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Open Access Journal of Microbiology & Biotechnology
ISSN: 2576-7771

An Assessment of Alternative Therapeutic Options for the Treatment of Prolonged Zoonotic Fungal Infections in Companion Animals

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Research Article

Volume 4 Issue 3

Received Date: July 01, 2019

Published Date: July 25, 2019

DOI: 10.23880/oajmb-16000149

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Abstract

Zoonotic disease is an ongoing issue, becoming more prominent as more microbial species display antimicrobial resistance. The role of companion animals in society is also increasing as therapy dogs become the mainstay for many special needs persons. In order to ensure public health safety, it is essential to determine the extent of resistance amongst zoonotic pathogens and where possible to suggest novel treatment options to counteract such resistance. Fungal species are increasingly recognised as the causative agent in numerous incidents of canine morbidity. Therefore, the development of new, safe and effective chemotherapeutic agents is essential to prevent and control mycotic disease. Chronic incidents of cutaneous candidiasis as diagnosed in the cases used for this study are particularly high risk for zoonotic transmission. Studies described herein evaluate the resistance of these canine isolates of Candida species to common antifungal agents and identify levels of resistance using recognised in vitro methods. Results show high levels of resistance to amphotericin B, fluconazole and caspofungin for *Candida albicans* and *Candida krusei* isolates. This clinical resistance was more prominent in isolated species compared to control species. Novel compounds namely phendione and Roussin Black salts show promising antifungal activity with minimum inhibitory concentrations determined.

Keywords: Zoonotic; Fungal; Fatal; Inactivation; Resistance

Introduction

Anthropozoonosis (zoonotic) diseases are transmitted from animal to human hosts often resulting in pathogenesis. Zooanthroponosis however, describes diseases which transmit from human to animal hosts where, both are of increasing importance as multidrug resistant infections are becoming the mainstay. Indeed, zooanthroponosis may contribute to the increasing rates of mycosis in companion animals as pathogens transmit from close contact. Such diseases include infections

resulting from numerous microbial species of bacterial (Staphylococcus, Streptococcus), viral (influenza A virus), fungal (Candida) and protozoal (Cryptosporidium) origin. Previously, the largest incidences of morbidity and mortality in companion animals were from bacterial and viral infections, however fungal infections have become increasingly prevalent [1]. So much so, dermal and systemic fungal infections in companion animals currently represent a routine problem for veterinarians. Furthermore, fungal infections (mycosis) in humans are common and often fatal, by end of the 1990s fungal infections had become the seventh leading cause of human morbidity resultant from infectious disease [2]. Such mycotic disease may be acute, sub-acute or chronic in nature manifesting as dermatitis, pneumonia, meningitis or osteomyelitis. Fungal pathogens are a major concern for immunocompromised persons such as AIDS patients or persons on immunosuppressive therapy. Epidemiology studies show that patients with advanced AIDS harbour fluconazole-resistant *C. albicans* in their oral cavities [3]. For animal species however, the recognition and identification of fungal disease is often only a post mortem discovery [4]. The treatment and control of invasive fungal infections in animals traditionally relied on the use of amphotericin B with the azoles, fluconazole and itraconazole being introduced in the last decade. Drug resistance in fungal species such as Candida is an emerging problem with resistance to these antifungals a common occurrence in infected animals [5]. Furthermore, amphotericin B has issues with animal toxicity, poor absorption from the gastrointestinal tract (GIT), drug/drug, and drug/food interactions, while the azole drugs also present with erratic absorption patterns [6] making these drugs less than efficient oral antifungal agents. Consequently, the prognosis for infected animals remains poor with mortality rates exceeding 80%. This drug and multi drug resistance is also seen in fungal biofilm communities due to their slow growth rate and the reduced rate of therapeutic penetration into the biofilm matrix [7]. The formation of biofilm structures in the chronic stages of infection in dogs has been identified in many chronic fungal infections such as periodontitis, otitis media and endocarditis [8]. Therefore, there is a need for novel antifungal agents for the treatment of systemic and dermal fungal infections in companion animals and the prevention of zoonotic transmission. Such drugs should have antifungal activity, improved GIT absorption while having improved biocompatibility to the animal itself in comparison to the antifungal therapeutics currently in use. Given the pathogenic and zoonotic potential of these microorganisms, this study aims to assess the antifungal activity of a range of synthesised chemotherapeutic agents on veterinary isolates of Candida species. The findings of this study may help identify novel antifungal therapeutics for use in animal cases of candidiasis. Candida species under study were isolated from infected dogs presenting with prolonged dermal or systemic infections.

Methodology

Amphotericin B (Amp B), fluconazole and caspofungin were sourced from Sigma Aldrich, Dublin, Ireland. All therapeutic agents were made to stock solutions by dissolving the drug powder in adequate volumes of dimethyl sulfoxide (DMSO) (wgt/vol) with the exception of Roussin Black Salt (RBS) which is a polar compound and therefore, dissolves in sterile phosphate buffered saline (Sigma Aldrich, Dublin, Ireland). Once completely dissolved with no precipitation evident, working concentrations of all test agents where then made by diluting the stocks in sterile 0.01 M phosphate buffer, containing 0.0027 M KCL and 0.137 MNaCl at a pH of 7.4 (PBS).

Drug synthesis and Characterization

Reagents and solvents for synthesis of MIT, phendione and Roussin's black salt (RBS) were purchased from Sigma Aldrich, Fisher and Apollo Scientific and were used as supplied. NMR spectra were measured using a Bruker Avance III (400 MHz). Chemical shifts (δ) are expressed in parts per million (ppm) relative to TMS (tetramethylsilane, internal standard). Coupling constants (J) are expressed in Hertz, multiplicities of signals are reported as doublets (d) and doublet of doublets (dd). High resolution mass spectrometry was performed in University College Dublin by Kevin Conboy. Melting points were measured on a Stuart MP/SMP10/ melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer Spotlight 400N FT-IR spectrometer using a UATR accessory.

Mitonafide (MIT, 2-(2-(dimethylamino)ethyl)-3-nitro-1H-benzo[de]isoquinoline-1,3(2H)-dione): To a mixture of 3-nitro-1,8-naphthalic anhydride (300 mg, 1.23 mmol) in toluene (24 mL) in a round bottomed flask equipped with a Liebig condenser was added ethanol (6 mL), followed by N,N-dimethylethylenediamine (161 µL, 1.46 mmol). The mixture was heated under reflux for 40 minutes, allowed to cool to room temperature, filtered and the volatiles removed in vacuo. The resulting dark brown residue recrystallised from hot ethanol (10 mL), to

afford the pure product as a red-brown solid (140 mg, 36 %).

1H NMR (400 MHz, CDCl3) δ/ppm: 2.33 (6H, s), 2.66 (2H, t, J = 6.8 Hz), 4.34 (2H, t, J = 6.8 Hz), 7.93 (1H, dd, J = 8.4 and 7.4 Hz), 8.42 (1H, dd, J = 8.4 and 0.8 Hz), 8.77 (1H, dd, J = 7.4 and 0.8 Hz), 9.12 (1H, d, J = 2.2 Hz), 9.29 (1H, d, J = 2.2 Hz); 13C NMR (100 MHz, CDCl3) δ/ppm: 38.6, 45.8, 56.9 (C-2), 123.2, 124.3, 124.7, 128.9, 129.1, 130.2, 131.0, 134.5, 135.6, 146.3, 162.6, 163.2; υmax/cm^{-1} (ATR): 3080, 2779, 1706, 1662, 1598, 1541, 1509, 1435, 1417, 1344, 1327, 1290, 1238, 1173, 1159, 1142, 1075, 1049, 1019, 963, 942, 912; HRMS (m/z, ES): calcd for C16H16N3O4 [M + H]+ 314.1141, found 314.1131; Melting point: 139-140°C (Lit. 139-140°C) [9].

Phendione (1,10-Phenanthroline-5,6-dione): 1,10-Phenanthroline (5.010 g, 27.8 mmol) was dissolved in concentrated sulfuric acid (30 mL). Sodium bromide (2.520 g, 24.5 mmol) was added portion-wise followed by concentrated nitric acid (15 mL). The resulting dark brown solution was heated at reflux for 5.5 hours. The reaction mixture was allowed to cool to room temperature and was poured over ice (500 mL). It was neutralised with aqueous sodium hydroxide (400 mL, 3 M), and extracted with DCM (3 x 100 mL), dried over anhydrous sodium sulfate and the volatiles were removed to give a brown amorphous solid. Recrystallisation from toluene and methanol (4:1) gave the product as an orange solid (1.95 g, 33 %). 1H NMR (400 MHz, DMSOd6) □/ppm: 8.98 (dd, 2H, J = 4.8 and 2.0 Hz), 8.38 (dd, 2H, J = 7.6 and 2.0 Hz), 7.66 (dd, 2H, J = 7.6 Hz and 4.8 Hz); 13C NMR (100 MHz, DMSOd6) δ/ppm: 197.3, 176.8, 153.4, 151.3, 134.7, 128.1, 124.2; υmax/cm^{-1} (ATR): 1686, 1566, 1437, 1379, 735, 720; Melting point: 260-261°C (Lit. 260°C).

Roussin black salt: Sodium Nitrite 7.2 g (0.10 mol) wash charged to 33 mL of distilled water and stirred until dissolved. In a separate flask 8 mL of a 22% solution of Ammonium sulphide was added to 24 mL of distilled water. The resulting solutions were added together in a three neck flask resulting in a pale yellow solution. This was heated at reflux until the solution turned a dark cherry-red. A Ferrous Sulphate solution was prepared by adding Iron Sulphate heptahydrate 16 g (0.05 mol) and 120 mL of distilled water. This solution was mixed until the solid had dissolved to give a pale green-blue solution. Once the original solution had turned a dark cherry red the iron sulphate solution was charged. The resulting solution turned black instantly. The solution was heated and stirred throughout. Ammonium Hydroxide 22% 20 mL was then added in small portions while stirring. Once this addition was complete the solution was heated at reflux for 10 min. The solution was then filtered hot with the filtrate retained. The resulting black solution was allowed to stand at room temperature overnight. The solution was then filtered and a black crystalline solid was collected and dried under vacuum to give 1.00 g (1 mmol) of $NH_4[Fe_4S_3(NO),]$. The resulting compound was used without further purification. υmax/cm^{-1} (cm^{-1}) 1780, 1740, 1700, 1605 cm^{-1}. Melting point: 198°C (Lit. 198 to 200°C).

Animal Morbidity and Clinical Symptoms

Canine patient 1: an intact female 4-year-old fox terrier cross displaying chronic pruritus for >1 month, erythema along the ventral abdomen with musty odour evident. Also presented with a circular patch of inflamed skin with an oily discharge in a large area over the pelvis and an oily coat extending along the back to the neck. Very swollen, painful and discharging paws which were also very pruritic. Initial treatment consisted of malaseb wash (miconazole and chlorhexidine) (Dechra Veterinary Products, Uldum, Denmark) twice weekly and 250 mg of cefalexin (antibiotic) twice daily for 3 weeks. Skin scrape using a blunted scalpel blade and microbial culture identified *Candida krusei* dermal infection via selective agars and PCR amplification. Diagnosis, chronic cutaneous candidiasis with the zoonotic causative agent *Candida krusei*.

Canine patient 2: 6-year-old intact Jack Russell terrier with similar but not identical presenting signs. Symptoms include severe chronic pruritis with “greasy” skin along the back and inflamed skin along ventrum and legs. Skin flaking excessively and similar “musty” odour evident. Initially prescribed amoxicillin (12.5 mg/kg) with clavulanate acid (3.124 mg/kg) and prednisolone twice daily and received fipronil, s-methopene (flea treatment) followed by imidacloprid and moxidectin (topical flea and mange treatment). No alleviation of symptoms occurred. Treated with topical neomycin (antibiotic), triamcinolone (glucocorticoid) and nystatin (antifungal) once daily for 2 weeks and washed with malaseb twice weekly for 6 weeks. Skin scrape and microbial culture identified *Candida albicans* via selective agars and PCR identification. Diagnosis of cutaneous candidiasis with the zoonotic *Candida albicans* as the causative agent.

Canine patient 3: 4-year-old terrier cross presenting with pyometra and nephritis, dull, pale, diarrhoeic and foul smelling urine. Initial treatment prescribed 10 days of marbofloxacin (2 mg/kg daily) and amoxicillin with

clavulanic acid, also received rehydrating drip (IV) for the first 3 days. Urine sample and microbial culture identified *Candida krusei* via selective agars (confirmed by PCR). Diagnosis kidney infection with the zoonotic *Candida krusei*.

Fungal Isolation, Identification, Culture and Maintenance

Collected samples of infection (skin scrapes) were inoculated in sabouraud dextrose broth (Cruinn Diagnostics, Dublin, Ireland) and incubated at 30°C for up to 72 hours before streaking onto sabouraud dextrose agar (Cruinn Diagnostics, Dublin, Ireland). In the case of the prolonged canine kidney infection, a urine sample was obtained and inoculated onto sabouraud dextrose agar followed by incubation at 30°C for up to 72 hours. Individual colonies were streaked for isolation and Candida species identified based on their morphological characteristics, biochemical profile and growth on CHROMagar-Candida (CHROMagar, Paris). As CHROMagar medium includes chromogenic substrates, at least 48-hour incubation period is necessary for the full colour development of Candida spp., with *C. albicans* isolate displaying green pigmented colonies and *C. krusei* isolates appearing as fuzzy, pink coloured colonies. Identity was confirmed via colony polymerase chain reaction (PCR). Strains were stored and cultured in sabouraud dextrose broth/agar at 30°C and identity confirmed via gram stain prior to each experimental set up.

PCR identification: A single Candida colony (DNA template) was picked from a 48-hour culture using a sterile micropipette tip and suspended in 100 µl sterile deionized water. Fungal primers ITS1-F 5'-CTT GGT CAT TTA GAG GAA GTA A-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (Sigma Aldrich, Dublin, Ireland) were used for direct amplification of intergenic spacer regions (ITS) of Candida rDNA. Direct colony PCR was performed in a total reaction volume of 20 µl, containing 17 µl red Taq 1.1x master mix (VWR, Dublin, Ireland) 1 µl ITS1F, 1 µl ITS4 and 1 µl of selected colony suspension. DNA amplification was performed in a thermo cycler (VWR, Dublin, Ireland) using the recommended parameters. Clean-up and gene sequencing of PCR products was completed by Source Bioscience (Waterford, Ireland).

Scanning Electron Microscopy of Candida Isolates

SEM was used to examine cell morphological of Candida species and to confirm species identification and cell cycle stage. Untreated skin isolates of *C. albicans* and *C. krusei* were prepared by aseptically transferring a single colony into 10ml sterile sabouraud broth, followed by incubation at 30°C under rotary conditions (125 rpm). After 24-hour incubation period, cells were harvested via centrifugation (4000rpm, 15min) and washed with sterile PBS prior to fixing with formalin. Yeast cells were subsequently dehydrated by a sequential immersion (10 min) in water/ethanol (50, 70, 80, 90, 100%) and ethanol/HMDS (50, 75, 100%), and left to dry overnight in a desiccator at room temperature. Samples were then sputter-coated with gold and SEM imaged (Hitachi S-4800).

Antifungal Testing

Antifungal susceptibility patterns of isolated Candida strains to amphotericin, caspofungin, fluconazole, mitonafide, phendione and Roussin black salt were assessed via disc diffusion (Kirby-Bauer) method, with the most effective drug concentrations selected for further study in the inhibition of growth assay. Two reference strains, *Candida krusei* ATCC 14243 and *Candida albicans* ATCC 10231, were included in each test for comparative sensitivity analysis to disease isolates. In addition, the effect of DMSO on Candida growth was evaluated by testing the different concentrations without the antifungal agent to negate the effect of DMSO induced toxicity, as it was used for drug dissolution.

Kirby Bauer assay: The Kirby-Bauer assay was performed to evaluate the sensitivity or resistance of isolated Candida spp. to all test agents. A test inoculum of each strain was prepared from a pure culture grown in sabouraud broth for 16 hours to enter the log phase of growth. Test samples were centrifuged at 10,000 rpm for 10 minutes and the pellet re-suspended in sterile phosphate buffered saline and a serial dilution performed to determine a viable cell count. All fungal cell densities were adjusted to $1x10^6$cfu/ml in sterile PBS to maintain osmotic normality of the cells. Subsequently, 300 µL of this microbial suspension were transferred onto triplicate sabouraud agar plates and spread with a sterile L-shaped spreader (Cruinn Diagnostics) to ensure even disruption across the agar surface. Agars were prepared per manufacturer's instructions in deionized water at room temperature and poured to a depth of 4 mm. Immediately before inoculation, media was checked to ensure it was moist but free of water droplets on the agar surface and the petri dish lids. Antimicrobial disks (6mm) were immersed in each test solution at varying concentrations for 15 seconds with excess solution allowed to drip off the disk. Subsequently, discs were placed on the inoculated

plate at a rate of 1 disc per plate, followed by incubation at 30°C for 48 hours. Zones of inhibition were then measured and recorded in millimetres using a vernier calliper.

Inhibition of fungal growth: Inoculums of each test Candida spp. were prepared in sabouraud broth which was adjusted to meet the varying concentrations for each antifungal agent. The solutions were then seeded with a single colony of separate Candida test strains and incubated under rotary conditions (125rpm) at 30°C for 24 hours. Controls contained sabouraud dextrose broth only. Subsequently, 100 µl of the diluted solution was aseptically serially diluted and spread on sabouraud dextrose agar plates in triplicate. All plates were incubated inverted for 48 hours at 30°C. Surviving colonies were counted and reported as log10 cfu/ml compared to an untreated control.

Statistics

All the experiments were performed three times with three plate replicates for each experimental data point providing a mean result for each test species and antifungal susceptibility (+/-standard deviation). The log10 inhibition of growth was calculated as the log10 of the ratio of the concentration (cfu/ml) of the non-treated (N0) and treated (N) samples [log10 (N0/N)]. Student T tests were conducted to determine significance levels ($p<0.05$) of bacterial susceptibility to treatment using Minitab 16 (Minitab Ltd, Coventry, UK).

Results

Scanning electron microscopy was employed to confirm species identity and growth phase of Candida species. SEM (Figure 1) revealed typical colony morphology for both strains of yeast with well-defined structures and smooth membrane surfaces. Yeasts of the Candida genus are unicellular fungi which reproduce by generating daughter cells by a process of budding, separating at sites of septation. Typically, morphological characteristics of Candida budding cells manifest as oval, round, or cylindrical shapes as seen in Figure 1 (indicated by arrow). SEM images show the elongated cells of *C. krusei* compared to the round cells of *C. albicans*, both of which are attached to the adjacent cells, typical morphology for these species [10]. Figures 1a and 1b depict actively reproducing cells of *C. albicans* and *C. krusei* skin isolates, respectively.

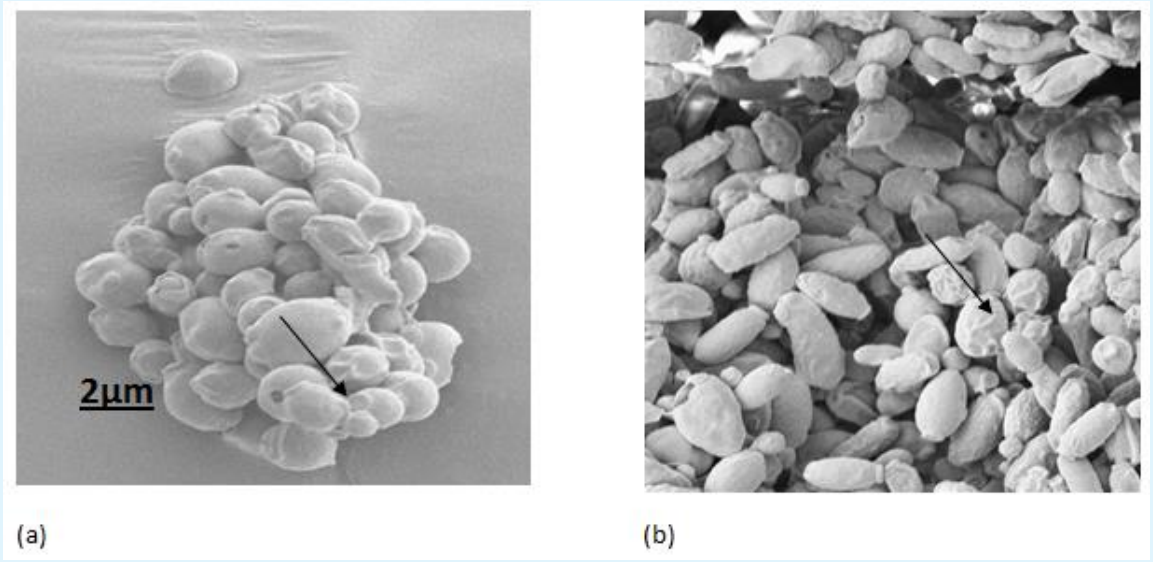

Figure 1: Scanning electron microscopy of a) *C. albicans* and b) *C. krusei* isolated from chronic cutaneous candidiasis of canine dermis, arrow indicating budding yeast cells.

Results show (Figure 2) the susceptibility of fungal test species to three common antifungal drugs as determined by the Kirby Bauer assay. Amp B (Figure 2a) provided a significant zone of inhibition at 2.5 ppm which

did not increase greatly with an increase in concentration for all test species. At 2.5 ppm the kidney isolate of *C. krusei* proved most sensitive followed by *C. albicans* ATCC and *C. krusei* skin isolate. At concentrations of 12.5 ppm all inhibition plateaued and did not increase at concentrations exceeding this, up to 50 ppm (data not shown). All *C. krusei* strains showed similar susceptibility to treatment with the isolated species comparable to the reference strains (ATCC) at 6.25 ppm however, a concentration of 12.5 ppm provided a greater cell death of *C. krusei* ATCC and the kidney isolate. *C. krusei* skin isolate displayed some increase in resistance to Amp B as the concentration increased, showing that higher concentrations do not provide increased cell death. *C. albicans* skin isolate was the only species which showed an increase in zone diameter with an increase in Amp B concentration, increasing from 10 to 12 mm with a concentration increase to 12.5 ppm. At 6.25 and 12.5 ppm both *C. albicans* strains proved more resistant to treatment than *C. krusei*. Fluconazole (Figure 2b) showed a slight decrease in cell death for *C. albicans* comparative to Amp B at 2.5 ppm with similar results at 6.25 and 12.5 ppm. The skin isolate of *C. albicans* showed the same sensitivity to Amp B and fluconazole at all test concentrations, being more resistant than the ATCC strain to both antifungals. All *C. krusei* strains proved more sensitive to fluconazole than Amp B. *C. krusei* kidney isolate was more sensitive than the other krusei strains to fluconazole at 2.5 ppm and this plateaued at 6.25 ppm with no further dose dependent increase in cell death. Fluconazole gave a 12 mm zone of inhibition of *C. krusei* skin isolate at all test concentrations and proved the most sensitive test species to treatment. A dose dependent significant increase in zone diameter was observed for *C. krusei* kidney isolate, producing a 13.5 mm zone at 12.5 ppm. Both *C. albicans* strains and *C. krusei* kidney isolate showed greater sensitivity to caspofungin treatment than the two other drugs tested with a significant increase observed at 6.25 and 12.5 ppm (Figure 2c). The zone of inhibition for the *C. albicans* ATCC strain increased from 11 mm at 2.5 ppm to 14.5 mm at 6.25 ppm and did not increase with a further increase in concentration. While *C. albicans* skin increased from 11 mm with Amp B to 15.5 mm following exposure to caspofungin at the same concentration (12.5 ppm). No significant difference in sensitivity to caspofungin was seen for *C. albicans* ATCC, *C. albicans* skin *C. krusei* ATCC at a concentration of 2.5 ppm but differences were observed above this concentration. *C. krusei* skin isolate proved more sensitive at this lower concentration with a significant increase in zone diameter at 12.5 ppm compared to 6.5 ppm.

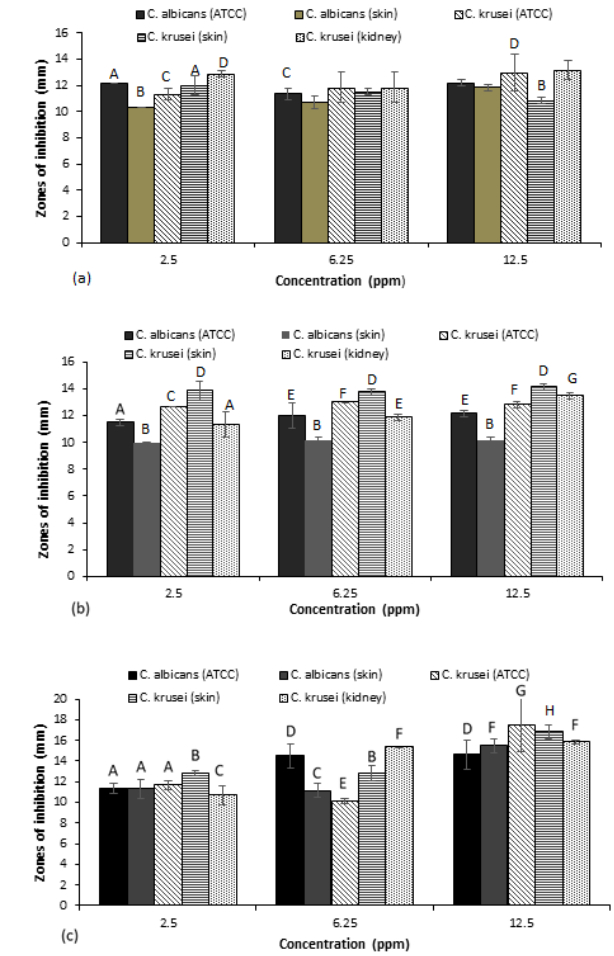

Figure 2: Antifungal susceptibility testing of Candia species (reference and isolate) to (a) amphotericin B, (b) fluconazole and (c) caspofungin as determined by the Kirby Bauer disk diffusion assay (+/- S.D). A, B, C, D, E, F, G and H denote significant difference at $p<0.05$.

The krusei kidney isolate proved most resistant to caspofungin at 2.5 ppm, with an increase in zone diameter as the antifungal concentration increased to a maximal 15.8 mm at 12.5 ppm. A concentration of 12.5 ppm for all antifungal agents provided the greatest zones of inhibition for all test species. At this concentration of caspofungin, the order of sensitivity (from highest to lowest) is C. krusei ATCC, C. krusei skin, C. krusei kidney, C. albicans skin and C. albicans ATCC. Compared to Amp B where the order is as follows: C. krusei ATCC, C. krusei kidney, C. albicans (both strains) and C. krusei skin. Fluconazole provided the following order of sensitivity at

this concentration: C. krusei skin, C. krusei kidney, C. krusei ATCC, C. albicans ATCC and C. albicans skin isolate. As displayed in Figure 2, caspofungin proved the most effective antifungal therapeutic agent of the three drugs tests, followed by fluconazole and Amp B at this optimal concentration of 12.5 ppm.

Figure 3 depicts the sensitivity of all test strains to the novel test compounds phendione, mitonafide and RBS. Results indicate that for phendione (Figure 3a), a 10 mM (2x103 ppm) concentration was needed to produce zones of cell death comparable to the antifungal agents. Additionally, there was a dose dependent increase in cell death for all test species up to 50 mM, where the zones produced were significantly greater than those observed with the antifungal agents. At 10 mM, phendione provided a zone diameter of 15, 12, 14, 23.5 and 22 mm compared to 12.5 ppm caspofungin giving a zone of 14.6, 15.5, 17.5, 16.8 and 15.8 mm for *C. albicans* ATCC, *C. albicans* skin, *C. krusei* ATCC, *C. krusei* skin and *C. krusei* kidney isolate respectively. With an increase in concentration (to a max of 50 mM) however, the rate of cell death increased in a dose dependent manner (not observed with the antifungal therapeutics). Specifically, 50 mM provided a zone diameter of 24, 19, 22.5, 31 and 32 mm for *C. albicans* ATCC, skin isolate, *C. krusei* ATCC, skin and kidney isolate respectively, a significant increase for all test species. *C. albicans* skin isolate proved most resistant to treatment with phendione, followed by *C. krusei* ATCC, *C. albicans* ATCC, *C. krusei* skin and kidney isolates.

All test strains showed a decreased sensitivity to mitonafide (Figure 3b) compared to phendione (and all the antifungal drugs) with a maximal zone diameter of 12, 10, 2.5, 6 and 6 mm achieved for *C. albicans* ATCC, *C. albicans* skin, *C. krusei* ATCC, *C. krusei* skin and *C. krusei* kidney isolates respectively at 50 mM. At this concentration the order of sensitivity was *C. albicans* ATCC, skin isolate, *C. krusei* isolates with *C. krusei* ATCC proving most resistant to mitonafide. RBS (Figure 3c) provided consistent low level inactivation of all strains (comparative to phendione) as determined by the Kirby Bauer assay. Additionally, there was no significant increase in zone diameter with increasing RBS concentrations for all strains up to 0.4 mM, indicating that increasing the concentration of RBS does not provide increased antifungal activity when assessed by this method. A maximal zone of 12 mm was obtained at the highest concentration (0.4 mM) for the most sensitive strain, *C. krusei* kidney isolate. The *C. albicans* strains proved slightly more resistant to RBS followed by *C. krusei* ATCC. As evident from figure 3, phendione provided the highest levels of antifungal activity exceeding that of all antifungal therapeutics tested (Figure 2) followed by RBS and lastly mitonafide (neither of which proved more yeasticidal than the antifungal drugs).

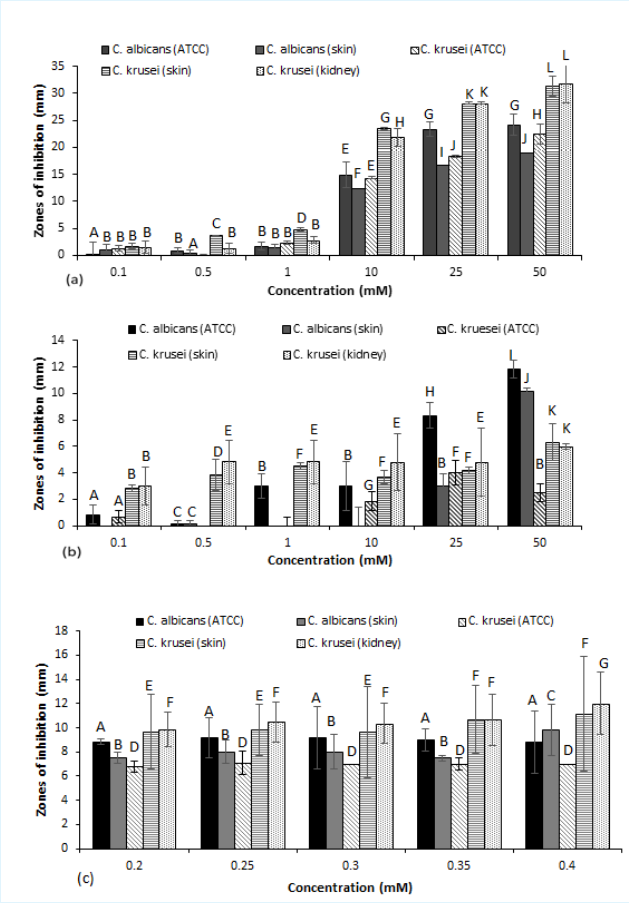

Figure 3: Antifungal susceptibility testing of Candia species (reference and isolate) to (a) phendione, (b) mitonafide and (c) RBS as determined by the Kirby Bauer disk diffusion assay (+/- S.D). A, B, C, D, E, F, G, H, I, J, K and L denote significant difference at $p<0.05$.

Figures 4 and 5 depict the log inhibition of cell growth of the antifungal therapeutics and novel compounds respectively. Amp B (Figure 4a) provided the greatest level of inhibition at a concentration of 25 ppm and this did not increase in a dose dependent fashion. Similarly, as seen with the Kirby Bauer assay a plateau effect was evident at all concentrations exceeding 25 ppm. The ATCC strain of *C. albicans* proved most sensitive to inhibition with ca. 3 log10 decrease in viable cell number compared

to the untreated control followed by *C. albicans* skin (2.5 log10), *C. krusei* ATCC (2.4 log10), *C. krusei* kidney (1.9 log10) and *C. krusei* skin (1.7 log10).

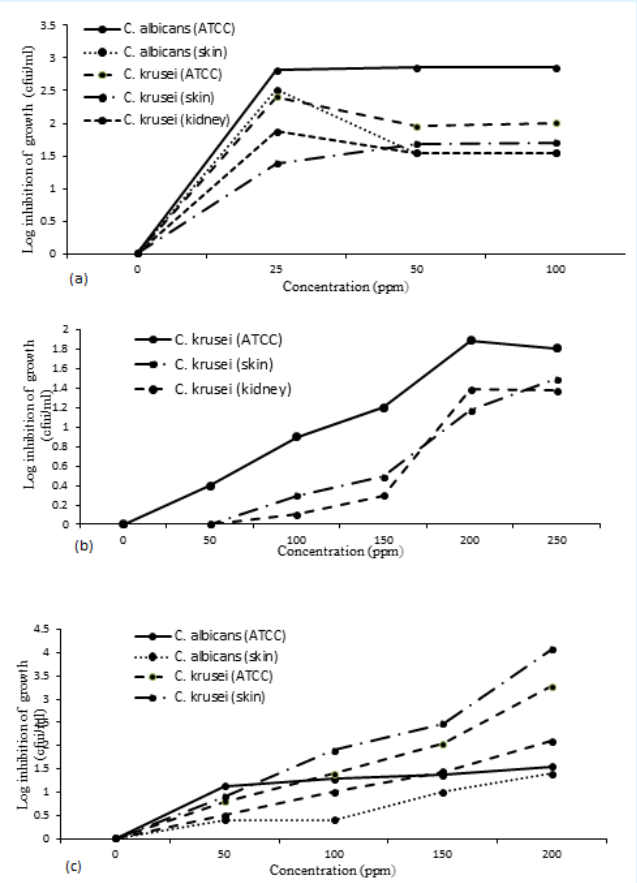

Figure 4: Antifungal inhibitory testing of Candia species (reference and isolate) to (a) amphotericin B, (b) fluconazole and (c) caspofungin as determined by log inhibition of cell growth (cfu/ml) at 24 hours ($p<0.05$).

It must be noted however, that while 25 ppm of AMP B provided the maximal inhibition found above for both C. albicans strains, C. krusei ATCC and kidney isolate, 50 ppm was required however, for maximal inhibition of C. krusei skin isolate. C. krusei skin isolate proved the most resistant to AMP B inhibition with the lowest inhibition achieved overall. Fluconazole did not provide any inhibition of either C. albicans species at the concentration range tested indicating complete resistance to this drug. Furthermore, for all C. krusei strains (Figure 4b) a marginal decrease in cell inhibition is evident compared to AMP B, with a maximal 1.8, 1,5 and 1.4 log10 cfu/ml decrease in viable cell number achieved with 200 ppm fluconazole (significantly higher dose than AMP B). This data indicates a strong resistance to fluconazole for all test species. Caspofungin also proved more ineffective at inhibiting cell growth (Figure 4c) compared to AMP B, with 200 ppm again required to achieve maximal inhibition. Caspofungin did inhibit both C. albicans strains, although to a lesser extent than AMP B, with a max inhibition of 1.5 and 1.4 log10 cfu/ml for the ATCC and skin strains respectively at 200 ppm. This concentration also provided a 3.2, 4 and 1.4 log10 cfu/ml inhibition of C. krusei ATCC, skin and kidney strains. Again all strains proved more resistant to caspofungin requiring a significantly higher concentration (200 ppm) to obtain similar levels of inhibition to 25 ppm AMP B. Caspofungin however, proved more effective than fluconazole for all Candida species. Therefore, for cell growth inhibition the order of effectiveness is AMP B, caspofungin and fluconazole, with the reference ATCC strains proving more sensitive than their isolated counterparts.

Phendione provided complete inhibition of both *C. albicans* strains and *C. krusei* skin isolate at 5 mM with a ca. 7 log10 cfu/ml loss of viable cells compared to the untreated control. At concentrations exceeding this up to 50 mM no increase in inhibition was possible, but some cell viability did manifest with a 1 log10 recovery of cell numbers at 25 mM (Figure 5a). *C. krusei* ATCC and kidney isolate proved more resistant to inhibition with a maximal 6.5 log10 inhibition of both species with 10 mM phendione. For both these strains however, there was significantly less viable cells evident at the higher concentrations. Mitonafide (Figure 5b) also provided significant inhibition of all test species compared to the untreated control. For all Candida species a maximal ca. 6.2 log10 cfu/ml inhibition of growth was achieved at 10 mM with *C. krusei* kidney isolate proving slightly more sensitive to mitonafide. (6.6 log10 inhibition). At higher concentrations inhibition did not increase and complete cell inhibition of any species was not achieved with this drug as seen with phendione. RBS (Figure 5c) also possesses the ability to inhibit fungal cell proliferation at the concentration range tested. For *C. albicans* strains, *C. krusei* ATCC complete inhibition (ca. 7 log10 cfu/ml) at 0.4 mM was achieved by RBS. *C. krusei* skin and kidney isolates proved more resistant to inhibition with a maximal 2.4 and 3.2 log10 cfu/ml inhibition occurring respectively at 0.4 mM RBS. With increasing concentrations no further inhibition of yeast cells occurred. Based on inhibition of cell growth phendione

proved the most antifungal agent of those tested providing significantly greater inhibition than AMP B, fluconazole and caspofungin.

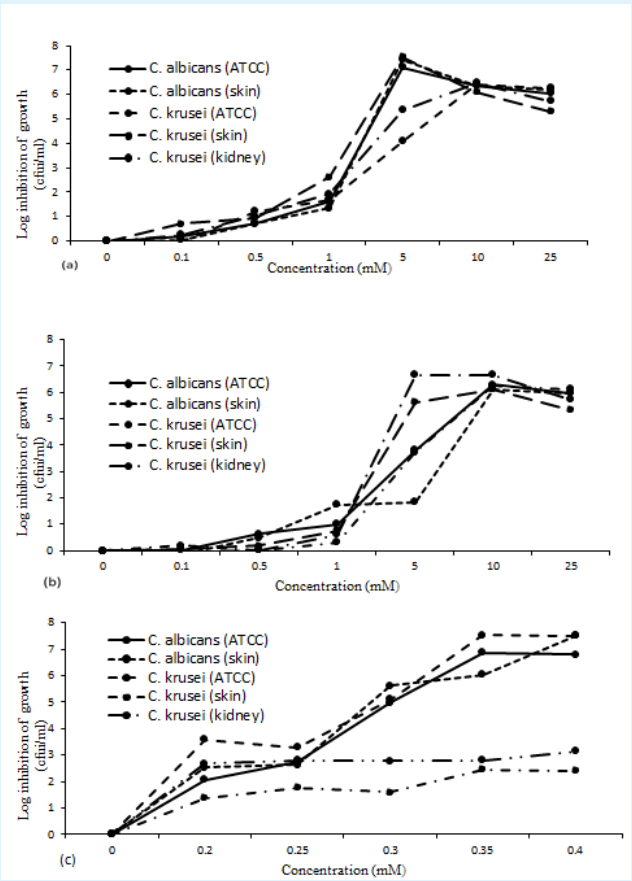

Figure 5: Antifungal inhibitory testing of Candia species (reference and isolate) to (a) phendione, (b) mitonafide and (c) RBS as determined by the log inhibition of cell growth (cfu/ml) at 24 hours ($p<0.05$).

Discussion

Chronic infections of companion animals represent an ongoing issue for veterinarians and a potential reservoir for zoonotic microbial species. Standard practice in treating cutaneous and systemic infections in canine animal's typical aims to target bacterial causative agents, where prescribing antibiotics such as cephalexin or amoxicillin is the primary course of action when patients present with a range of symptoms. Additionally, prednisolone is often prescribed to treat pyoderma or hypersensitivity in the animal. As demonstrated by this study for all three cases the causative agent was determined to be fungal with both *Candida albicans* and *Candida krusei* identified. This study suggests that the presence of a fungal causative agent is often initially overlooked in diagnosing infections in animals, allowing chronic conditions to manifest. Increasing evidence proposes that acquired resistance to antifungals such as amphotericin B and the azoles in Candida species may be an emerging and underdiagnosed risk. The findings of this study also suggest this, with caspofungin having the highest effect on all strains via the Kirby Bauer assay followed by fluconazole and lastly amphotericin B. For cell inhibition however, AMP B proved most effective followed by caspofungin and fluconazole. For treating human candidiasis AMP B is the primary drug of choice and in certain cases is combined with other antifungals such as azoles. Fluconazole is the azole of choice for local and systemic fungal infections. *Candida krusei* however, possess intrinsic resistance to this therapeutic [11]. Antifungal drug resistance can be measured as the minimum inhibitory concentration (MIC) that inhibits the growth of the fungus under standardized methods in vitro. MIC tests are used in resistance surveillance and in the comparative testing of new antimicrobial agents [12]. Complete fluconazole resistance was identified in this study for *Candida albicans* in the inhibitory assay and a decreased sensitivity was evident for all *C. krusei* strains under test. For this study no MIC was determined for any of the three antifungal agents up to 250 ppm (0.25 mg/ml), a significantly greater concentration than that reported by Eksi et al., 2013 [13]. This study reports less than 1 ppm MICs for both Candida species exposed to AMP B and caspofungin and less than 65 ppm for fluconazole. It must be noted however, that Eksi et al. (2013) [13] focused on human isolates using a micro dilution assay. Concentrations exceeding those under test are not realistic as antifungal drugs are known to cause toxicity to non-fungal cells. Consequently, success rates for AMP B use in veterinary medicine are difficult to assess due to the high proportion of patients incapable of tolerating an adequate antifungal dose [6]. AMP B toxicity manifests as vomiting, nausea, hypotension, nephrotoxicity and renal failure in chronic cases with daily doses of 35 mg [14]. AMP B is not absorbed from the gastro intestinal tract when taken orally and is therefore given by IV for systemic fungal infections at a dose of 1 mg/10 ml or 0.1 mg/ml [15]. Caspofungin is administered by IV at a dose of 70 mg/per day for the treatment of Candida infections with MICs ranging between 0.015 and 4 ppm [16] depending on the species. The increasing number of invasive and severe fungal infections is associated with the use of broad spectrum antibiotics and

the increasing use of long term immunosuppressant drugs including chemotherapeutics [6]. The resistance seen in this study may relate to the administrating of antibiotic drugs to the animals before diagnosis was made. Candia species possess efflux pumps, a non-selective resistance mechanism which may have been activated in the presence of these xenobiotic compounds. Both reference ATCC strains proved more sensitive than their isolated counterparts in the inhibition assay suggesting that some environmental factor may have contributed to the resistance. The presence of this resistance in Candida species in companion animals is a serious concern as the possibility for human transmission is high risk. Virulence factors allow fungal species to colonise, invade and establish a pathogenic hold in the animal. The formation of fungal biofilms is once such virulence factor which resists the host immune system and antimicrobial therapy. The potential of cutaneous Candida infections to cross the dermis and enter the systemic circulation resulting in organ mycosis also increases it pathogenicity. The ability of Candida species to produce proteases, and hyphae allow for this tissue penetration and adherence to epithelia cells [17]. Furthermore, the prescribing of prednisolone to the patient may reduce the immune response of the animal and result in iatrogenic Cushing's or Addisons disease. A potential option for the treatment of such aggressive species is to combine or replace antifungal agents with novel products exhibiting antifungal activity such as those described herein. Phendione provided the highest level of antifungal activity for both assays and as such represents the best option from this study. Additionally, phendione provided complete inhibition of fungal growth for *C. albicans* with an MIC of 5 mM identified. Studies have reported the antibacterial activity of phendione compounds against Pseudomonas planktonic and sessile cells [18]. The lipophilic nature of this drug is believed to contribute to its antimicrobial activity, where DNA binding may play a role in its toxicity. RBS is a polar compound which has long been known for its antibacterial activity. Studies report the antibacterial activity of RBS against Pseudomonas, Listeria and Clostridium due to lysis of the cell membranes [19]. RBS also shows potential for antifungal use, with an MIC of 0.4 mM identified for both *C. albicans* strains and *C. krusei* ATCC. Both *C. krusei* isolates showed a greater level of resistance however, with a max inhibition of 2.4 and 3.2 log10 cfu/ml inhibition for *C. krusei* skin and kidney respectively. The findings of this study therefore demonstrate its aggressive antifungal activity against veterinary isolates of Candida species. The clinical result of Candida infection in humans and animals relates to many factors including the immune status of the host, drug pharmacokinetics, food-drug and drug-drug interactions. Consequently, drug studies in-cooperating all these factors need to be conducted including biocompatibility testing on relevant canine cell lines before a full profile is complete. Concentrations of phendione and RBS which provide antimicrobial activity at sub-cytotoxic levels in canine cells needs to be established using cell culture systems in vitro.

Conclusion

Fungal infections of companion animals result in animal morbidity, economic pressure and risk of zoonosis to animal owners. As human and animal relationships increase an amplified incidence of zoonotic disease is inevitable. Resistance to antifungal drugs is an ongoing problem particularly for immunosuppressed persons where chronic morbidity may lead to fatalities. The presence of resistant species in companion animals such as those described herein, represents an often unrecognised route of disease transmission. Candida species represent a significant portion of infective agents of disease particularly in dermal cases, with a potential for penetration and systemic infection. The development of new treatment options is therefore essential for both animal and human incidents of infection in order to ensure public health safety and quality of life. Two drugs used in this study have shown potential as antifungal agents and should be further analysed to determine their ability to remain cell toxic irrespective of fungal virulence factors and resistance mechanisms.

Conflict of Interest

The authors declare there is no conflict of interest.

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