of electric parameters of the culture media caused by the growth of microorganisms; (2) various *Candida* species produce significantly different types of frequency curves in 1 or 2 days' culture period; (3) this difference can be identified by the partial least square technique. Using the proposed method, three species (*Candida albicans*, *Candida glabrata*, and *Candida tropicalis*) from a collection of 53 clinical strains of *Candida*, isolated from hospitalized patients, were identified with a classification rate of 98.1%. New proposed MSPQC-PLS method is simple, rapid and convenient to perform. It can identify clinical *Candida* species directly without passing through pure culture process. This will save identification time greatly. It could be popularized in clinical microbiology laboratories.

1. Introduction

Nosocomial candidiasis has become an important cause of morbidity and mortality [1–3]. Invasive candidiasis is the predominant fungal infection in the intensive care unit setting, neutropenic patients and post-surgery units [4–6]. *Candida* species Account for 70–80% of invasive bloodstream fungal infections, and they represent the fourth most common nosocomial bloodstream infection [7–9]. Among the *Candida* species causing invasive infections, *Candida albicans* is by far the species most commonly isolated, causing up to two-thirds of all cases of invasive candidiasis. *C. albicans*, *Candida tropicalis*, and *Candida glabrata* account for about 80% of fungal isolates encountered in the clinical laboratory [1,10]. Hence early identification of these commonly encountered *Candida* species is very important for doctor to do optimal antifungal therapy and patient management.

Commercially available identification systems used in routine were automatic identification system, such as ID32C of VITEK2, manual identification system like API *Candida*. These identification system were based on the identification of physiological and nutritional (sugar assimilation and/or fermentation, enzymatic tests) characteristics of pure yeast colonies which isolated from the raw clinical samples. So it usually need up to 5 days between the receipt of patient material and presentation of identification results to the clinician [11]. It is more serious for blood samples, because several days will be taken for positive reporting.

Some novel approaches for the rapid identification of clinically relevant microorganisms, including genotypic techniques base on PCR and spectroscopic techniques combined with chemometrics, were also performed on isolated pure colonies [12–17]. These techniques especially genotypic techniques showed some serious drawbacks, such as sensitivity to contamination and mutations, complicated protocol, reagent costs, etc., and are not up to now applied in routine.

MSPQC is a bioelectrochemical device based on the series piezoelectric quartz crystal (SPQC) sensor [18,19]. It attracted the attention of many analysts because of its sensitive response, low cost and easy operation. MSPQC system, which composed of eight single channels SPQC system, has been proposed to detect pathogens in clinical samples, to test susceptibility of clinical *Escherichia coli* isolates against ampicillin [20,21].

Chemometrics is the technique for extracting useful information from detected data. In this paper, MSPQC combined with PLS method was proposed for early identification of *Candida* species without preisolation of pure colonies from raw clinical species. This method was based on the fact that fingerprints response curve of *Candida* species while growing in our self-prepared culture media can be detected by MSPQC system, and these curves can be differentiated by the partial least square (PLS) technique effectively. For the reasonable explanation of those special frequency curves, the

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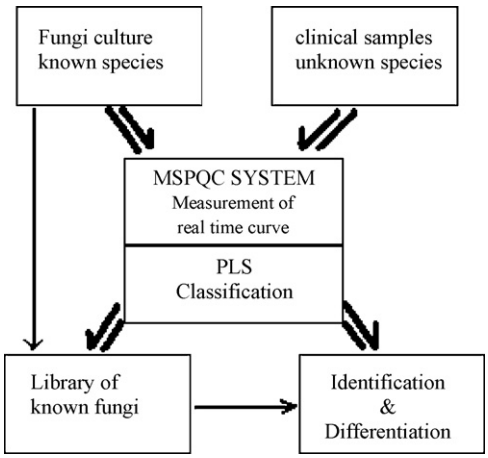

Fig. 1. Schematic representation of the MSPQC-PLS concept.

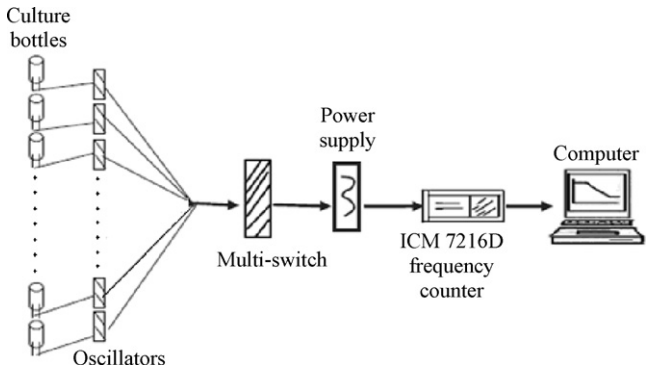

Fig. 2. Diagram of MSPQC system.

impedance characters of the three types of *Candida* species were studied by impedance analyzer.

The concept of the MSPQC-PLS system is shown in Fig. 1. First, real-time frequency curve of known clinical *Candida* species was detected by MSPQC system. These species and curves are made up of our library of known fungi. Then, the library was connected to a data processing and classification module via a specifically designed PLS algorithm. The PLS trained the data library to got a regression coefficient matrix which possess the ability of pattern recognition. The matrix was called "prediction machine". For an unknown clinical species, importing the data of frequent curve record by MSPQC system to the PLS system, the prediction machine will recognize the curve belong to which species' curve pattern, and give out the early identification result of *Candida* species.

2. Materials and methods

2.1. Strains

Fifty-three *Candida* strains provided by Xiangya Hospital of Central South University, Changsha, China, were isolated from different hospitalized patients and identified by standard identification system API20C.AUX (bioMerieux, France). Prior to use for identification, isolated strains were stored at room temperature in Sabouraud dextrose broth. For long preservation, these strains were frozen at −20 °C in distilled water.

2.2. Culture media

All the culture media used in our experiment were prepared by ourselves.

YC broth: beef extract, 2 g; yeast extract, 1 g; glucose, 40 g; YC nutrient fluid, 16 ml; ionized water, 1000 ml; pH 7.0–7.2.

Sabouraud dextrose broth: peptone 10 g, dextrose 40 g, distilled water 1000 ml; pH 5.4.

2.3. MSPQC system

The diagram of MSPQC is shown in Fig. 2. It is composed of 32 detection cells and 32 oscillator circuits. For single detection cell and oscillator circuit, an AT-cut 9 MHz piezoelectric quartz crystal coated with 0.5 mm silver disc at two sides was connected to the self-made TTL oscillated circuit and a pair of conductive stainless steel electrodes. The temperature of culture bottle was maintained at 37 ± 0.2 °C. 32 oscillator circuits share universal frequency counter, computer, and temperature controller system. The frequency outputs were switched alternately by multi-switches. Using the MSPQC, 32 samples can be tested at the same time. The on-line curves of frequency shift vs. culture time could be seen in screen by using self-developed software, so the growth of microorganism could be observed in real time. All frequencies and corresponding test times were autosaved by computer with TXT format.

2.4. MSPQC method for recording the frequency curve

Fungi were grown on Sabouraud dextrose agar plates at 37 °C overnight. The next day, the cells were harvested and suspended in 10 mL sterile water, the concentration were adjusted to 9.0×10^8 cfu/mL and 1.0×10^2 cfu/mL, respectively. 1 mL fungi suspension was transferred into culture bottles which contained 9 mL YC broth. Culture bottles were placed into MSPQC system and incubated at 37 ± 0.2 °C for a period of 1–3 days. At the same time, the on-line curves of frequency shift vs. culture time was recorded by using self-developed software. To avoid contamination, all glassware, culture media and equipment were sterilized. For comparison, the capacitance and resistance curves of fungi (9.0×10^8 cfu/mL) growth were recorded by HP-4192A LF impedance analyzer (Hewlett Packard) at 37 ± 0.2 °C.

2.5. Method for building the library of known fungi and creating the prediction machine

Each fungus sample can be represented by frequency shift data vector, $x = (x_1, x_2, x_3, \ldots, x_n)$, where x_n was the frequency shift measured by MSPQC system after frequency detect time(FDT). The FDT was defined as the time at which oscillating frequency shift of MSPQC changed rapidly [22]. If frequency shift was recorded at a interval of 10 min by MSPQC system, then x_1 was the frequency shift measured at 10 min after the FDT and x_2 was the frequency shift measured at 20 min after the FDT, x_n is the frequency shift measured at $n \times 10$ min after the FDT. Frequency shift data vector of total clinical collected fungi detected by MSPQC formed a data matrix. This data matrix is the library of known fungi. Every row of the data matrix represented one clinical sample. Then PLS was used to train the library. 70% of the total samples in library were selected randomly as the training set. Through the training, the characteristic of data vectors of each species was extracted by the PLS algorithm respectively and a $n \times 3$ regression coefficient matrix Y was construct (see Eq. (1)). Each column of the matrix Y denoted the characteristic of one type of species. So the number of fungi species need to be differentiated was equal to the column number of Y. The rest of 30% of the total samples were selected as the testing

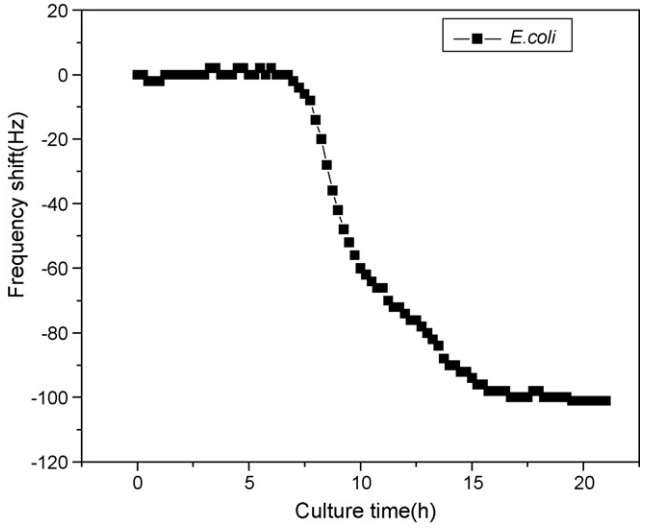

Fig. 3. Typical frequency curve of *E. coli*.

set to confirm the validity of the Y

$$\begin{bmatrix} x_{11}, x_{12}, \ldots, x_{1n} \\ x_{21}, x_{22}, \ldots, x_{2n} \\ \vdots \quad \ddots \quad \vdots \\ x_{m1}, x_{m2}, \ldots, x_{mn} \end{bmatrix}_{m\times n} \xrightarrow{PLStrainning} \begin{bmatrix} Y_{11}, Y_{12}, Y_{13} \\ Y_{21}, Y_{22}, Y_{23} \\ \vdots \quad \ddots \quad \vdots \\ Y_{n1}, Y_{n2}, Y_{n3} \end{bmatrix}_{n\times 3} \tag{1}$$

For an unknown sample X, a 1×3 vector Z was obtained by $X \times Y$ (see Eq. (2)). Sample X was regarded as the same species which show maximal value in vector Z. The regression coefficient matrix Y was called as "prediction machine".

$$\begin{bmatrix} X_1, & X_2, & \ldots, & X_n \end{bmatrix}_{1\times n} \times \begin{bmatrix} Y_{11}, Y_{12}, Y_{13} \\ Y_{21}, Y_{22}, Y_{23} \\ \vdots \quad \ddots \quad \vdots \\ Y_{n1}, Y_{n2}, Y_{n3} \end{bmatrix}_{n\times 3} = \begin{bmatrix} Z_1, & Z_2, & Z_3 \end{bmatrix}_{1\times 3} \tag{2}$$

2.6. Early identification of Candida species

The simulating clinical blood sample was prepared by adding 1 mL of 1.0×10^2 cfu/mL known *Candida* species to 1 mL human fresh blood. They were added individually to MSPQC bottle which contained 8 mL culture media. These culture bottles then were placed into MSPQC system and incubated at $37 \pm 0.2\,^{\circ}C$ for a period of 1–3 days. Then the real-time frequency shift curve was transformed to a data vector X and the simulating sample was identified by PLS system.

3. Results and discussion

3.1. Theory

MSPQC system is based on the monitoring electric parameter change of the culture media caused by the growth of microorganism. The theory basis for the SPQC can be represented by the following

$$\Delta F = \frac{\pi F_0^2 C_m (4\pi F_0 G_0 C_d + 4\pi^2 F_0^2 C_d^2 tg\theta - G_0^2 tg\theta)}{[G_0^2 - 2\pi F_0 C_0 G_0 tg\theta + 4\pi^2 F_0^2 C_d (C_0 + C_d)]^2} \Delta G \tag{3}$$

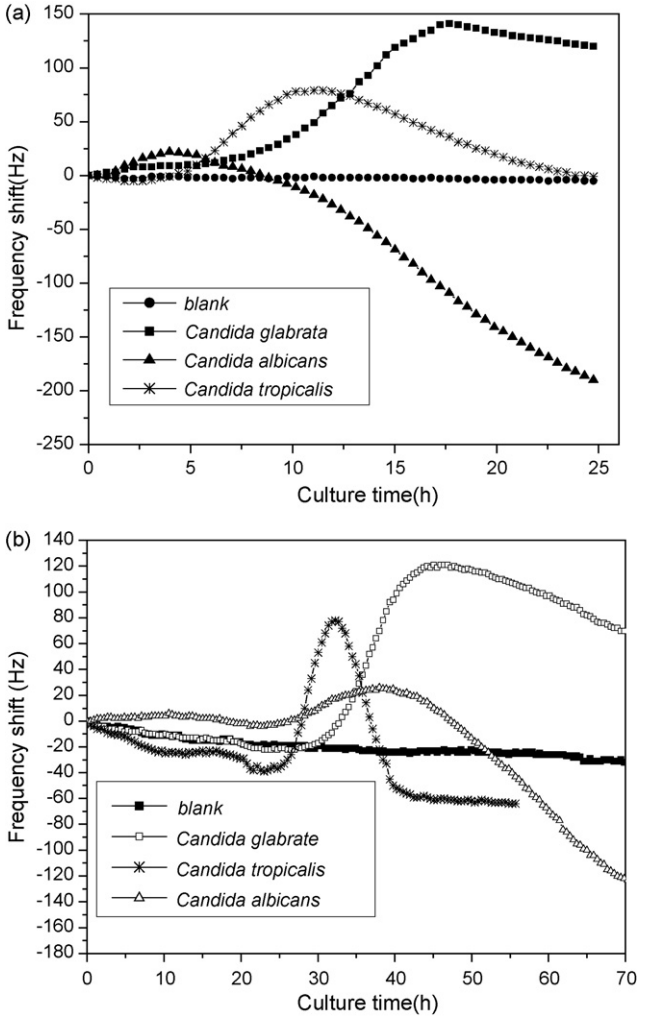

Fig. 4. Typical frequency curve of three species of *Candida* cultured in YC broth with the initial concentration: (A) 9.0×10^7 cfu/ml; (B) 1.0×10^1 cfu/ml.

where F_0 is the resonant frequency of the crystal, C_m and C_0 are the dynamic capacitance and static capacitance of the crystal respectively, C_d is the capacitance of solution which mostly based on the solution permittivity and parasitic capacitance between the leading wires of the electrode. G_0 is the solution conductance, θ is the phase shift. $\Delta F = F_i - F_0$, F_i is the oscillating frequency of crystal. From Eq. (3), the change of F is mainly dependent on solution conductivity and permittivity if other above-mentioned parameters are kept as constant. So the conductivity and permittivity change of the culture media caused by the growth of microorganism can be represented by the sensor frequency change. The typical frequency curve of *E. coli* detected by SPQC is shown in Fig. 3.

This curve was related to the metabolism of *E. coli*: at the first stage, oscillating frequency varied slowly because bacterium adjusted itself to new culture. After a few hours, bacterium came into an exponential growth stage and oscillating frequency started to decrease rapidly. Finally, when the growth of bacterium reached saturation, frequency arrived at the second flat stage. For the fungi, different species shows different way of metabolism. Further more, the same species in different culture medium, or at different stages of growth may also show different morphology and different ways of metabolism. These led to more specific diversity of MSPQC response in the grown process. These specific diversity were the basis of identification of fungi species.

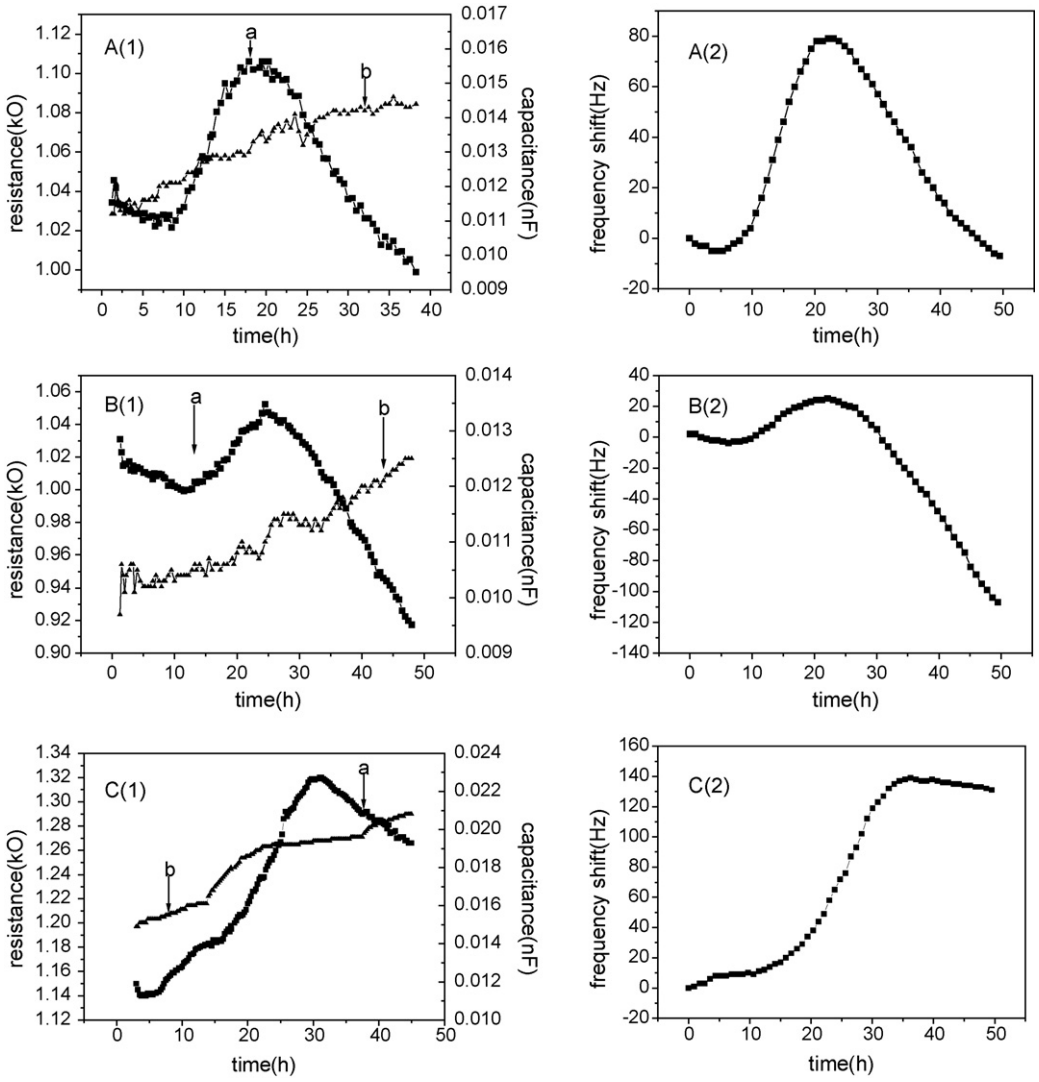

Fig. 5. The resistance curves[A(1)a, B(1)a, C(1)a], capacitance curves [A(1)b, B(1)b, C(1)b] and frequency shift curves [A(2), B(2), C(2)] between the two electrodes caused by the growth of three *Candida* species. A for *C. tropicalis*; B for *Candida albicans*; C for *C. glabrata*.

3.2. Typical frequency curve of three species of Candida

Typical frequency curves of three species of *Candida* (*C. albicans*, *C. glabrata*, *C. tropicalis*) in YC broth with high initial concentration are shown in Fig. 4(A). It showed clearly that three *Candida* species produced significant different type of frequency curve in 1 days' culture. For *C. tropicalis*, it showed a clear upward at first and then downward trend; *C. glabrata*, showed a clear upward trend; *C. albicans*, showed a clear downward trend after a unconspicuous upward trend. And from Fig. 4(B), it can be seen that *Candida* species gave out the same pattern of frequency curve no matter cultured with high initial concentration or low initial concentration. The only difference between the curve of high and low initial concentration was the FDT. For high initial concentration, the FDT of each *Candida* species was about 3–5 h (see Fig. 4(A)). For low initial concentration, the FDT of each *Candida* species was about 25–30 h (see Fig. 4(B)). Anyway, the three different species of *Candida* showed different pattern of frequency curve. Those diversity was easily recognized by PLS system.

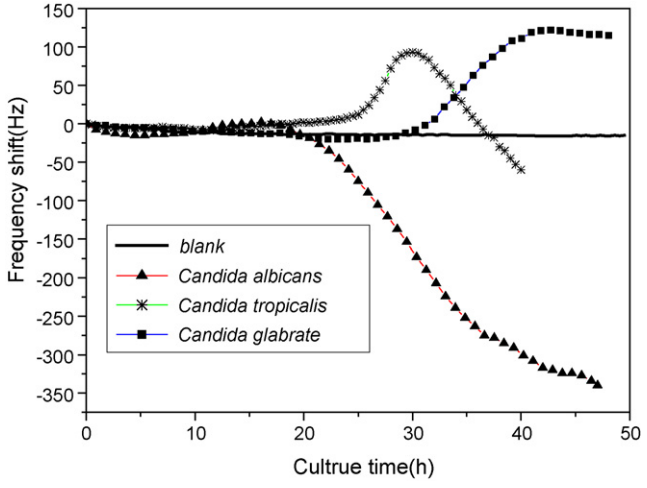

Fig. 6. Typical frequency curve of three species of *Candida* in simulating blood sample.

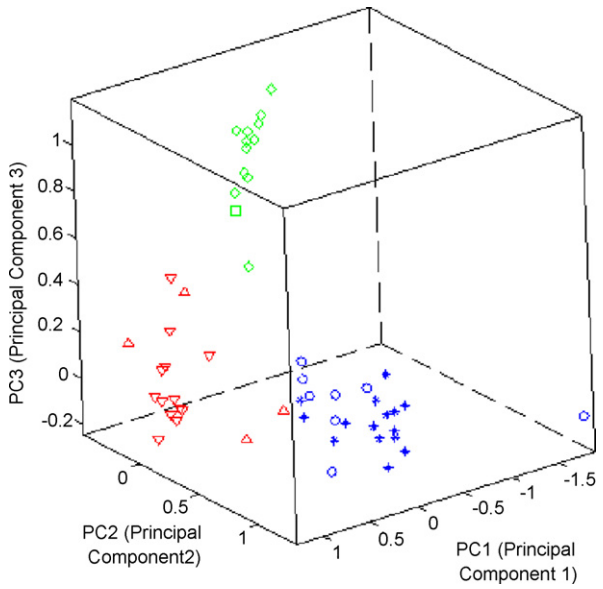

Fig. 7. Three-dimensional PLS plot showing discrimination of 53 samples. (▽) Training samples of *C. glabrata*. (△) Testing samples of *C. glabrata*. (○) Training samples of *C. albicans*. (✱) Testing samples of *C. albicans*. (◇) Training samples of *C. tropicalis*. (□) Testing samples of *C. tropicalis*.

3.3. Impedance changing curve of three species of Candida

Fig. 5 shows the resistance and capacitance change between the two electrodes of culture bottle while *Candida* species were growing in YC broth.

From Fig. 5, it can be seen that the capacitance between the two electrodes changed about 0.003–0.005 nF during the growing period of these *Candida* species. It means that there is no significant change in capacitance. But for the resistance between the two electrodes changed about 100 Ω and the pattern of the resistance changing curve is the same as the pattern of frequency shift curve produced by *Candida* species in comparative experiment. It clearly showed that the reason of producing those typical frequency curve was mainly related to the resistance changed by the growth of *Candida* species.

3.4. Typical frequency curves of three Candida species in simulating blood sample

As pathogens in raw clinical materials are always in low concentration, frequency curve of *Candida* species in simulating blood sample with low initial concentration were determined. From Fig. 6, it can be seen that the pattern of frequency curve was not affected by human blood. There is no significant difference in frequency curve between the simulating experiment and the low initial concentration experiment. This result means that raw clinical samples can be identified by proposed method directly without passing through pure culture process.

3.5. Construction of the prediction machine

A total of 53 clinical fungi were used to construct the prediction machine. All the fungi used here were pure culture species. Three types of frequency curve were identified. They were produced by *C. albicans*, which showed a clear downward trend after a unconspicuous upward trend, by *C. glabrata*, which showed a clear upward trend curve, by *C. tropicalis*, a clear upward and downward trend, respectively. Fig. 7 is a three-dimensional plot differentiated by PLS classification which was based on 53 samples of the three types of frequency curve caused by three species of *Candida*. Seventy percent of the 53 samples were used as the training set and the rest 30% were used as the testing set. From Fig. 7, it can be seen clearly that frequency curve produced by three types of fungi were successfully classified into three types by the PLS program.

Table 1
Identification result for 53 *Candida* species.

Candida species	Number of sample	Classification rate
C. albicans	23	100%
C. tropicalis	13	100%
C. glabrata	17	94.1%

Table 2
Identification result of early identification in simulating clinical blood samples.

Candida species	Number of sample	Prediction accuracy	Average identification time (h)
C. albicans	6	100%	46.5
C. tropicalis	6	83.3%	52.3
C. parapsilosis	5	100%	49.6

Table 1 shows very satisfying result. Only one *C. glabrata* sample was misclassified. The total classification rate of the 53 clinical samples was 98.1%. So the three types of clinically commonly encountered *Candida* species can be identified by proposed method in about 1 day.

3.6. Early identification of Candida species in simulating clinical blood samples

Simulating clinical blood sample were prepared by adding 1.0×10^2 cfu/mL dilutions of known *Candida* species to human fresh blood. A total of six *C. albicans*, five *C. glabrata*, six *C. tropicalis* were identified from the simulating blood samples. The PLS prediction machine which trained from 53 known fungi of the four *Candida* species was used to predict simulating clinic blood samples, the results are shown in Table 2. Only one *C. tropicalis* sample was misclassified. The average identification time of each *Candida* species was about 50 h. This is much shorter than the time needed in clinical routine. It means that several days can be saved using the new proposed method.

4. Conclusion

A MSPQC-PLS system was constructed to identify the most frequent *Candida* species encountered in human pathology with a single measurement. A "fingerprint frequency curve" of the sample measured by MSPQC can be recognized and classified by PLS. Pure cultures of clinical fungi can be identified by proposed method in about 1 day, with the classification rate of 98.1%. The identification of *Candida* species in simulating clinic blood samples showed that the proposed method has a potential capacity to identify *Candida* species directly without pure culture process. Proposed method enables early classification and differentiation of fungi at species level.

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