Prevalence of yeasts in diarrheic calves with molecular Identification of *C. albicans*


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Abstract

This study focused on determining the frequency and morphological identification of yeast involved in unresponsive calves’ diarrhea by antibiotics, with molecular identification of *C. albicans*. *C. albicans* the most frequently isolated yeast in this study and its Phospholipase B1 (*PLB1*) gene as a virulence factor. One hundred fecal samples from diarrheic calves were investigated, 49 strains of yeast were isolated. Thirty four different strains (69.3%) classified as *Candida* were identified based on phenotypic properties. The dominating species were *C. albicans* (20.4%) Followed by *C. glabrata* (16.3%), *G. candidum* (14.3%), *C. krusei* (12.2%), *C. tropicalis* (10.2%), *C. guilliermondii* (10.2%), *R. rubra* (8.2%) and *C. neoformans* (8.2%). seven out of ten strains phenotypically identified as *C. albicans* were confirmed by PCR using specific primers for the amplification of a fragment of the rRNA gene of *C. albicans*. Phospholipase B1 gene a virulence determinant of *C. albicans* was detected in all molecularly identified *C. albicans* isolates. The results of the present study indicate the importance of PCR as more rapid, effective and reliable tool for screening of yeast spp. than the phenotypic methods. When confronted with cases of undifferentiated calf diarrhea to avoid unnecessary and potentially harmful antibacterial therapy.

**Key words**: Diarrhea, Yeasts, *C. albicans*, Calves, PCR.

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1. Introduction

The neonatal diarrhea remains one of the main causes of calf morbidity and mortality causing major economic losses in many dairy and beef herds. Calves are at greatest risk of developing diarrhea during the first month of life, and the risk then decreases with age [1,2]. The mortality rate was reported to vary between 1.5% and 8% [1], although rates up to 25% have been also described [3]. Furthermore, diarrhea in young calves has been found to increase the risk of other diseases later in life [4].

Diarrhea in dairy calves has a multifactorial etiology, in which of a number of variables, including pathogen (viruses, bacteria, protozoa), animal (immunological and nutritional status), environmental and management factors (housing, feeding, hygienic conditions) play a role [1, 5]. Several infectious or nutritional factors may occur either alone or in synergy and among the different infectious agents. One of the most common causes of calf death is acute neonatal diarrhea due to pathogenic agents such as rotavirus, coronavirus, and Escherichia coli bacteria [1, 6, 7, 8, 9]. Enterotoxigenic Escherichia coli, Cryptosporidium parvum, rotavirus and coronavirus are usually seen as the most common infectious causes of neonatal calf diarrhea [10].

The fungi, particularly yeasts and moulds are always neglected, although they are well known to cause diseases of all animal species.
predisposing by their insensitivity to antibacterial antibiotics, so they usually flourish following prolonged antibiotic therapy.

Yeast and molds are sometimes associated with lesions in the stomach or intestines of scouring calves. These organisms are not considered a primary cause of scours, but rather secondary invaders. Often they are found when scouring calves are subject to overuse of antibiotics and very little was done to counteract dehydration by using fluids and electrolytes [11]. Calves with forestomach candidiasis have watery diarrhea, anorexia, and dehydration, with gradual progression to prostration and death [12]. Fecal samples and intestinal contents of pre-weaned calves contain yeasts, among which *Candida glabrata* has been shown to be the most prevalent [13, 14], the abomasums, the target organ for *C. glabrata* colonization. It has been suggested that the presence of yeasts in the gastrointestinal tract (GIT) might be associated with neonatal calf diarrhea [14] in naturally infected animals.

*C. albicans* Phospholipase B1 gene is considered important virulence determinants [15], and could potentially facilitate increased penetration of fungal hyphal elements by directly damaging host cell membranes. Identification of this increasing diversity of pathogens by conventional methods is often difficult and sometimes inconclusive [16]. Morphological features and reproductive structures useful for identifying isolated yeast and fungi may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology [17]. Molecular techniques utilizing amplification of target DNA provide alternative methods for diagnosis and identification [18]. PCR-based detection of fungal DNA sequences can be rapid, sensitive, and specific [19].

The aim of the present study was to isolate, identify, and determine the prevalence of yeast in fecal samples from calves suffering from diarrhea unresponsive to antibiotics treatment with molecular based identification of *C. albicans* isolates and its phospholipase B1 gene as a virulence determinant factor.

2. Material and Methods

2.1. Samples

Fecal material was collected from the rectum of calves by direct digital rectal stimulation using a disposable latex glove. A total of 100 fecal swabs were collected from 100 diarrheic neonatal calves, with ages up to 60 days. The age, clinical signs and case history for each sampled calf were recorded at sampling. The swabs were transported to the laboratory on ice and processed the same day. Samples were refrigerated (4°C) during transportation to the laboratory and kept at 4°C until processing. Fecal samples were incubated for 15 minutes at 25°C and homogenized feces were inoculated in 4.5 ml of Sabouraud's Dextrose Broth at pH 3.5, and then incubated at 37°C during 10 days. Thereafter, 50 µl of each broth culture was plated on Sabouraud’s Dextrose Agar (SDA) with Chloramphenicol (0.05 mg/ml).

The plates were incubated at 37°C and examined for growth at 24, 48 and 72 hours and at weekly intervals for 4 weeks, after which the plates showing no growth were considered negative [20]. The colonies were picked up and re-streaked on another SDA plate to get the pure cultures. These fungal isolates were studied for their cultural and morphological characteristics. The morphological characteristics were noted after staining with Gram's and Lacto phenol cotton blue stain. Isolates were identified by different sugar fermentation tests.

2.2. Identification of isolated yeast species

2.2.1. Morphological identification

Colonies appearing as yeast-like in morphology were examined by the Dalmau technique [21] for microscopic characteristics. A total of 55 (55%) yeast strains were preliminarily grouped based on their culture morphology and physiological characteristics. Yeast identification was performed taking into consideration morphological characteristics, like formation of chlamydoconidia, pseudohyphae and germinal tube development. Additional characteristics were also evaluated, such as growth in the presence of 0.1% cyclohexamide (Sigma-Aldrich), acidic pH tolerance, urea hydrolysis and carbohydrates assimilation and/or fermentation (glucose, maltose, trehalose, galactose, xylose, lactose, sucrose) accordingly to the methodology described by [18].

2.2.2. Molecular Identification of *C. albicans*

Total chromosomal DNA isolated using Analytik-jena extraction kit, Germany according to the manufacturer’s instructions from *C. albicans* was subjected to PCR amplification. The primer set was designed and
2.3. Detection of the PLB1 in C. albicans isolates

A 751 bp DNA fragment containing the PLB1 gene was amplified with two primers as described by [23]. The sequences of the oligonucleotide primers were as follows: 5′-TGTGCTCTCTCGGGGCGGCGCCG-3′ and 5′-AGATCATATTGCAACATCTAGTTAAATTC-3′. DNA samples were amplified in a total of 25 μl reaction mixture consists of 12.5μl PCR Master Mix (2X) Go Tag Green (Promega, USA), 1μl of each (10pmole) primers (Biobasic inc. company, Canada), 5μl template DNA and 5.5 μl nuclease-free water. The cyclic parameters for PCR amplification were initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified PCR product was electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA buffer. A 100-bp DNA ladder (Invitrogen, USA) was used as a molecular weight marker.

3. Results

3.1. Prevalence of yeast in calves with diarrhea

Out of 100 fecal samples analyzed from diarrheic calves, 49 yeast species were isolated (Table 1). Based on traditional methods (sugar fermentation, assimilation, culture on rice agar medium, tobacco agar medium and germ tube formation), for identification of different yeast species, it was found that Candida isolates were obtained in 69.3% (34/49) from which 5 different species were identified. The species most frequently found was C. albicans (20.4%) followed by C. glabrata (16.3%), C. krusei (12.2%) and C. tropicalis (10.2%), C. guilliermondii (10.2%). Other yeast species were isolated with an incidence 30.7% (15/49) including G. candidum (14.3%) R. rubra (8.2%) and C. neoformans with an incidence (8.2%).

3.2. Identification of C. albicans isolates using PCR:

In this study specific PCR based on species universal primer was used to confirm phenotypic identification of C. albicans. Out of ten PCR tested strains previously identified morphologically as C. albicans, seven strains yield predicted 175 bp DNA fragments (Fig. 1).

3.3. Detection of PLB1 gene of C. albicans by PCR:

For detection of the PLB1 gene (using a specific pair of primers amplifying 751bp) as the most important contributory factor for phospholipase activity of C. albicans, PCR produced a DNA fragment of 751 bp in all molecularly identified C. albicans strains (Fig. 2).

4. Discussion

Diarrhea is the most important disease in young calves and accounts for approximately 75% of the mortality of dairy calves within the first 3 weeks of age [24]. There is little information about mycotic diarrhea in calves and the prevalence of calves diarrhea related to fungi is usually low as compared with other agents. However, sometimes they have been associated with clinical disease in calves. Yeast and molds are sometimes associated with lesions in the stomach or intestines of scouring calves. These organisms are not considered a primary cause of scour but rather secondary invaders. Very often they are found when scouring calves are victims of overuse of antibiotics or sulfas when very little was done to counteract dehydration by using fluids and electrolytes.

The present study indicated that Candida species alone contribute 69.3% of the total yeast isolated from diarrheic calves. Among Candida isolates, C. albicans was the most frequently recovered species (20.4%) of all positive diarrheic samples. Our results confirmed the
findings reported by [25], these authors isolated C. albicans from the contents of the small intestine and from other organs from a calf that died from the disseminated candidiasis. A higher percentage of C. albicans isolation (25.9%) from calves with diarrhea had also been reported by [26].

In our study, C. glabrata were detected from calves with diarrhea in 16.3% from total yeast, which is in agreement with the results of previous studies [14, 27]. It is known that the phenotypic characterization of yeasts can lead to errors due to the fact that several species present similarities in their morphologies and biochemical/physiological characteristics used in the conventional identification of yeasts. Therefore, the most reliable tests for rapid identification of Candida spp. and, especially, for C. albicans are based on molecular techniques. In the present study, ten strains previously identified morphologically as C. albicans were tested using species universal primer that could distinguish individual C. albicans. Out of ten C. albicans strains, only seven strains yield DNA fragments of the predicted size. The PCR negative strain could be C. dubliniensis as the phenotypic methods for the identification of Candida spp. are often unable to discriminate C. albicans and C. dubliniensis, and no single phenotypic test has proven to be highly effective in the distinction between C. albicans and C. dubliniensis, and genotype tests may be necessary for definitive identification [28].

The validation of PLB1 gene as a virulence factor in C. albicans by using animal models of hematogenously disseminated and gastrointestinal Candidiasis was studied by [29], the PLB1 gene appears thus far to be the single most important contributory factor for phospholipase activity of C. albicans [15, 29]. Many authors like [30, 31, 32] used PCR for detection of phospholipase activity. In the current study, all isolates of genetically identified C. albicans were identified PLB1 positive by PCR. Candidal virulence factors such phospholipases have attracted interest as a possible means for developing novel therapeutic interventions against candidiasis [15]. Another potential use of fungal extracellular phospholipases, particularly candidal PLB, is as a diagnostic tool. Since it has been demonstrated that candidal PLB is released during the progression of candidal infection in murine models of candidiasis [29]. Phospholipase B possesses a number of advantages which make it attractive for development as a diagnostic tool: (i) it is a naturally secreted protein and not a cell wall or cytoplasmic component [33] and (ii) it is a purified well-defined antigen rather than a crude extract containing cell wall polysaccharide [34]. In conclusion, this study found a relatively higher incidence of yeast isolated from diarrheic neonatal calves in which Candida species especially C. albicans are the most common microorganisms among the mycotic agents. We suggest that the intensive antibiotic treatment predisposed to, or exacerbated, the Candida infections. The phenotypic methods for the identification of Candida spp., although simple and inexpensive, but these methods are time consuming and for more often unable to discriminate C. albicans and other Candida species. Therefore, the most reliable test for rapid identification of Candida species are based on molecular techniques. PLB1 provides new attractive therapeutic and diagnostic targets for mycotic diarrhea caused by C. albicans.

6. References


[26] H. S. Abd El-Hakeem, “Mycological studies on Cr. neoformans and other yeasts isolated from clinical cases and environment” M.V.Sc. thesis, Department of Microbiology, Faculty of Veterinary Medicine, Cairo Univ. 2005.


Fig. (1): Amplification of 175 bp by PCR for detection of C. albicans. Lane M: 100 bp ladder, lanes 1, 2, 3, 5, 7, 8 and 10 showed positive samples, while lane 4, 6 and 9 were negative, lane 11 was control negative.

Fig. (2): Amplification of 751bp by PCR for detection of PLB1 gene. Lane M: 100 bp ladder, lanes 1, 2, 3, 4, 5, 6 and 7 showed positive samples.

Table 1: Out of 100 fecal samples analyzed from diarrheic calves, 49 yeast species were isolated

<table>
<thead>
<tr>
<th>Yeast isolates</th>
<th>Fecal samples</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td>C. albicans</td>
<td>10</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>8</td>
</tr>
<tr>
<td>C. krusei</td>
<td>6</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>5</td>
</tr>
<tr>
<td>C. guilliermondii</td>
<td>5</td>
</tr>
<tr>
<td>G. candidum</td>
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<tr>
<td>R. rubra</td>
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<tr>
<td>C. neoformans</td>
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<tr>
<td>Total</td>
<td>49</td>
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