VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 19, Number 3, 2019

DOI: 10.1089/vbz.2018.2321

Genetic Diversity Among *Candida albicans* Isolated from Humans and Cattle with Respiratory Distress in Egypt

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Abstract

As human populaces develop, they are progressively squeezed into higher living densities. The same is true for horticulture and animals expected to bolster these communities. Despite the high potential for zoonotic transmission, connections among humans and cattle have been understudied; however, *Candida albicans* remains the most important medical mycosis. The genesis of the mycobiome can vary, and interactions between humans and cattle are progressively being perceived as a key interface for disease transmission. α*INT1* is a unique gene from *Candida albicans*; hence, it has been used for detection as well as intraspecific and interspecific phylogenetic analysis of *C. albicans* collected from human patients and cattle with pulmonary distress in urban–rural populations. A total of 1,921 specimens were examined by direct microscopy and culture to recover yeast associated with human infection. Identification was performed by micromorphology using an API 20C AUX system. The fungal species identified in bovine nasal specimens were *Alternaria* species (15%), *Penicillium* species, and *C. albicans* (6.7%). Other fungal species, such as *Aspergillus niger*, *Torulopsis* species, *Mucor* species (5%), *Aspergillus flavus*, *Fusarium* species, *Trichosporon* species (3.3%), *C. rugosa*, *C. tropical*, and *Saccharomyces* species (1.7%), were also isolated. In human sputum specimens, *C. albicans* (20%) and *C. parapsilosis* (2.7%) were the only reported yeast species in our samples. The four identified *C. albicans* species (two human and two cattle) were subjected to α*INT1* gene sequence analysis, which confirmed major phylogenetic relationships among human and cattle isolates. This finding highlights the public health importance of bovines as a potential source for *C. albicans* zoonotic transmission to humans in an urban–rural community. Additionally, the close relationship between circulating *C. albicans* strains recorded in Egypt and the United States indicates the possible cross-species transmission of *C. albicans* between imported foreign and native cattle breeds.

Keywords: pulmonary mycobiome, *Candida* spp., human, cattle, phylogeny, internal transcribed spacer region

Introduction

Quick and impromptu urban development undermines practical improvements when a fundamental foundation is not created or when approaches are not implemented to guarantee that the advantages of city life are impartially shared. Today, despite the preference for urban areas, urban areas are more unequal than rural zones, and several million of the world's urban poor live in substandard conditions. In a few urban areas, spontaneous or poorly overseen urban extension has prompted rapid sprawl and pollution. Urbanization can result in creation of a wellspring of pathogenic microorganisms through the release of human sewage into water environments (Girones et al. 2010).

By examining microorganisms in water, it is conceivable to distinguish potential pathogens. For example, species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Aeromonas hydrophila*, *Staphylococcus aureus*, *Salmonella* sp., *Enterococcus*

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sp., *Streptococcus* sp., and *Bacteroides* sp. have been found in urban environments and most of these are related to fecal material from people and animals (Savichtcheva et al. 2007, Gonzalez et al. 2010, Willems et al. 2011).

Waste from ranches, such as animal and human fertilizers, straw and cotton waste, and offal from butchering cows (excrement of animals in the prebutchering period, including substances from the intestinal tract and other natural solids), is exceedingly tainted with pathogenic microorganisms and therefore poses risks to animals and people. Many moulds, including both pathogenic and toxigenic varieties, have been found in waste from poultry, dairy animals, pigs, and other animals. These include *Aspergillus*, *Penicillium*, *Candida*, *Fusarium*, and *Mucor* species, which are commonly observed in human and veterinary clinical practice and can be identified by their form and manner of spore production, along with the characteristic appearance of hyphae and mycelium (Haley and Callaway 1978, Koneman et al. 1997, Laemmlen 2001, Larone 2002).

Studies of fungi have been ongoing for more than a century, beginning with the first mycological article published in 1852 (Hassall 1853, Chiee and Ewart 1878). The connotation of microbiome changed in 2010 when the term mycobiome (a combination of the words mycology and microbiome) was first used to refer to the fungal microbiome (Ghannoum et al. 2010). In our recent search of PubMed (2014–2017), the term mycobiome appeared in 50 publications of studies in many fields, such as those focused on humans, soil, or produce, indicating its acceptance in the literature. The genesis of mycobiome pathogens can vary; however, interactions among humans, pets, and food and peridomestic animals are progressively being perceived as key interfaces for disease transmission (Daszak et al. 2000, Meerburg et al. 2009, Rabinowitz and Conti 2013, Bublitz et al. 2014).

As human populaces develop, they are progressively squeezed into higher living densities. The same is true for horticulture and animals expected to bolster these communities. Despite the high potential for zoonotic transmission, connections among humans and domesticated and peridomestic animals are still understudied. In contrast to the amount of work done on *C. albicans*, there are relatively few studies examining non-*C. albicans Candida* (NCAC) species. Therefore, this investigation provides information on the current state of knowledge regarding the identification and epidemiology of selected emerging agents and less common agents associated with opportunistic mycoses (Pfaller and Diekema 2004). This study emphasizes the diversity of the mycobiome isolated from humans and cattle with pulmonary distress in an urban–rural population as *C. albicans* and organisms that are genetically related to it remain poorly investigated when they are isolated from humans and contact cattle sources.

Materials and Methods

Research area

The study was conducted in the Governorate of Al-Menoufiya in Egypt, which is located in the northern part of the country in the Nile Delta to the south of Gharbia governorate and 81 km to the north of the capital Cairo (30.52°N 30.99°E at an altitude of 9.0 m). The average temperature varies between −3°C and 48°C, with an average rainfall of 40 mm^3 and a rainy season from October to March.

Although the area of study (2,543.03 km^2) has gone through a process of rapid urbanization over the past three decades, according to population estimates from 2015, the majority of residents in the governorate live in rural areas, with an urbanization rate of only 20.6% compared with a mean rate of 43.14% in Egypt as a whole (CAPMAS 2016). Of an estimated 3,941,293 people residing in the governorate, 3,128,460 (79.4%) people live in rural areas, as opposed to only 812,833 (20.6%) in urban areas (CAPMAS 2016). Much of the population in the Governorate of Al-Menoufiya relies on livestock, cotton, maize, and wheat, as well as farming of vegetable crops, such as potatoes and green beans, for subsistence.

Samples

Human sputum samples were obtained from 120 patients by medical staff from the outpatient clinic at Al-Menoufiya Hospital for Chest Diseases over a two-month period (September–October 2016). The chosen patients showed respiratory signs, including cough, sneezing, nasal discharge, and respiratory distress. During the same time period, 400 cattle nasal swabs were collected from eight randomly selected farms within a 5-km radius of the hospital. The cattle were diagnosed as having respiratory distress with nasal discharge, cough, and sneezing. The 520 samples were sent to the Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Al-Menoufiya, Egypt, for mycosis identification.

Direct examination, culture, and identification of isolates

As described in detail previously (Abia-Bassey and Utsalo 2006, Masoud-Landgraf et al. 2014), wet preparations of swabs and sputum were mounted in 10% KOH and examined microscopically. All samples were inoculated onto Sabouraud dextrose agar (SDA; Oxoid Ltd., Basingstoke, United Kingdom) plates supplemented with refobacin (gentamicin) (40 mg/L) and chloramphenicol (100 mg/L) (Oxoid Ltd.) to prevent or minimize bacterial growth. The plates were examined for growth of yeast cells 24–48 h after being incubated aerobically at 35–37°C. Pure growth of ≥20 yeast colonies was regarded as significant. When mixed cultures were obtained, yeast colonies were subcultured into fresh medium to isolate pure colonies.

Morphological features were observed on Czapek yeast extract agar with 2% malt extract agar and yeast extract sucrose agar (HiMedia Laboratories Pvt. Ltd., India) incubated in the dark at 25°C for 7 days. Identification of gross morphological characteristics and a detailed study of microscopical and biochemical features of the isolates were performed. *Candida albicans* was identified based on the following microscopic features: thick-walled oval yeast cells with pseudomycelium, germ tube formation in human serum at 37°C, and chlamydospore formation on rice extract agar with 1% Tween 80 (FUJIFILM Wako Chemicals). Other *Candida* spp. and *Trichosporon* spp. that were isolated were identified based on their morphological characteristics and carbohydrate assimilation patterns using the API 20C AUX system (bioMérieux, Marcy L'Etoile, France) and ID 32 C (bioMérieux) commercial kits, respectively.

PCR confirmation for C. albicans *isolates*

To confirm the morphological results, molecular identification of *C. albicans* isolates was performed by PCR by targeting the species-specific α*INT1* gene in *C. albicans*. DNA was isolated from *C. albicans* strains using the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany), as described by the manufacturer.

The following primers were used for PCR detection of the α*INT1* gene in *C. albicans*: Forward LH1 5′- AGC CAC AAC AAC AAC AAC AAC TCT -3′ and Reverse LH2 5′- TTG AGA AGG ATC TTT CCA TTG ATG -3′, which amplify a 344-bp fragment (Abia-Bassey and Utsalo 2006). The primers were synthesized by the Chromogen Company (South Korea). PCR reactions were performed in a Gradient Thermal Cycler (S-1000 thermal cycler; Bio-RAD). The reaction mixture (total volume of 50 μL) contained 25 μL of Dream Green PCR Mix (DreamTaq Green PCR Master Mix (2X) Fermentas, Waltham, MA), 5 μL of target DNA ranging from 1 to 500 ng, and 2 μL of each primer (at 10 pmol/μL) with the addition of sterile distilled water to 50 μL.

PCR amplification was performed as described by Lim and Lee (2000), with the following conditions: (1) a 3-min initial step at 93°C, (2) followed by 30 cycles at 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and (3) a final extension step at 72°C for 10 min. Amplification products and the GeneRuler 100-bp DNA Ladder (Fermentas, Waltham, MA) were separated by electrophoresis on a 1.5% w/v agarose gel (Agarose; Sigma) and stained with ethidium bromide for visualization with a transilluminator to observe amplified DNA in the gel and compare it with molecular weight markers.

Sequencing and phylogenetic analysis of C. albicans *isolated from humans and cattle*

PCR products were cleaned with the GeneJET™ PCR Purification Kit (Thermo, Germany) according to the manufacturer's instructions. The purified PCR products were shipped to GATC Biotech (GATC Biotech AG, Konstanz, Germany) for sequencing using forward and reverse primers.

DNA sequencing of the *aINT1* gene was conducted in both directions, and a 324-bp consensus sequence was used for nucleotide (nt) analysis. The original sequence was trimmed to remove vague nt sequences that are usually present at the beginning of the sequencing reaction using the BioEdit sequence alignment editor (Masoud-Landgraf et al. 2014). Identification of the homology between nt and amino acid sequences from the studied *C. albicans* DNA sequences and other *Candida* published in GenBank was completed using the BLAST 2.2 search program (National Center for Biotechnology Information (NCBI), www.ncbi.nlm.nih.gov).

Alignment of the obtained nt and amino acid substitutions in the *C. albicans* sequences published in GenBank was done using MegAlign, DNASTAR, Lasergene®, Version 7.1.0. Phylogenetic trees were constructed using MegAlign for sequence tree reconstruction using a neighbor-joining method based on ClustalW (Lim and Lee 2000). Sequence divergence and identity percentages were calculated by MegAlign. Human and cattle isolates were submitted to the GenBank database and given the following accession numbers: KU852506 (Cand-EWE.3.EG2015), KU852507 (Cand-EWE.5.EG2015), KU852508 (Cand-EWE.7.bov.EG2015), and KU852509 (Cand-EWE.8.bov.EG2015).

Ethical approval

(1) Regarding human samples: The medical staff of Al-Menoufiya Chest Hospital collected all the human samples. The research details and risks of participation in this study were explained to all participating patients, and verbal consent was obtained before sample collection.
(2) Regarding animal samples: All animal samples were collected according to the institutional animal care and use committee (IACUC) regulations for the faculty of veterinary medicine, University of Sadat City, Egypt (approval number: vusc-011-1-18).

Results and Discussion

Fungi that cause diseases in animals are either endemic fungi, which are geographically restricted and can potentially cause serious infections in immunocompetent animals, or opportunistic fungi, which are widely distributed in the environment or live as body commensals and convert to secondary pathogens under predisposing conditions. These fungi cause diseases in hosts with compromised defenses or in animals receiving prolonged immunosuppressive or antibacterial therapy, those that have used indwelling devices or intravenous catheters, or those that are members of extreme age groups, where the fungi gain access to the host through the respiratory tract, alimentary tract, or intravascular devices (Hall 1991).

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased significantly over the past three decades in an era of increasing immunosuppression (Thompson et al. 1994, Pfaller and Diekema et al. 2004), and these pathogens are associated with excessive morbidity and mortality (Pfaller and Diekema et al. 2004). Of the fungi regarded as human pathogens, members of the genus *Candida* are most frequently recovered from human fungal infections. Of the *Candida* species isolated from healthy and diseased humans, *C. albicans* is the most prevalent (Lass-Flörl 2009, Tewari 2009). However, while mycological studies have shown that *C. albicans* represents over 80% of isolates from all forms of human candidosis (Tewari 2009) in the last two decades, the number of infections due to NCAC species has increased significantly (Kauffman et al. 2000, Calderone 2002, Samaranayake et al. 2002).

The apparent increased involvement of NCAC species in human candidosis may partly be related to improvements in diagnostic methods, such as the use of chromogenic media with the ability to differentiate *Candida* species as well as introduction of molecular techniques in routine diagnosis of fungemia (Manzano-Gayosso et al. 2008, Ruan and Hsueh 2009).

The genus *Aspergillus* is one of the best studied genera of filamentous fungi largely because of the medical (*A. fumigatus* and *A. terreus*), food spoilage (*A. flavus* and *A. parasiticus*), and industrial (*A. niger*, *A. aculeatus*, and *A. oryzae*) relevance of some of its species (Liguori et al. 2009). The fungal genus *Aspergillus* is of critical importance as it contains important pathogens in humans and animals (Liguori et al. 2009). Although *Aspergillus* is the most common mould causing fungal sinusitis, other fungi such as *Alternaria* can also be involved. The genus *Alternaria* contains several species that cause opportunistic human infections (human alternarioses) that are frequently associated with hypersensitivity

Table 1. Distribution of Fungal Isolates Recovered from Human and Cattle Specimens

	Cattle samples		*Human samples*	
	No.	*%*	*No.*	*%*
Fungal isolates				
Aspergillus niger	3	5	0	0
Aspergillus flavus	2	3.3	0	0
Mucor species	3	5	0	0
Penicillium species	4	6.7	0	0
Alternaria	9	15	0	0
Fusarium	2	3.3	0	0
Yeast species				
Candida albicans	2	3.33	2	5.71
C. parapsilosis	0	0	1	2.9
C. rugosa	1	1.7	0	0
C. tropical	1	1.7	0	0
Torulopsis spp.	3	5	0	0
Trichosporon spp.	2	3.3	0	0
Saccharomyces spp.	1	1.7	0	0

pneumonitis, bronchial asthma, and allergic sinusitis and rhinitis (Silva et al. 2012). *Fusarium* species are well-known plant pathogens and food contaminants that have also appeared as one of the most important groups of medically significant fungi and are associated with high morbidity and mortality rates (Pastor and Guarro 2008, De Vries et al. 2017).

Some commonly encountered saprophytes in human and veterinary clinical practice, including *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp., have been implicated as causative agents in the diseases candidiasis and thrush, mucormycosis, and opportunistic pneumonia (Laemmlen 2001, Larone 2002, Nucci and Anaissie 2002, Consigny et al. 2003). Zygomycosis (mucormycosis) is caused by moulds from the order Mucorales, which is the largest group of Zygomycetes (Pattron 2006). The order Mucorales includes the important genera *Absidia* spp., *Mucor* spp., and *Rhizopus* spp., which are of significance in human and animal diseases (Pattron 2006, Borchers et al. 2017). Of the 520 specimens collected, 95 (9.3%) were positive for diversity in their mycobiome (Table 1 and Fig. 1). As illustrated in Table 1, the most frequently isolated fungal species in bovine nasal specimens included *Alternaria* species (15%), *Penicillium* species, and *C. albicans* (6.7%). Other fungal species, such as *Aspergillus niger*, *Torulopsis* species, *Mucor* species (5%), *Aspergillus flavus*, *Fusarium* species, *Trichosporon* species (3.3%), *C. rugosa*, *C. tropical*, and *Saccharomyces* species (1.7%), were also isolated.

With respect to human sputum specimens, *C. albicans* (20%) and *C. parapsilosis* (2.7%) were the only reported yeast species in our samples; these species were also recovered by Park et al. (2016) from clinical samples at higher prevalence rates (64.0% and 5%, respectively), in addition to *C. tropicalis* (18.1%), *C. glabrata* (8.6%), *C. krusei* (0.7%), and other *Candida* species (1.9%). Non-*Candida* species, including *Trichosporon* species (1.2%), were also frequently isolated from clinical samples in this previous study.

Animals are hosts to numerous fungal pathogens, most of which can also infect humans. For *C. albicans*, in particular, studies have shown genetic evidence that animals, including cattle, could serve as sources of human *C. albicans* infection (Edelmann et al. 2005). However, the zoonotic transfer of *C. albicans* strains requires close contact between humans and animals (Edelmann et al. 2005 and Wrobel et al. 2008). Another study showed that bovine *C. albicans* isolates harbored virulence genes implicated in clinical cases of human candidiasis, which emphasizes the potential zoonotic risk of bovine *C. albicans* isolates (Mousa et al. 2016). These studies highlight the role of cattle as a potential reservoir for zoonotic *C. albicans* isolates.

Sequencing and phylogenetic analysis of C. albicans *isolates of human and bovine origin*

The α*INT1* gene was targeted for sequence and similarity analysis with selected temporally and geographically matched *C. albicans* isolates from humans (two isolates) and bovines (two isolates) in Egypt. α*INT1* plays a critical role in *C. albicans* cell adhesion and virulence (Gale et al. 1998) and it was chosen for its uniqueness to *C. albicans* isolates (Gale et al. 1998, Abia-Bassey and Utsalo 2006). BLAST sequence analysis for isolate homology showed a high degree of similarity between the Egyptian isolates and two *C. albicans* isolates from humans in the United States (*C. albicans*-

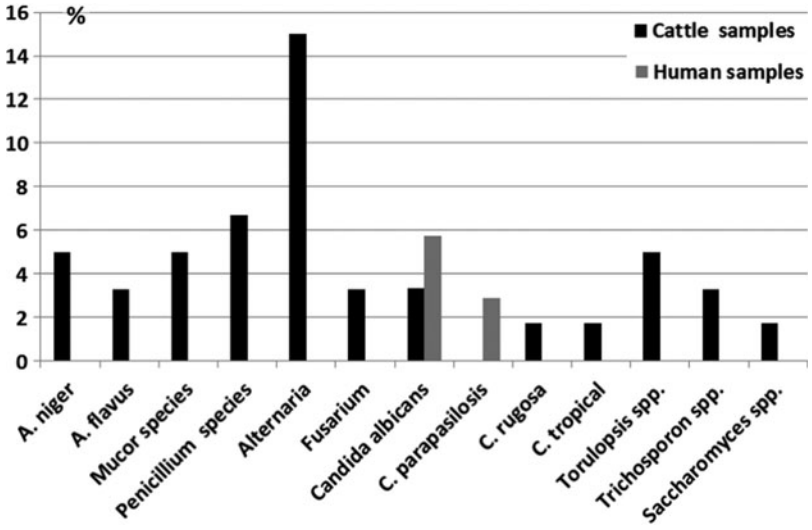

FIG. 1. Distribution of fungal isolates recovered from human and cattle specimens.

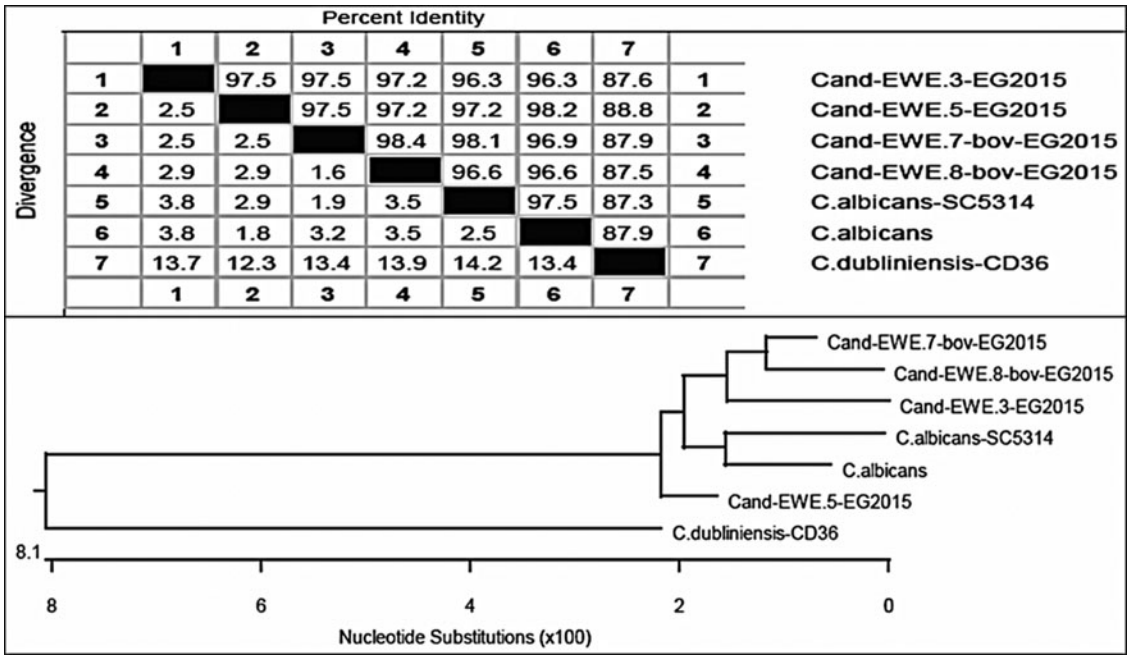

FIG. 2. Nucleotide sequences of *Candida albicans* isolates from cattle and humans.

SC5314 and *C. albicanis*-10261), with 96.3% to 98.4% nt sequence identity and 94.3% to 98.1% amino acid substitution identity, respectively. However, a distinct divergence (87.5–88.8% and 84–86.3% identity for nt and amino acid sequences, respectively) was observed when comparing *C. albicans* isolates with *C. dubliniensis*, a *Candida* spp. that is phylogenetically close to *C. albicans* (Figs. 2 and 3).

Additionally, amino acid sequence alignment showed a clear segregation between *C. dubliniensis* and all tested *C. albicans* isolates, with at least 14 amino acid substitution differences between the two *Candida* species (Fig. 4). This inter- and intraspecies segregation was also confirmed by a phylogenetic neighbor-joining tree, which showed that there are two main clusters divided into seven clones. The first

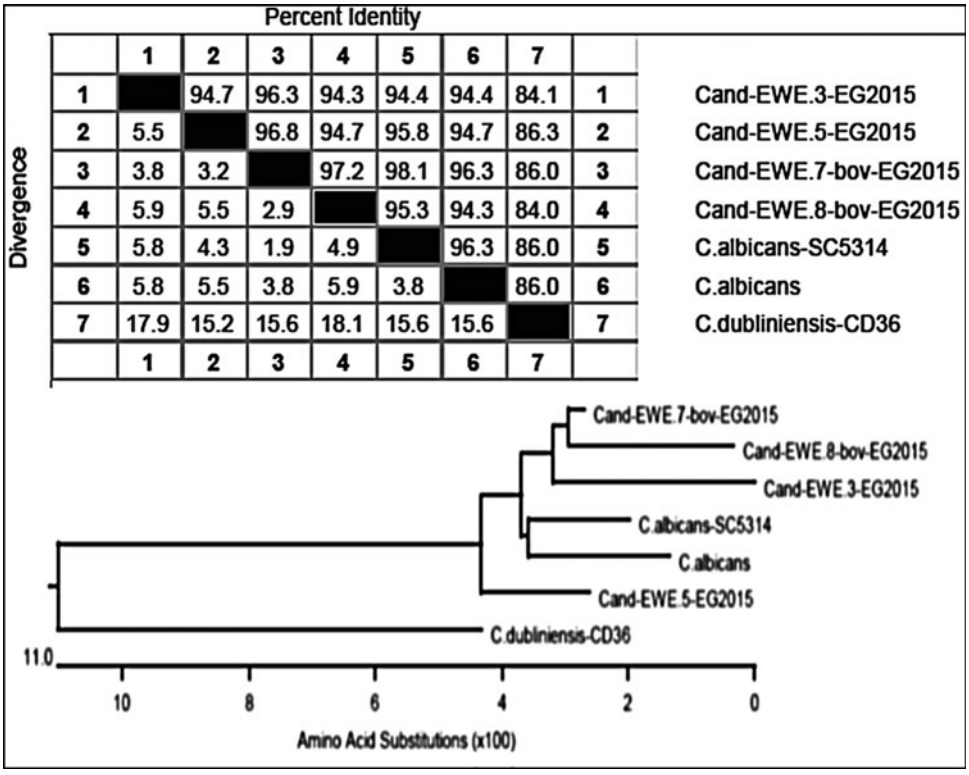

FIG. 3. Amino acid sequences of *C. albicans* isolates from cattle and humans.

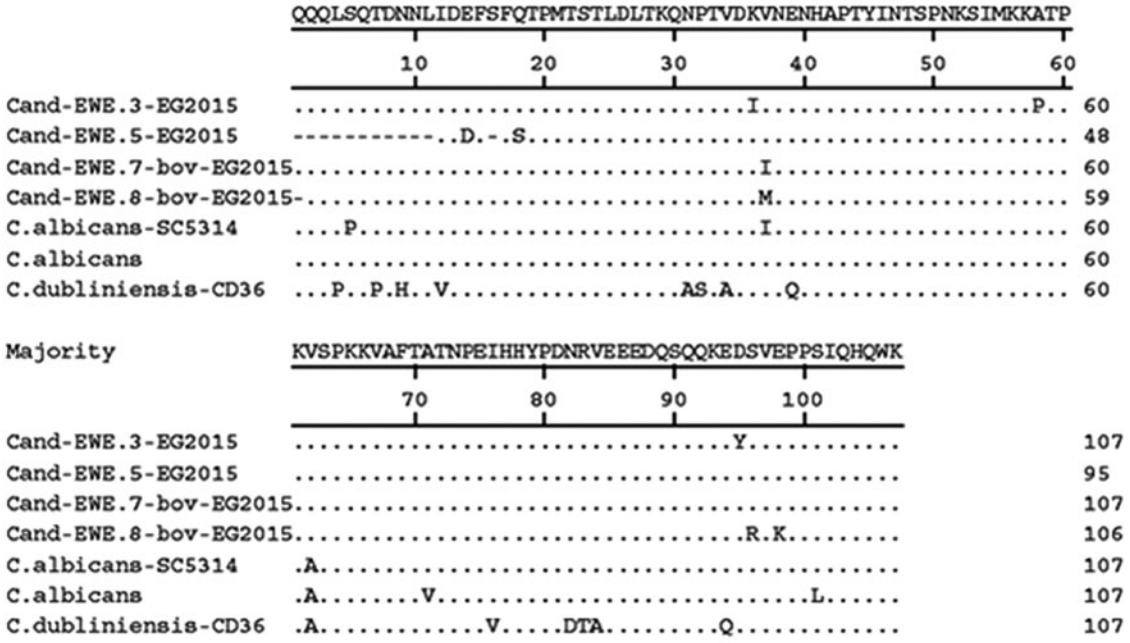

FIG. 4. Amino acid sequence alignment of *C. albicans* isolates from cattle and humans. *Dots*, residues that match the majority consensus.

cluster consisted of six isolates: four isolates from Egypt, including two human isolates (cand-EWE.3.EG2015 and cand-EWE.5 EG2015) and two bovine isolates (cand-EWE-7-bov-EG-2015 and cand-EWE-8-bov-EG-2015), and two isolates from human samples from the United States (*C. albicans*-SC5314 and *C. albicans* strain 10261). The second cluster contained only one human isolate from the United Kingdom (*C. dubliniensis*-CD36); this is illustrated in Figs. 2 and 3. These findings support the use of α*INT1* not only as a marker for detection of *C. albicans* infections, as proposed in the study by Lim and Lee (2000), but also as a marker to study inter- and intraspecies genetic relationships in *C. albicans*, as demonstrated by our results.

A high degree of similarity was shown between the two bovine isolates, with only the following 4 amino acid variations in the cand-EWE-8-bov-EG-2015 isolate: glutamine (Q) deletion at position 1, isoleucine (I)/methionine (M) replacement at position 37, serine (S)/arginine (R) replacement at position 96, and glutamate (E)/lysine (K) replacement at position 98 (Fig. 3). Interestingly, close homogeneity was reported between a human isolate (cand-EWE.3.EG2015) and a bovine isolate (cand-EWE-7-bov-EG-2015), with the following 4 amino acid variations in the cand-EWE.3.EG2015 human isolate: lysine (K)/isoleucine (I) replacement at position 36, isoleucine (I)/valine (V) replacement at position 37, alanine (A)/proline (P) replacement at position 58, and aspartate (D)/tyrosine (Y) replacement at position 95.

The other human isolate (cand-EWE.5 EG2015) showed a marked amino acid sequence variation from the two bovine isolates and the cand-EWE.3.EG2015 human isolate owing to the following three main alterations in the cand-EWE.5 EG2015 isolate: the deletion of 12 amino acids at positions 1–11 and 16, glutamate (E)/aspartate (D) replacement at position 14, and glutamine (Q)/serine (S) replacement at position 18. Moreover, clustering patterns in the phylogenetic tree showed that one of the human isolates (cand-EWE.3.EG2015) had a closer relationship with both bovine isolates than with the other human isolate (Figs. 2 and 3). These findings may indicate a close genetic relationship between human and bovine *C. albicans* isolates as well as possible cross-species transmission in the study region or potentially a partial indication of a single source of infection and a close relationship.

Edelmann et al. (2005) declared that *C. albicans* isolates from animal species were as closely related to human strains as human strains were to one another. The authors added that there is no species specificity for *C. albicans*, meaning that one possible reason for genetic divergence between human and animal isolates is due to a low frequency of cross-species transmission. In line with this result, Wrobel et al. (2008) attributed the higher similarity between pet–human and cow–human *C. albicans* isolates to the closer contact between humans and indoor-raised pets than between barn-raised cows and the human population, which elucidates the roles of and connection between community/habits and the *C. albicans* genetic groups recovered from humans.

C. albicans isolates from humans and cattle were obtained from the Menofia governorate, the current study area, because this is an agricultural governorate with high-density human–animal populations, and many residents in rural dwellings live in close proximity to their owned cows. Additionally, a recent study in the same governorate showed a relatively high prevalence of *C. albicans* in bovine milk samples (Mousa et al. 2016). These conditions may increase the human–bovine transmission rate of *C. albicans* in the region and explain the high similarity between human and bovine isolates in this study.

The close relationship between the circulating *C. albicans* strains recorded in Egypt and the United States may be due to possible cross-species transmission of *C. albicans* between imported foreign and native cattle breeds, a risk that has been recently highlighted in the aftermath of global animal trade

and movement, either in the form of live animals or as meat and meat by-products.

Conclusions

This study reported high prevalence rates of *C. albicans* in respiratory discharge from humans and cattle suffering from respiratory distress in Al-Menoufiya Governorate, Egypt. Phylogenetic analysis of the α*INT1* gene from *C. albicans* isolates showed a high degree of genetic relatedness between human and bovine isolates in the study region. These findings highlight for the first time the public health importance of bovines as a potential source for *C. albicans* zoonotic transmission to humans in the study region. Furthermore, the study recommends the use of the α*INT1* gene as a marker to study inter- and intraspecies genetic relationships between *C. albicans* isolates from animals and humans. Further investigations are required to elucidate potential routes and risk factors associated with the human–bovine transmission cycle, which could help increase community awareness and provide a suitable prevention strategy for the potential zoonotic transmission of *C. albicans* in the study region.

Acknowledgments

The authors extend their appreciation and sincere thanks to the Chief Director and the staff members at the Al-Menoufiya Hospital for Chest Diseases for supplying clinical information and samples.

Author Disclosure Statement

No competing financial interests exist.

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