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Molecular typing and genetic relatedness of 72 clinical *Candida albicans* isolates from poultry

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ABSTRACT

Candida albicans is the most prevalent opportunistic fungus of humans and animals. While most studies focus on human isolates, they rarely focus on poultry isolates. In this study, *C. albicans* strains were recovered from poultry in the southern Hebei Province (China) and identified. Molecular typing and analyses were performed to understand the molecular epidemiology and genetic relatedness of the strains. The fungi were isolated from live birds with presumed candidiasis or their corpses. The isolates were identified based on morphology, differential medium culture, and rDNA internal transcribed spacer sequencing. The identified *C. albicans* strains were analyzed by ABC genotyping and multilocus sequence typing. Clonal groups were identified using the eBURST (version 3.0) software, and an UPGMA phylogenetic tree was constructed using the MEGA (version 6.06) software. Overall, 72 isolates were divided into three genotypes (A, B, and C), 48 novel sequence types (STs), five groups with 10 singletons, and four clades. Results indicated that candidiasis is common in poultry in the southern Hebei Province, and that the genetic composition of the *C. albicans* poultry population from the area is relatively complicated. Based on the eBURST analysis for the STs in this study and others, we suggest that *C. albicans* poultry isolates were relatively independent but not completely separated from human isolates. The strains with the same or closely related genotypes but recovered from both birds and humans could have transferred and evolved between the two types of host.

1. Introduction

Candida albicans is the most prevalent opportunistic fungus in both humans and animals. Healthy individuals are usually resistant to the *C. albicans* infection, but individuals whose immunity is impaired by certain physiological and pathological factors are susceptible. When homeostasis of a host is unbalance, the fungus increases in quantity and virulence and transforms from a harmless commensal to a pathogen capable of infecting the host. This fungus infects mammals (including humans), birds, and other organisms, when it occurs on the skin, mucous membranes, and further in the blood and viscera (Pfaller and Diekema, 2007; Kim and Sudbery, 2011).

With the increase of environmental pollution, radiotherapy, chemotherapy, and the use of wide-spectrum antibiotics, hormones, and immunosuppressant in recent years, *Candida* infection (candidiasis) rates have increased in both humans and animals. In a study by the National China Hospital Invasive Fungal Surveillance Net, 814 isolates of yeast-like fungi were recovered from monitored sites at 12 tertiary hospitals in China between August 2009 and July 2010. Among 814 strains, *Candida* accounted for 90.5% (737/814) of the isolates, and *C. albicans* was the most frequently isolated species of the genus (38.3% isolates, 282/737) (Wang et al., 2012). In another clinical study, involving four tertiary hospitals in China, *Candida* accounted for 92% of all fungal isolates obtained between March 2012 and December 2013; *C. albicans* accounted for 35% of these isolates (Dong et al., 2015). Our recent investigation of poultry farms located in the southern Hebei Province in China revealed that candidiasis occurs in the broiler, layer, duck, pigeon, quail, and ornamental birds (Liu et al., 2015). Typically, the disease persisted in every batch of broiler chicken in the same farm for several years and, once, the morbidity and mortality of a batch reached 30% and 14%, respectively (detailed in the Supporting materials: complementary Table 1). Moreover, the pathogen may survive and proliferate in the poultry farms for prolonged periods of time because of its pronounced environmental adaptability, and result in a

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successive invasion in birds under permissive circumstances. Even when the disease-associated mortality is not high, the productivity of the poultry farm decreases and the production cost increases. In addition, candidiasis is a zoonotic disease and, therefore, a pathogen originating in different sources (human and animals) may infect other host species (Hayashi et al., 1989). Therefore, the study of animal isolates of *C. albicans* is also important. To date, studies have mainly focused on the candidiasis in human but rarely in animals, especially in poultry. Animal candidiasis research in China is primarily limited to disease diagnosis, treatment, and review, but in-depth studies on pathogenic biology, molecular epidemiology, molecular typing, and pathogenesis are still lacking.

In the present study, we recovered and identified *C. albicans* strains from several types of poultry, from birds suffering from candidiasis-like disease in a defined geographic area (the southern Hebei Province) to understand the general epidemiology of poultry candidiasis in this area. ABC genotyping and multilocus sequence typing (MLST) of the isolates, together with statistical analysis, were conducted to establish the genetic relatedness between novel sequence types (STs), and between the novel and pre-existing STs. We also determined the dominant clonal groups, STs, and founders in the area; described the correlation between the ABC and MLST genotyping results; and analyzed whether the difference of genotypes related to their different source regions and host birds.

2. Materials and methods

2.1. Specimen collection

Sampling sites were selected in areas where poultry farms were most concentrated, based on the distribution of these farms, in the southern Hebei Province (China). The 42 sites covered 11 counties or cities in the Shijiazhuang, Xingtai, and Handan districts. Sampling was performed between June 2015 and May 2017, and was based on clinical and autopsy symptoms. The material from suspected sick birds was sampled by oropharyngeal swabs, anal swabs, and by crop lavage, as previously described (Brilhante et al., 2010). Dead birds were dissected, and their crops, glandular stomachs, muscular stomachs, and intestines were removed. The organs were cut open in Petri dishes and the inner surfaces of the viscera were wiped with sterile swabs. The specimens were sealed within sterile zip-lock plastic bags, tagged, recorded of epidemiological data (detailed in the Supporting materials: complementary Table 1), and taken back to lab for use.

2.2. Strain isolation

Swab samples were individually immersed in 1 mL of sterile saline in test tubes (15 mL), which were then agitated gently for two minutes. Aliquots (100 μL) of the suspension liquid from the swab samples and the lavage liquid were smeared evenly onto the YEPD agar medium containing chloramphenicol (0.5 g L^{-1}), and then incubated at 30 °C. Colonial growth was monitored daily. Approximately 3 d after the inoculation, the suspected colonies were inoculated on PDA-gentamicin (25 mg L^{-1}) medium by streak plate. Then, 3 d after incubation under the same conditions, colonial characteristics were evaluated before smearing the culture onto a glass slide, staining with Gram stain, and microscopic evaluation. Yeast-like colonies were inoculated in the Sabouraud medium in test tubes (15 mL), and incubated at 30 °C for 3 d before use.

2.3. Morphological evaluation

The suspected strains were inoculated onto YEPD agar, Sabouraud agar, and corn meal Tween-80 agar media, and incubated at 30 °C (Garcia-Hermoso et al., 2007; Brilhante et al., 2010). Yeast-like colony forms were observed after 3 d, and filamentous colony morphology after 5 d. Hyphae, blastospores, germ tubes, and chlamydospores were observed under an optical microscope (400×).

2.4. Differential culturing

Differential culture of the suspected strains was performed using CHROMagar *Candida* chromogenic media, as previously described (Odds and Bernaerts, 1994). The suspected strains were separately inoculated in the marked areas on Petri dishes and incubated at 28 °C for 72 h, and the colonial colors were differentiated. Jade green colonies were identified as *C. albicans*, and colonies of other colors were negative.

2.5. Internal transcribed spacer (ITS) rDNA sequencing

Genomic DNA of strains preliminarily identified as *Candida* using approaches described in Sections 2.3 and 2.4 was extracted using a fungal DNA extraction kit (produced by Beijing Solarbio Science & Technology Co., Ltd). The ITS1-5.8S-ITS2 rDNA sequences were amplified using a pair of universal primers, ITS1 (5′-TCCGTAGGTGAACC TGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), in a polymerase chain reaction (PCR). PCR products were purified and then sequenced by Shanghai Sangon Biological Engineering Technology Service Co., Ltd. The rDNA-ITS sequences of the evaluated strains were submitted to BLAST search at the NCBI website (http://blast.ncbi.nlm.nih.gov) for online alignment-based identification of strains with the highest similarity. Identical species were defined as sharing 99% or more identity in sequence comparisons of the evaluated and known strains (Landeweert et al., 2003). Using the BioEdit (version 7.0.9) and MEGA (version 6.06) programs, the Neighbor-Joining (NJ) phylogenetic tree was constructed by aligning the rDNA-ITS sequences of *C. albicans* isolates, and other *Candida* species.

2.6. ABC genotyping

ABC genotyping of the isolates was performed as described by McCullough et al. (1999).

2.7. MLST

Following the recommendations of Bougnoux et al. (2003) and Tavanti et al. (2003), the MLST analysis was based on introns of seven housekeeping genes of *C. albicans*, namely, AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13, and ZWF1b. The primer sets and their amplicon lengths were described in detail in the above-mentioned references. The used PCR reaction system and amplification program for the seven gene segments were as previously described (Chen et al., 2006; Moorhouse et al., 2016).

The sequencing data (by Shanghai Sangon Biological Engineering Technology Service Co., Ltd) were revised according to standard intron sequences of the seven housekeeping genes. The revised sequences were submitted to an MLST database (https://pubmlst.org/calbicans/) and allele numbers were retrieved for each strain. When no corresponding number was identified, novel allele numbers were obtained from the database curator after sequence submission. Seven allele numbers were assigned to each strain, and formed an allelic profile in the specified order. The allelic profiles were then re-submitted to the MLST database to determine the STs. When no corresponding STs were found in the database, novel ST numbers were obtained from the database curator after allelic profile submission (Alastruey-Izquierdo et al., 2013).

2.8. eBURST analysis

As described by Feil et al. (2004), STs and their allelic profiles in the MLST database were submitted in TAB to an online program eBURST (version 3.0; http://eburst.mlst.net/) for computational analysis; to