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A rapid qPCR method to investigate the circulation of the yeast *Wickerhamomyces anomalus* in humans

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SUMMARY

The yeast *Wickerhamomyces anomalus* has been proposed for many biotechnological applications in the food industry. However, a number of opportunistic pathogenic strains have been reported as causative agents of nosocomial fungemia. Recognition of potentially pathogenic isolates is an important challenge for the future commercialization of this yeast. The isolation of *W. anomalus* from different matrices and, recently, from mosquitoes, requires further investigations into its circulation in humans. Here we present a qPCR protocol for the detection of *W. anomalus* in human blood samples and the results of a screening of 525 donors, including different classes of patients and healthy people.

KEY WORDS: Wickerhamomyces anomalus, opportunistic pathogen, fungemia, qPCR, mosquito vectors.

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Wickerhamomyces anomalus (also designated as *Pichia anomala*, *Hansenula anomala* and *Candida pelliculosa*) is an ascomycete yeast well known for its antimicrobial properties, that have led to this microorganism being considered a biocontrol agent (Walker, 2011). Some examples of applications of *W. anomalus* are: in food/feed biopreservation (Olstorpe and Passoth, 2011), as a probiotic additive (Zuo *et al.*, 2013) and as a volatile aroma enhancer in wine making (Swangkeaw *et al.*, 2009). This yeast has been isolated from different habitats or matrices like food and feed systems (Walker, 2011) and even from insects (Toki *et al.*, 2012, Ricci *et al.*, 2011a). The identification of antimicrobial *W. anomalus* strains in *Anopheles* and *Aedes* mosquitoes have led this yeast being proposed also in the biocontrol of malaria (Cappelli *et al.*, 2014, Ricci *et al.*, 2011b) and other mosquito-borne diseases (Ricci *et al.*, 2011a).

With regard to food safety aspects, *W. anomalus* is classed at biosafety level 1 by the European Food Safety Authority (De Hoog, 1996). In addition, there are no reports in the literature on hazardous mycotoxin formation or allergic reactions to the spores from this yeast. However, a number *W. anomalus* strains have been isolated from humans, raising the possibility that this yeast is an emerging opportunistic pathogen (Hazen, 1995, Kalkanci *et al.*, 2010). In particular, reports over the last decades have highlighted an increase in nosocomial yeast bloodstream infections, particularly pediatric outbreaks, due to uncommon species, including *W. anomalus* (Taj-Aldeen *et al.*, 2014). *W. anomalus* infections have been reported in cancer patients (Thuler *et al.*, 1997), infants and premature neonates (Aragão *et al.*, 2001, Oliveira *et al.*, 2014), and in

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patients in surgical intensive care units (Kalenic *et al*., 2001). Possible explanations for the emergence of opportunistic pathogenic yeasts could be the increase in the number of patients at risk, such as immunocompromised persons, and the contribution of more sensitive diagnostic methods. However, the reasons for the increased incidence of fungemia from uncommon pathogenic yeasts are not completely clear. Differentiating non-pathogenic strains from potential opportunistic pathogens is necessary for future biotechnological applications of *W. anomalus* (Passoth *et al*., 2011). In addition, the recent isolation of a *W. anomalus* strain from different mosquito species (Ricci *et al*., 2011a) has set a new alert, considering that hematophagous insects might play a role in spreading this microorganism.

Here, we present a qPCR protocol for the detection of *W. anomalus* using species-specific primers previously designed (Huang *et al*., 2012), aimed at improving the current procedures for the rapid diagnosis of infections by this yeast. This optimized qPCR protocol was applied in a screening study to estimate the circulation of *W. anomalus* in human blood samples from different classes of patients (including immunocompromised) and from healthy donors.

We carried out a retrospective analysis using previously collected blood samples. A total of 525 donors were examined by qPCR (Table 1): 243 blood samples, collected between January 2011 and November 2012 from critically ill patients (adult and pediatric patients with suspicion of sepsis) admitted to the Intensive Care Units and Haematology Units (ICUs & HU) at the St. Orsola Malpighi University Hospital of Bologna; 183 blood samples, collected at the S. Matteo Hospital of Pavia in the context of another study, from 20 HIV, 61 HCV, and 19 HIV/HCV positive patients, and 83 healthy donors; 99 blood samples from additional 81 healthy donors collected at St. Orsola Malpighi, 10 malaria patients collected at Spedali Civili di Brescia (Institute for Infectious and Tropical Diseases, IITD) and 8 workers at the insectary of the University of Camerino (Unicam). All of the samples included in the present study were obtained and analysed in strict accordance with the current European and Italian rules on informed consent.

The results of this screening for *W. anomalus* (*Wa*) in blood samples required the development of a highly specific and sensitive qPCR assay. For the protocol optimization 12 yeast isolates (eight representing *W. anomalus*; four representing other yeast species) were used as control strains: *Wa*F17.12, *Wa*M9.11, *Candida* sp. and *Pichia* sp. isolated from the malaria vector mosquito *An. stephensi* (Ricci *et al*., 2011b); the environmental strains *Wa*ATCC 996603 and *Wa*UM3 (Polonelli *et al*., 1997), *Saccaromyces cerevisiae* ATCC 2601 and *Williopsis saturnus* var. *mrakii* (Guyard *et al*., 2002); four clinical isolates of *W. anomalus* from four neonates hospitalized in an intensive care unit (Kalkanci *et al*., 2010). In the qPCR standard curve setup, a target sequence of 218 bp of β-tubulin

TABLE 1 - *Summary of the screening for Wickerhamomyces anomalus (Wa) in human blood samples.*

Human samples and health or disease status	*Samples origin*	*Wa-qPCR assay results positive/total*
Critically ill patients	St. Orsola Malpighi/ICUs & HU, University Hospital of Bologna	0/243* (36 pediatric cases; 207 adults cases)
HIV positive patients	S. Matteo, Hospital of Pavia	0/20
HCV positive patients	S. Matteo, Hospital of Pavia	1/61
HIV/HCV positive patients	S. Matteo, Hospital of Pavia	0/19
Malaria patients	Spedali Civili di Brescia/ IITD	0/10
Donors exposed to mosquito bites at insectary	Unicam	0/8
Healthy blood donors	S. Matteo, Hospital of Pavia	0/83
Healthy blood donors	St. Orsola Malpighi, University Hospital of Bologna	0/81
Total		1/525

*All of these patients displayed at least two signs among the criteria of the systemic inflammatory response syndrome (Kaukonen *et al*., 2015).

gene was amplified using the specific primers SpWanom-170F (5'TTATCCATCCACCAATTG3') and SpWanom-374R (5'GGAACTAAGTTCACAGCTA3') (Huang *et al*., 2012). The primers were previously tested against yeast genomic DNAs from all of 12 isolates listed above. The expected amplicon was obtained from all eight *W. anomalus* isolates tested (two isolated from mosquito, two environmental and four clinical isolates), while no amplification was obtained from the other four yeast isolates, representing other species (*Candida* sp., *Pichia* sp., *Saccaromyces cerevisiae* ATCC 2601 and *Williopsis saturnus* var. *mrakii*).

To evaluate the amplification efficiency of the selected primers, the PCR product from *Wa*F17.12 was cloned into a plasmid vector (pGEM-T Easy Vector System, Promega, Wisconsin, USA) and the amount of the recombinant plasmid was determined. We carried out a standard curve, using as DNA templates eight serial dilutions (10^{-1}) of the recombinant plasmid, corresponding to around $2.3x10^7$ – 2.3 gene copies per µl of reaction. qPCR amplifications were carried out with the β-tubulin gene primers (200 nM each) using Sybr Green Master Mix (Fermentas, Burlington, Canada) in a final volume of 25 µl. Amplification cycling conditions were as follows: 10' at 95°C; 1' at 95°C, 1' at 52°C, 30" at 72°C for 40 cycles. A final step for the melting curve analysis from 65°C to 95°C (increment of +0.5°C) was performed and the relative dissociation curve displayed a single specific dissociation peak at 74.50°C. The obtained standard curve showed high sensitivity, up to around 2.3 gene copies per µl of PCR reaction were detected.

The qPCR protocol was then validated mixing *W. anomalus* cells with human blood samples. We tested the strain *Wa*F17.12 and the four clinical isolates of *W. anomalus*. The yeasts were grown in YPD medium and incubated overnight at 28°C at 110 rpm to obtain an optical density 1.0 ($3.2x10^7$ cells/ml). Yeast cells were collected by centrifugation at 3500 rcf, 4°C for 10' and diluted in sterile water. Eight serial dilutions from 3.2 $x10^7$ cells to 3.2 cells were obtained in a final volume of 200 µl of human blood samples from a healthy donors (in triple replicates for each isolates). Total DNAs were extracted using the Jet Quick Blood DNA Spin Kit (Gentaur, Löhne, Germany) and suspended in 50 µl of sterile water. Fifty ng of the purified DNAs were used as templates for qPCR analysis at the same conditions described for the standard curve set-up. The relative dissociation curves confirmed the same melting temperature as the standard curve, while no specific peak was detected in the negative control reactions carried out with pure blood samples. The sequence analysis of the amplified product confirmed 100% homology with the GeneBank *W. anomalus* partial sequence of ß-tubulin gene available in GeneBank (JQ734945.1). The qPCR assay was able to detect up to around 16 yeast cells per ml of blood sample, for all the tested isolates (*Wa*F17.12 and four *W. anomalus* clinical isolates).

After the validation of the *Wa*-qPCR assay, we proceeded to screen the 525 selected blood samples. The DNA from these samples was obtained as described in Epis *et al*. (2012) and quantity and quality of the extracted samples were checked by spectrophotometer. Each sample was tested in triplicate (both undiluted and at a 1:10 dilution) and the positive control (DNA from *Wa*F17.12 strain) was included. The template DNAs were subjected to the *Wa*-qPCR assay at the amplification conditions previously described. In addition, an amplification control was performed by running a parallel PCR test targeting the human Beta-2-microglobulin. Beta-2-microglobulin amplification was carried out using a new primer set, Bmicr2F (5'CTCCGTGGCCTTAGCTGTG3') and Bmicr2R (5'TTTGGAGTACGCTGGATAGCCT3') (250 nM each) that amplifies a 69 bp fragment, following the conditions described for *W. anomalus* detection except for the annealing step (60°C for 30").

The present study analysed a heterogeneous group, including healthy donors and different types of patients (critically ill adults and children, HIV and HCV patients, malaria patients), blood donors exposed to mosquito bites and healthy donors, in order to have an overview of the yeast circulation in humans exposed at different types of risk. The screening revealed an almost complete negativity of the analysed samples, with the exception of a single HCV patient that tested positive (3.9 copies of ß-tubulin gene per µl of reaction) at the *Wa*-qPCR

assay (Table 1). Sequence analysis of the amplified product revealed the perfect match of the amplified fragment with the β-tubulin gene from *W. anomalus* (JQ734945.1). This newly obtained sequence was deposited in EMBL-EBI under the accession number LN680997. In addition, to confirm the positivity of this sample and the negativity of 30 samples (a sub-sample, casually selected), we performed a nested-PCR targeted at 18S rRNA gene, for the specific *W. anomalus* detection following the protocol published in Ricci *et al.,* 2011b. Application of this further method to the positive sample and to 30 samples that were negative at the first PCR confirmed our results.

Our results support the view of *W. anomalus* as an organism considered safe for healthy individuals and are coherent with the evidence of an uncommon opportunistic yeast (Taj-Aldeen *et al*., 2014). As stated in a recent review of the literature, *W. anomalus* infections have been reported occasionally and the occurrence of a few hundred positive cases (single cases and outbreaks) worldwide is estimated over a period longer than twenty years (Kalkanci *et al*., 2010). We found a single case out of 353 immunocompromised patients (St. Orsola Malpighi, 243; Spedali Civili di Brescia, 10; S. Matteo Hospital, 100) and 164 healthy donors screened. Interestingly, no positivity was found in the malaria patients (exposed to malaria vector mosquito bites in Burkina Faso) and in the workers at the insectary (exposed to *Anopheles* and *Aedes* mosquitoes bite and to *W. anomalus* cultures). Nevertheless, the recognition of potentially pathogenic isolates is required for any future biotechnological applications of this yeast as a biocontrol agent. Our quantitative assay aimed at performing rapid mass screenings may effectively contribute to further investigations necessary for a better understanding of the epidemiology of *W. anomalus* infections.

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