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PROFILING AND SPECIATION OF *CANDIDA* SPECIES BY USING HICHROME AGAR FROM VARIOUS CLINICAL AND POSTMORTEM SAMPLES IN LIVESTOCK AND POULTRY

G. KALAISELVI[1]*, G. BALAKRISHNAN[2], RAMAN[3], R.RAMYA[4], R. SAAHITYA[4] AND C. SOUNDARARAJAN[5]

Central University Laboratory, Center for Animal Health Studies
Tamil Nadu Veterinary and Animal Sciences University, Chennai, T.N., India
[1,2,4,]Central University Laboratory, Centre for Animal Health Studies, Chennai, T.N., India
[3]ADIU, Tiruvannamalai, Tamil Nadu, India
[5]Centre for Animal Health Studies, TANUVAS, Chennai, T.N., India

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***Key words**: Candida species, Candida albicans and non Candida albicans, Livestock and poultry, ABST, Biofilm*

Abstract–Animal Candidiasis is associated with oral and upper respiratory disease, pyothorax, ocular lesions, intestinal disease and urocystitis in dog and cats and causes arthritis in horses and mastitis and abortion in cattle. The present study was undertaken to determine the prevalence of *Candida* species among various clinical and post mortem samples of livestock and poultry. All samples were collected using aseptic precautions. After receiving in the Central University laboratory, the samples were inoculated onto both Blood agar and MacConkey agar, XLD agar and SDA agar and plates were incubated at 37 °C for 24 hours aerobically. The colonies appeared as smooth, pasty, opaque, white or beige were suspected as *Candida* colonies and Gram stain was done for confirmation. The speciation of the *Candida* isolates was performed by inoculating it on Hicrome Candida differential agar. The Hicrome agar was prepared as per the manufacturer's instructions and incubated at 37 °C for 24 hours aerobically. The antibiotic sensitivity test was performed with Highrome agar plate. The pure culture of *Candida albicans* were streaked in hichrome agar plate and antibiotic disc were placed in appropriate distance and incubated at 37 °C for 24 hours aerobically. Biofilm study of yeast was performed with Sterile 96-well polystyrene plates which were inoculated with 200 µl bacterial suspension (105 CFU/ml) in BHI medium and incubated at 37 °C for 24 hrs without shaking. Species identification of Candida was done by the morphology and color of the colonies. The *Candida albicans* produce light green colonies, *C. tropicalis* metallic blue colonies, C.krusei produces purple fuzzy colonies, and *C. glabrata* white to cream-colored colonies. Out of 298 *Candida* isolates, *C. Albicans* was the most common species in 210 (70.46%) strains. The remaining 88 (29.5%) strains showed *Non-albicans Candida*. Out of 88 *Non-albicans Candida* isolates, *Candida* isolates, 20 were *C. tropicalis* (23%), 28 were *C. glabrata* (32%), 18 were *Candida parapsilosis* (20%) and 22 were *C. krusei* (25%) respectively (Table 1). Out of 298 isolates from oronasal swab (HVS), the most common species was *C. Albicans* followed by *C. tropicalis, C. glabrata, Candida parapsilosis* and *C. krusei*. Among the 16 urine samples, the most common species was *C. Albicans* followed by *C. tropicalis*, *C. glabrata,* and *C. krusei* were isolated.

INTRODUCTION

Candidiasis is a one of the common yeast infection localized in mucocutaneous disease of livestock and poultry. In the recent years, the incidence of mycotic infections has progressively increased among the livestock and poultry. Fungi once considered as nonpathogenic or less virulent are now recognized as a primary cause of morbidity and mortality in immune compromised and severely ill patients and animals (Amit *et al*., 2015)

C albicans is an opportunistic pathogens t of the nasopharynx, GI tract ear and external genitalia of many species of animals. Some immuno suppressive diseases, drugs, disruption of mucosal integrity, indwelling intravenous or urinary catheters and administration of antimicrobials are the predisposing factors for candida infection. The

([1]Assistant Prof., [2]Prof. and Head, [3]Assistant Director, [4]Assistant Prof., [5]Director)

candida most frequently infects birds involving the oral mucosa, esophagus and crop and superficial infections limited to the mucous membranes of the intestinal tract in pigs and foals. The systemic candidiasis has also reported in cattle, calves, sheep, and foals associated with secondary to prolonged antibiotic or corticosteroid treatment.

Animal Candidiasis is associated with oral and upper respiratory disease, pyothorax, ocular lesions, intestinal disease and urocystitis in dog and cats and causes arthritis in horses and mastitis and abortion in cattle. The clinical signs are variable and nonspecific and are often more associated with the primary or predisposing conditions than with the candidiasis itself. In birds, crop and esophageal lesions are circular white ulcers with raised surface scabs that produce thickening of the mucosa and easily removable pseudomembrane. The candida infected chicks are listless and have decreased feed intake and growth rate. Gross lesions of the skin and mucosae in other species are generally single or multiple raised circular white masses covered with scabs. The organism can penetrate keratinized epithelium and cause marked thickening of the mucosae of the tongue, esophagus, and rumen. In case of calves with fore stomach candidiasis have watery diarrhea, anorexia, and dehydration, with gradual progression to prostration and death. The Porcine candidiasis affects mostly oral, esophageal, and gastric mucosa with diarrhea and emaciation the most consistent clinical signs. Foals with oral and esophageal candidiasis may show an almost terrycloth-like texture to the tongue and oral mucosa. The gastro intestinal mucocutaneous candidiasis may have a characteristic sour or yeasty odor. Urinary candidiasis may occur in cats and rarely in dogs, particularly those with perineal urethrostomies or indwelling urinary catheters.

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and reproductive system and are responsible for various clinical manifestations from simple mucocutaneous overgrowth to invasive infections like bloodstream infections which is due to their great adaptability to different host environment (Das *et al*., 2016). In early years *C. Albicans* accounted for more than 80% of all *Candida* isolates recovered from yeast infections but recently *Non-albicans Candida* (NAC) species have been recovered with increasing frequency (Deorukar *et al*., 2018 and Dharmeshwari *et al*., 2014) so isolation and prompt identification of the infecting organism to the species level is essential to optimize the early antifungal therapy as certain species like *C. krusei* are inherently resistant to antifungal azole drugs (Deorukar *et al*., 2014 and 2018). The several chromogenic substrates containing culture media have been developed for differentiating Candida species. Hicrome agar is a differential media that allows selective isolation of yeasts and identifies colonies of *C. Albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis* and helpful for early diagnosis is essential for initiating appropriate therapy (Forbes *et al*., 2007). The present study was undertaken to determine the prevalence of *Candida* species among various clinical and post mortem samples of livestock and poultry.

MATERIALS AND METHODS

This fungal isolation studies study was conducted in the Central University Laboratory, Centre for Animal Health studies, TANUVAS, Chennai for a period of 2 years April 2021 to June 2023.

The study includes *Candida* isolates from various clinical samples and post mortem samples of livestock poultry sent routinely to the Central University Laboratory. All samples were collected using aseptic precautions. After receiving in the Central University laboratory, the samples were inoculated onto both Blood agar and MacConkey agar, XLD agar and SDA agar and plates were incubated at 37 ^{o}C for 24-48 hours aerobically. Colonies that appeared smooth, pasty, opaque, white, or beige were suspected as *Candida* colony and Gram stain was done for confirmation.

The growth obtained on SDA was further subjected to Gram staining and germ tube test. The Germ tube test was done to differentiate *C. albicans* and *C. dublinenses* from other *Candida* species. The isolated colony of Candida was suspended in 0.5 ml of serum and was incubated at 37 ^{0}C for 3 hours. A drop of this suspension was placed on a microscope slide and examined for the presence of germ tubes. The speciation of the *Candida* isolates was performed by inoculating it on Hicrome Candida differential agar. Hicrome agar was prepared as per the manufacturer's instructions and incubated at 37 ^{o}C for 24 hours aerobically.

The antibiotic sensitivity test was performed based on the method described by Jangla *et al.* (2018) with Highrome agar plate. The pure culture of *Candida albicans* were streaked in hichrome agar plate and antibiotic disc were placed in appropriate

distance and incubated at 37 °C for 24 hours aerobically.

Biofilm study of yeast was performed with Sterile 96-well polystyrene plates which were inoculated with 200 µl bacterial suspension (105 CFU/ml) in BHI medium and incubated at 37 °C for 24 hrs without shaking. Each strain of yeast was evaluated in triplicate number. Medium was removed from the wells, and washed three times with 200 µL sterile distilled water. The plates were air-dried for 45 min and the adherent cells stained with 200 µl of 0.1% crystal violet solution. The dye was removed and the wells washed four times with 300 µl of sterile distilled water to remove excess stain After 20 min. The dye incorporated by the cells forming biofilm was dissolved with 200 µl of ethanol/acetone (80% /20%) and the absorbance of each well was measured spectrophotometrically at 570 nm.

RESULTS

Species identification of Candida was done by the morphology and color of the colonies. The *Candida albicans* produce light green colonies, *C. tropicalis* metallic blue colonies, *C. krusei* produces purple fuzzy colonies, and *C. glabrata* white to cream-colored colonies (Figure 3,4,5,6). A total number of 252 *Candida* were isolated from various clinical and post mortem samples of dog, cat, poultry, lion, tiger, elephant, cattle, horse, sheep and goat, rabbit and macaw. All isolates grew well on Hicrome Candida differential agar after 24 hours of incubation at 37^{R}°C. Most of the isolates were from High oral and nasal swab (n=253) followed by postmortem samples crop and esophagus, stomach, intestine (n=305) Urine (n=16), viginal and cloacal swab (93), ear swab (n=85), ocular swab (n=35), Blood (n=185), Cerebro spinal fluid (n=03), brain tissue -(n=15) (Table 1). Out of 298 *Candida* isolates, *C. Albicans* was the most common species in 210 (70.46%) strains. The remaining 88 (29.5%) strains showed *Non-albicans Candida*. Out of 88 *Non-albicans Candida* isolates, *Candida* isolates,20 were *C. tropicalis* (23%), 28 were *C. glabrata* (32%), 18 were candida *parapsilosis* (20%) and 22 were *C. krusei* (25%) respectively (Table 1). Out of 298 isolates from

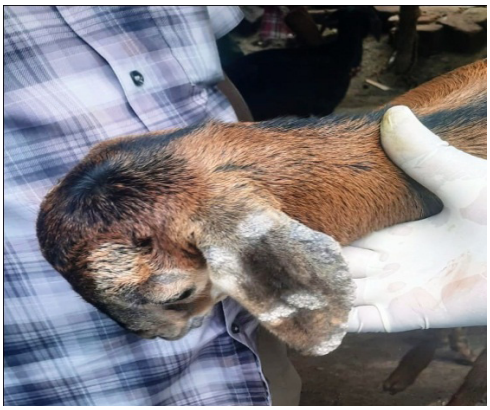

Fig. 1. Concurrent infection of *Candida albicans* and trichophyton

Fig. 2. *Candida* affected visceral organs in post mortem

Table 1. Species wise distribution of *Candida* isolates from various clinical and postmortem samples.

S. No.	Types of specimen	Number of samples screened	Type of *Candida* species					Total Positive
			C. albicans	*C. tropicalis*	*C. krusei.*	*C. glabrata,*	*C. parapsilosis*	
1	Oronasal swab	253	71	*8*	*3*	*5*	*1*	88
2	crop and esophagus,stomach, intestine, liver, spleen and kidney	305	78	7	4	11	3	103
3	Urine samples	16	6	1	3	2	1	13
4	Vaginal and cloacal swab	93	8	2	2	3	2	17
5	Ear swab	85	10		4	1	8	23
6	Ocular swab	35	10			5	3	18
7	Blood	185	24	2	5			31
8	CSF fluid	3	1					1
9	Brain tissue	15	2		1	1		4
	Total	**990**	**210**	**20**	**22**	**28**	**18**	**298**

oronasal swab (HVS), the most common species was *C. Albicans* followed by *C. tropicalis, C. glabrata, Candida parapsilosis* and *C. krusei*. Among the 16 urine samples, the most common species was *C. Albicans* followed by *C. tropicalis, C. glabrata,* and *C. krusei* were isolated. The candida stock culture prepared by using Hichrome agar slant and SDA slant (Figure 7). The antibiotic senstivity study shows senstivity toward genatmicin and chloramphenicol (Figure 8). The Biofilm study was performed with pure candida culture and which shows strong surface attachment (Figure 9)

DISCUSSION

Identification of *Candida* strains to the species level is important because of their variation in their ability to cause infection in animals and also in their susceptibility to antifungal and antibacterial agents. The species level of yeast identification is mandatory for epidemiological purpose and laboratory diagnosis of yeast infection in animals (Kaur *et al.,* 2016; Kumar *et al.,* 2013; and Pacynska *et al.,* 2013). The Hicrome Candida differential agar medium accurately identifies the important *Candida* species namely *C. Albicans, C. tropicalis, C. glabrata, C. dubliniensis,* and *C. krusei* based on their color and morphological character, patterncy of growth (Amit *et al.,* 2015, Kumar *et al.,* 2013 and Mathumathi *et al.,* 2018). In this present study, the rate of isolation of non Candida albicans was 29.5% and the isolation rate of *C.Albicans* was 70.46% and some of the samples shows more than two species of candida. The *Non-albicans Candida* accounted for 29 % of the isolates and the commonest species was Out of 88 *Non-albicans Candida* isolates, 20 were *C. tropicalis* (23%), 28 were *C. glabrata* (32%), 18 were *Candida parapsilosis* (20%) and 22 were *C. krusei* (25%)

Candida albicans is considered to be the common species causing human as well as animal diseases. Recently increase in the isolation rate of Non *albicans Candida* species, primarily *Candida tropicalis, Candida glabrata, Candida krusei* and *Candida parapsilosis*. This rise in *Non-albicans Candida* species has been associated with significant morbidity and

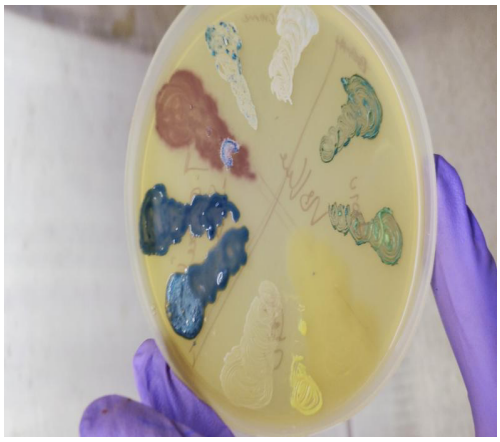

Fig. 3. *Candida* species in hi-chrome agar isolated from ear swab of animals

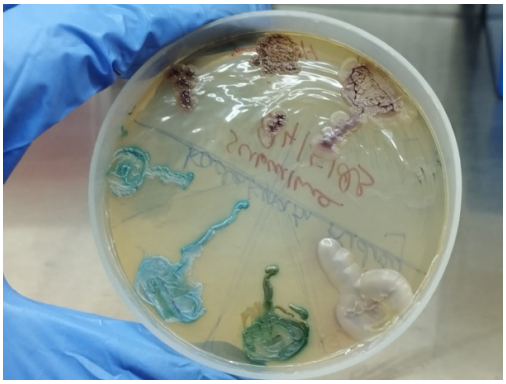

Fig. 4. *Candida* species in hi-chrome agar isolated from vaginal swab of animals

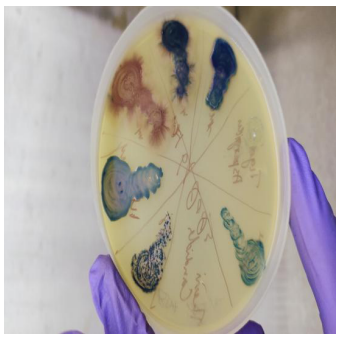

Fig. 5. *Candida* species in hi-chrome agar isolated from CSF fluid and brain tissue of animals

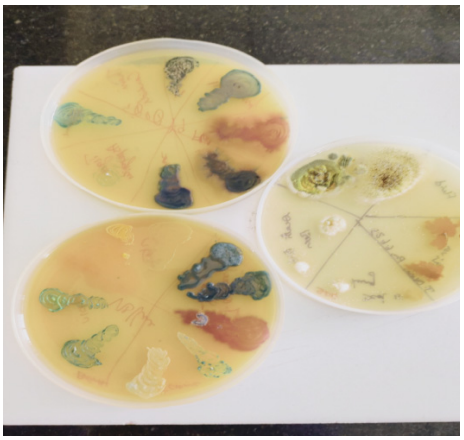

Fig. 6. *Candida* species in hi-chrome agar isolated from post mortem samples of animals

mortality. Hence, identification of species level *Candida* becomes necessary for the initiation of early and effective therapy (Manjunath *et al.*, 2012 and Das *et al*., 2016). As NAC species significantly vary in their prevalence among different countries and health-care setups within a country. The species identification plays an important role in the formulation of therapeutic guidelines and Sabouraud dextrose agar (SDA) is widely used for the isolation of all yeast species from a clinical specimen in most of the diagnostic laboratory but sabouraud dextrose agar is not a differential medium for yeast and various species of yeast growth cannot be easily distinguished from each other. The germ tube test is used to differentiate *C. Albicans* and *C. dubliniensis* from other *Candida* species in many laboratory. The hichrome agar based test may lead to false positive and false negative results (Sardi *et al*., 2013 and Sankari *et al*., 2019). The conventional methods like sugar fermentation and sugar assimilation tests used for the speciation of *Candida* are very time consuming. The PCR based molecular confirmation are very expensive and available only at advance laboratory centers. The Chromogenic agar based speciation of candida is a rapid method to differentiate different *Candida* species which contains enzymatic substrates that are linked to chromogenic compounds. When a specific enzyme cleaves the substrate, the chromogenic substances produce color (Vijaya *et al*., 2011 and Soumya *et al*., 2016). The action of different enzymes produced by yeast species results in color variation which is useful for the presumptive identification of some yeast infection and chromogenic medium is it greatly facilitates the detection of specimens containing a mixture of yeast species though there were no mixed cultures in the recent study (Rudrappa *et al*., 2018 and Rao *et al*., 2019). The prompt detection of such clinical scenarios of multiple yeast etiology may be an aid for early appropriate treatment decisions (Samyuktha *et al*., 2017 and Sankari *et al*., 2019). In the present study, frequently isolated candida species in animals was candida albicans, *C.tropicalis* followed by *C. glabrata* and *C. krusei*. Many other studies have also shown the preponderance of *C. tropicalis* over other NAC species (Saxena *et al*., 2014; Shettar *et al*., 2012; Shwetha, 2015 and Vigneshkanna

Fig. 7. *Candida* species in hi-chrome agar slant and SDA slant culture

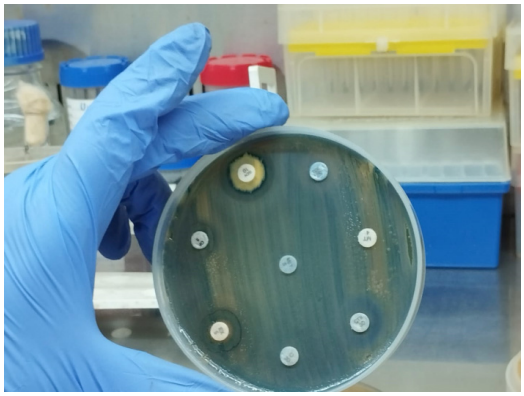

Fig. 8. ABST in hi-chrome agar for candida species

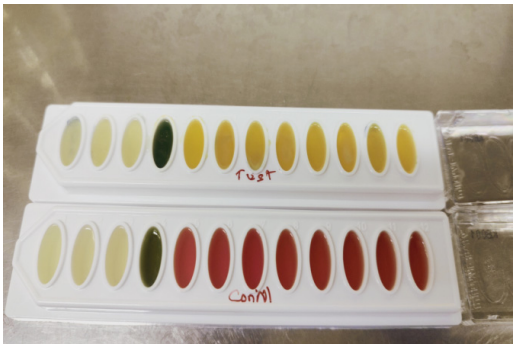

Fig. 9. Sugar fermentation test of *Candida* species in Himedia Biochemical test kit shows fermentation of manitol, dextrose, sucrose, arabinose

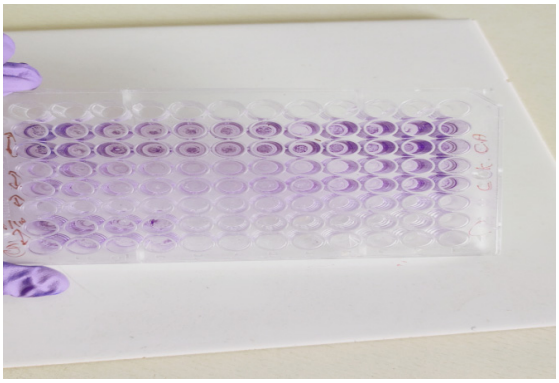

Fig. 10. Biofilm study of *Candida* isolates with 1% crystal violet shows strong biofilm formation

et al., 2017). In this preliminary study, cconventional methods for the identification of *Candida* species by using Hichrome agar and sugar fermentation and assimilation tests were used for isolation and speciation of candida species. Hence, other molecular confirmation and identification of virulence gene needed for accurate drug development in future.

CONCLUSION

Identification of *Candida* up to species level is very important in the early management of Candidiasis. Recent years *Candida* species are increasingly associated with invasive Candidiasis in livestock and poultry which differs from *C. Albicans* with respect to epidemiology and antifungal susceptibility. The present study indicates that the candida albicans and non *Candida albicans* has emerged as an important cause of infections even in animals and not ignored as non-pathogens and contaminants. In future in-depth detailed study needed for development of effective drugs against candidiasis in animals.

What does the study add to the existing knowledge?

The current study results are also important for local monitoring of different *Candida* species among the livestock and poultry which also helps in planning appropriate anti yeast drug development and treatment and selection of disinfection for removing environment contamination. Hicrome agar based isolation and identification is a simple, rapid and inexpensive method for identification of *Candida* species and is suitable for laboratories with limited resources. The major pathogenic species like *C. Albicans*, *C. tropicalis*, *C. glabrata,* and *C. krusei* are easily differentiated by their color and colony morphology within a short time by using Hichrome agar culture.

Conflict of interest: None

Ethical permission: Not applicable

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Author's contribution

Dr. G. Kalaiselvi: Contributed for isolation of candida species, Study design, Literature search, Data collection, statistical analysis, manuscript preparation, editing and review.

Dr. G. Balakrishnan: Contributed for Study design, manuscript preparation, editing and review

Dr. R. Saheethya: Contributed for post mortem sample and clinical sample collection from livestock, pet animals and poultry

Dr. R. Ramya: Contributed sample collection, literature collection and review

Dr. Raman: Contributed for sample collection from the field and sending to laboratory

Dr. C. Soundarajan: Contributed for Study design, manuscript preparation, editing and review

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