**Candida spp. in Lower Respiratory Tract Secretions – A Ten Years Retrospective Study**

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**Abstract**

**Introduction**: Lower respiratory tract secretions (LRTS) like sputum and tracheal aspirates are frequently sent to the microbiology laboratory from patients with various respiratory pathologies. Improper collection techniques can lead to false-positive results, resulting in improper therapy. **Aim of the study**: To determine the percentage of contaminated samples sent to the microbiology laboratory, to establish the prevalence of *Candida* spp. in non-contaminated samples and therefore, the presence of *Candida* spp. originating in lower respiratory tract infections. **Material and Methods**: A 10-year data survey was conducted to assess the differences in *Candida* prevalence from contaminated versus non-contaminated samples, assessed and categorised by Bartlett grading system, and to emphasise the importance of quality control for potentially contaminated samples. The data were analysed according to gender, age, referring departments, and the species of *Candida*. For the statistical analysis, Kruskal-Wallis and Fisher tests were used, and the alpha value was set for 0.5. **Results**: The prevalence of *Candida* spp. in all analysed samples was 31.60%. After excluding the contaminated samples, the actual prevalence was 27.66%. Of all sputum samples, 31.6% were contaminated. Patients aged more than 40 years old were more prone to provide contaminated sputum samples. *C. albicans* is more prevalent in non-contaminated sputum samples. In both sputum and tracheal aspirates, the chances of identifying a single species are higher than the chances of identifying multiple species. **Conclusions**: The study emphasises the importance of assessing the quality of sputum samples because of the high number of improperly collected samples sent to the microbiology laboratory.

**Keywords**: Candida, lower respiratory tract infections, contamination, laboratory diagnosis

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**Introduction**

*Candida* spp. are frequently isolated from respiratory tract secretions, but, as they normally colonise the oral cavity, the microbiological examination's clinical importance is questionable. Because *Candida* spp. are opportunistic pathogens, infections occur mainly in immunocompromised hosts. The higher risk population includes patients admitted to Intensive Care Units (ICU) because they have an impaired immune system and are exposed to various medical devices and invasive manoeuvres. Collecting lower respiratory tract secretions (LRTS) for the microbiological examination is a widely used diagnostic tool that can help identify *Candida* spp. However, interpreting the value of such examinations proves to be challenging: the yeast cells can be harmless commensal inhabitants of the respiratory tract, or they can express increased pathogenicity, by disseminating to various host niches to cause infections. The case fatality ratio of invasive infections in ICU is 45.9%, making *Candida* spp. infections a significant health problem [1].

Sample collection techniques have a high impact on the accuracy of a laboratory diagnosis. The collection
of sputum, in particular, poses challenges for patients and medical workers, and often saliva mislabelled as sputum is sent to a laboratory. Using sputum quality indicators such as the Q score or Bartlett score, samples contaminated with saliva can be ruled out after the microscopic examination [2].

Nevertheless, if a lower respiratory tract infection (LRTI) is indeed present, a sample may be ruled out because of the supplementary contamination with saliva by following the Q score alone. It is well known that culturing saliva will not provide enough evidence for diagnosing LRTIs, but despite this, the number of contaminated samples sent to laboratories is still high. Endotracheal aspirate samples are more qualitative than sputum, but endotracheal tubes are colonised quickly by oropharynx bacteria and yeast cells.

The oral mycobiome composition is dynamically modified over time, and underlying diseases can lower the threshold for opportunistic infections. Candida spp. are notorious for being part of the oral mycobiome, and the colonisation state of the oral cavity is often associated with deteriorating general health, medications, and denture wearing [3]. The whole picture of the clinical implications of the Candida spp. colonisation is not entirely understood, although there is an association between oral colonisation and the onset of infections, especially in hospitalised elderly patients [4]. Patients with prolonged hospitalisation and risk factors for invasive infections might benefit from early and appropriate antifungal therapy [5]. The economic impact of Candida spp. colonisation in patients discharged alive from the hospital was an added 12908 euros/patient, while in Spain a Candida spp. infection brings an added 21075 euros/patients in costs [6]. In the USA, Candida spp. are ranked the fourth most common pathogen responsible for bloodstream infections and the third most common in the ICU [7]. The mortality rate of candidemia ranges from 28% in young patients to 45% in elderly patients [8]. Recently, Candida spp. associate increased mortality in internal medicine yards [8, 9], raising serious concerns about new risk factors and bringing into the light the necessity for diagnostic algorithms that can help select patients who would most benefit from preventive antifungal treatment.

With a reported global emergence of non-albicans species [10, 11], monitoring fungal infections is crucial, especially for ICU patients, as intubation and mechanical ventilation are risk factors for Candida spp. colonisation.

The study aimed to:
- Determine the percentage of contaminated samples sent to the microbiology laboratory.
- Establish the prevalence of Candida spp. in non-contaminated samples and therefore, the presence of Candida spp. originating in lower respiratory tract infections.

## Materials and Methods

From the complete data base of reports of the Microbiology Laboratory, Mures Clinical County Hospital, between Jan 1, 2009, to 31th of December 2018, all reports on respiratory tract samples, sent to and processed in the microbiology laboratory were scrutinised for Candida spp. growth. Of these, 3199 sputum and tracheal aspirates were selected for inclusion in the study. Incomplete reports were excluded.

The sputum samples were further scrutinised for the sample’s quality and assessed using the Bartlett (Q) grading system. The presence of leukocytes, mucus and squamous epithelial cells was observed under the microscope in 10 low power fields (LPF) (<10 neutrophils/LPF = 0; 10-20 neutrophils/LPF = +1; > neutrophils/LPF = +2; the presence of mucus = +1; 10-25 squamous epithelial cells/LPF = -1; > 25 squamous epithelial cells/LPF = -2). A final score value of less than 1 indicates significant salivary contamination of sputum, and these samples were considered and designated as “contaminated”. A final score of 1 and above was considered to indicate a “non-contaminated” sputum sample, with marks of inflammation.

The tracheal aspirates were considered non-contaminated products for microbiological analysis, due to the aseptic way of harvesting.

The non-contaminated sputum samples plus all tracheal aspirates showing Candida spp. were considered relevant for diagnosing lower respiratory tract infection associated with Candida spp. (Figure 1).

Candida spp. were identified by culturing on Sabouraud agar. The samples that showed fungal growth were then subcultured on selective chromogenic agar for species identification. In selected cases, an automated system of identification (VITEK 2C) was used.

The results were analysed considering gender, age, referring departments, Candida species (C. albicans, C. non-albicans, and C. albicans + C. non-albicans).
Patients were categorised into the different risk groups according to the wards to which they had been admitted (Oncology, Critical Care, medical wards – Gastroenterology, Cardiology, Nephrology, Internal Medicine, Infectious Diseases), and surgical wards – General Surgery, Plastic Surgery).

The obtained data were analysed using the Kruskal-Wallis test to compare mean values and the Fisher test for nominal data. The alpha value was set for 0.05.

**Results**

The percentage of contaminated sputum samples testing positive for *Candida* spp. was 31.60% (164 out of 519 analysed sputum samples). Of the total of lower respiratory tract samples (sputum plus tracheal aspirates), the prevalence was 35.76% (1144 out of 3199 samples). After excluding the contaminated samples, the recalculated prevalence of *Candida* spp. in lower respiratory tract samples was 27.66%.

The prevalence in all lower respiratory tract samples ranged from a minimum of 26.94% in 2012 to a maximum of 43.45% in 2011 (Figure 2).

The total number of samples tested positive for *Candida* spp., 613 (53.58%) were sputum samples, and 531 (46.42%) were tracheal aspirates. Based on the quality of sputum samples, 164 (31.6%) were assigned a Bartlett score <1 and were excluded from further analysis, while 355 (68.40%) were assigned a Bartlett score ≥ 1 and considered non-contaminated.

From the total number of samples:
- 607 (53.06%) were collected: 531 (86.72%) tracheal aspirates and 76 (13.10%) sputum samples;
- 366 (31.99%) sputum were collected from patients admitted to medical wards;
- 114 (9.97%) sputum were collected from oncologic patients;
- 39 (3.41%) sputum were collected from surgical wards;
- For 18 samples (1.57%), the location could not be established.

In LRTS considered to be non-contaminated, a statistically significant difference was observed between the average age of the patients that showed colonisation with *C. albicans* and *C. non-albicans* in LRTS (Kruskal-Wallis Test; p < 0.05) (Table 1).

Comparing tracheal aspirates with sputum samples that showed *Candida* spp. growth, statistically significant differences were observed between patients aged less or more than 60 years old, between *C. albicans* and *C. non-albicans*, as well as between the number of identified yeast species (Fisher test; p< 0.05) (Table 2). The chance of identifying *Candida* spp. is higher in patients...
aged over 60 years old, especially from tracheal aspirates. *C. albicans* is more prevalent in sputum samples.

Comparing tracheal aspirates with sputum samples that showed *Candida* spp. growth, statistically significant differences were observed between patients aged <= or > than 60 years old, between *C. albicans* and *C. non-albicans*, as well as between the number of identified yeast species (Fisher test; p < 0.05) (Table 2). *Candida* spp. are more prevalent in male patients, and are more often identified in sputum samples. The chances of identifying one species are greater in both sputum and tracheal aspirates.
Patients aged more than 40 years old were more prone to provide contaminated sputum specimens (Fisher test; p<0.05) (Table 3). No statistical significance was observed between patients aged less than or equal to or greater than 60 years old or between patients aged less than 80 or equal to or greater than 80 years old (Fisher test; p>0.05).

Comparing the contaminated sputum samples, with the sputum samples considered non-contaminated, statistically significant differences were observed between the diagnosis of C. albicans and non-albicans species, as well as in the number of identified isolates (Fisher test; p<0.05) (see Table 3).

Significantly, more albicans species were found in non-contaminated sputum samples, mostly as single species.

**Discussion**

Pneumonia with Candida spp. is considered a secondary effect: usually an immunocompromised patient has a primary infection site (e.g. the skin, the gastrointestinal tract) from which Candida cells disseminate through the bloodstream and infect the lungs. Because the fungal serology often remains negative, these cases are seldom considered, and the definitive diagnosis is made post-mortem, in the pathology department [12]. However, in a two-year study conducted in an adult ICU, no single case of confirmed Candida pneumonia was reported [13].

Despite the frequent identification of Candida spp. in respiratory tract secretions, pneumonia with Candida spp. is rare, and positive cultures are most probably due to colonisation [13, 14]. Should these results be reported? If yes, how should they be interpreted? Barenfanger and al. (2003) showed that over 70% of the clinicians do not consider Candida spp. in respiratory tract secretions and rarely initiate antifungal therapy [15]. The mortality in patients with positive cultures with Candida spp. was not influenced by antifungal therapy, so some researchers consider that a limited identification can decrease the length of stay in the hospital, reduce costs, and limit the unnecessary usage of antifungal agents [15, 16]. On the one hand, higher antifungal usage is associated with increased resistance rates [17], supporting the necessity of limiting unnecessary antifungal treatment. On the other hand, the state of colonisation with Candida spp. is associated with prolonged hospitalisation in ICU and increased costs, probably because of the toxic effect of antifungal drugs [18].

Over half of the samples included in the current study came from the hospital's ICU, from patients with impaired immune systems that are, theoretically, prone to Candida spp. pneumonia. The rarity of such reported cases in the literature suggests that a positive LRTS sample indicates the state of colonisation with Candida spp. Although the respiratory tract was considered aseptic in the past, the bronchial tree has a characteristic mycobiota that plays a role in the development of lung pathology [19]. The respiratory tract's mycobiota is still highly
enigmatic, so the yeast cells come, most probably, from the oral cavity [20]. The high prevalence of *Candida* spp. in LRTS raises questions about the collection methods used for collecting such samples, and positive results are difficult to interpret from a clinical perspective.

In the current study, even after excluding the samples considered contaminated, according to the Bartlett score, the prevalence of *Candida* spp. in respiratory tract secretions was high. How effective are the protocols used to assess sputum quality? *Candida* spp. associated pneumonia is extremely rare. Nevertheless, in the current study, the number of *Candida* spp. isolates was high, in spite of all contaminated samples being excluded.

Multiple criteria can be used in a laboratory for judging the quality of sputum samples [21–23], with different reproducibility rates [2]. An accurate and reliable microbiological examination is more than desirable. Although initially it was thought that sputum Gram stain could not provide enough information in the absence of culturing the sample, this might not be entirely true. Gram staining can play a key role in diagnosing pneumonia, and the Bartlett score can predict true lower respiratory tract infections. However, regarding whether samples are contaminated or not, there is only a moderate agreement when the results of Gram stain and sputum sample-culturing are compared [24].

In a study conducted by Gunasekaran et al. (2019), 40% of the analysed sputum samples were of good quality, as assessed by the Bartlett score [24]. In a study by Fukuyama et al. (2014), the percentage of good quality samples was 71.3%. However, the sputum samples in this study were not obtained exclusively by expectoration, but also by nasotracheal suction. In some centres, clinicians are trained in microbial examination. They can perform a Gram stain in their routine clinical practice, which raises the number of good quality samples sent to the laboratory [25]. In our study, a comparable percentage (68.4%) of the sputum samples was of good quality and shown to be non-contaminated, although clinicians are not trained in microscopical screening methods in Romania.

Compared with bacterial isolates, the presence of *Candida* spp. in respiratory tract secretions, might be associated with systemic inflammation, and it might imply a poorer clinical outcome [18]. This may be due to the β-glucan, a fungal cell wall component that can activate the immune response [21]. However, in a prospective observational study on 598 patients, Williamson et al. (2011) contradict the association of inflammation with the presence of *Candida* spp. in respiratory tract cultures. Patients with positive cultures for *Candida* spp. had similar inflammation levels as the control group, but their clinical evolution was worse [18]. Terraneo et al. (2016) concluded that even if *Candida* spp. in RTS is associated with the initial severity of the cases, its identification does not influence the outcomes, regardless of the antifungal usage [22]. The association between *Candida* colonisation and inflammation and its impact on the medical cases’ clinical evolution remains highly controversial.

The mycological examination of LRTS can help predict the risk of developing severe *Candida* infections as a Colonization Index (CI) (number of positive sites / number of tested sites) greater than 0.5 indicates an increased risk of deep-seated fungal infections and may justify antifungal therapy. Blood cultures are never taken into consideration while calculating the CI [23, 26, 27].

It is well known that cross-kingdom relationships between bacteria and fungi can impact the host metabolism and immunity, hence playing a role in the onset and development of diseases [28]. For patients admitted to ICU, *Candida* spp. airway colonisation might increase the risk of developing ventilator-associated pneumonia (VAP) with multi-drug resistant bacteria. It might even worsen the evolution of VAP regardless of the primary causative agent [29]. For example, *Candida* spp. is often isolated next to *Pseudomonas aeruginosa*. A study on mice showed that *C. albicans* inhibited reactive oxygen species (ROS) production in alveolar macrophages and the rats that were colonised with *C. albicans* had an increased risk of developing VAP with *P. aeruginosa* [30]. In mixed biofilms, the interspecies competition between *C. albicans* and *P. aeruginosa* results in increased virulence factors and an enhanced mutability [31]. However, the relationship between *C. albicans* and *P. aeruginosa* is a complicated one: in an *in vitro* dual environment, they mutually suppress a biofilm development, which can be defined as antagonism. *In vivo*, either species can alter colonisation by the other, favouring or inhibiting disease [32, 33]. Some authors consider the usage of antifungals for reducing the risk of VAP in patients colonised with *Candida* spp. [34]. However, such studies have severe limitations, and clinical trials are needed to become a standard of practice.
In a prospective study on 3648 patients with candidemia, Pfaller et al. (2012) [35] the 90-day survival rate was 61.3%, candidemia occurring mainly in immunosuppressed hosts, i.e. patients with concurrent bacterial infections, corticosteroid therapy, mechanical ventilation, diabetes, and patients that underwent non-implant related surgeries during hospitalisation. Whether the source of the infection is endogenous or exogenous is debated in the literature. Cross-contamination between colonised and un-colonised patients is not documented, so invasive Candida spp. has an endogenous start point. Calculating the CI can help practitioners detect patients at high risk for developing candidemia [27]. In studies of autopsy-proved invasive Candida spp., ante-mortem blood cultures were positive in 21-71% of the cases. Nonculture diagnostic methods are not ready to be implemented into the current standard of practice, and blood cultures are an excellent diagnostic method for invasive infections, at the moment [36]. In this context, calculating the CI might have clinical value, especially for patients with impaired immune systems.

The risk of developing Candida infections increases with the number of underlying comorbidities like HIV infection, malignancies diabetes mellitus, chronic obstructive pulmonary disease, chronic liver disease, and severe heart failure. Also, the previous usage of antifungal and/or antibiotic drugs, corticosteroids, undergoing surgical procedures, parenteral nutrition, dialysis, mechanical ventilation, or septic shock favour the onset of infections [37]. The oral colonization with Candida spp. in diabetic patients may play a role in how the immune system will later respond to glucose intake [38]. Next to candidiasis, oral diseases are the most common infections in diabetic patients [39] and diabetic patients with poor glucoregulation are more often carriers of Candida spp. [40].

The present study emphasises the improper collection of sputum, as the number of saliva samples sent to the laboratory is still high. Although sputum should be sterile in the lack of infections, in reality, the collection of sputum through expectoration permits microorganisms, living in the oral cavity, to contaminate LRTS. Patients admitted to an ICU are often intubated, and around 45% of the intubated patients became colonised with Candida spp. after 48 hours [41]. It is accepted that prolonged ICU hospitalisations, the number of invasive manoeuvres a patient is receiving, bacterial sepsis, and exposure to broad-spectrum antibiotics are risk factors for Candida spp. colonisation [42].

The oral mycobiome composition has received scant attention, mainly because many fungi are difficult to culture. Although the composition and, most importantly, the oral mycobiome’s role is a work in progress, Candida spp. are the most studied and generally considered the most important of the mycobiome. The oral mycobiome changes with time, and it is influenced by factors such as the salivary flow rate, the Body Mass Index, and geographical background [32]. To colonise the oral cavity, the yeast cells have to first attach to epithelial cells. Proteins like als 1-7, als 9, eap1, hwp1, pga1 mediate the attachment process, thus playing a pivotal role in colonising a host niche. To increase the survival chances, Candida cells can form biofilms, complex heterogeneous structures from which the fungal cells can detach and generate novel communities in other body niches [43].

Endotracheal tubes become contaminated with oral cavity flora within twenty-four hours of intubation, and a longer incubation time increased the proliferation rate of microorganisms on such devices [44]. As proven in vitro, Candida spp. can form biofilms in a short timeframe: in approximately eleven hours microcolonies can be observed. The intermediate phase of biofilm formation takes 12-30 hours, and the maturation stage takes approximately 38-72 hours [45]. In vivo, biofilm formation starts even sooner, within eight hours [46]. After the adhesion to a substrate, the fungal cells proliferate and are further engulfed in an extracellular matrix. As opposed to planktonic cells (free-floating cells), sessile cells (part of the biofilm) are more resistant to various host defence mechanisms, either by altering the host’s immune response or by exhibiting epigenetic resistance to antimicrobials. The cells released from a preformed biofilm have an increased virulence [43, 47, 48]. If neutrophils release NETs (extracellular traps) in response to planktonic Candida cells, the extracellular matrix impairs NET production [49]. C. albicans cells dispersed from biofilms are developmentally distinct compared to planktonic cells: they have enhanced virulence-associated characteristics like adherence and filamentation. They are transcriptionally reprogrammed to acquire nutrients and metabolise alternative carbon sources [50, 51].

As aging is associated with changes in the oral cavity’s ecology, due to medications, denture wearing, or a deteriorated general health status, the increased number of LRTS samples collected from patients aged over 60 years old meets the expectations. The oral microbi-
ome of the elderly consists of a high level of staphylococci, Candida spp., lactobacilli, and actinomycetes [3].

**Conclusions**

The sample collection methods play a crucial role in ensuring that patients are correctly diagnosed and provided with the appropriate treatment. Given the high percentage of contaminated sputum samples, the study results emphasize the significance and need for high-quality procedures when collecting specimens. Furthermore, the results indicated the feasibility and benefits of microscope screening methods in respiratory tract secretions' routine workouts. Every clinical case should be judged on its own merits.

**Author Contributions**

Conceptualization, C.N.C., A.D.M., F.T., A.M.; methodology, C.N.C., A.D.M., A.M.; validation, C.N.C., A.S., A.D.M., A.M., B.K, F.T., C.V., I.P.; formal analysis, C.N.C., A.S., A.D.M., A.M., B.K; investigation, C.N.C, B.K., C.V., I.P.; resources, C.N.C., A.D.M., A.M., F.T.; data curation, C.N.C, B.K., C.V., I.P.; writing—original draft preparation, C.N.C., A.D.M., A.M.; writing—C.N.C., A.S., A.M.; visualization, C.N.C., A.S., A.D.M., A.M. B.K, F.T., C.V., I.P.; supervision, A.M.; project administration, CNC, A.M.; funding acquisition, C.N.C. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**Ethical Approval**

The ethical approval was waived by the Ethics Committee of Mures County Clinical Hospital, Decision no 15596/19.10.2018 in view of the retrospective nature of the study and all the procedures being performed were part of the routine care.

**Data**

Data available on request from the authors.

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