

Evaluation of *In Vitro* Synergistic Interaction of Miconazole and Polymyxin B Against Clinical Strains of *Malassezia pachydermatis*

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Abstract: *Malassezia pachydermatis* is a yeast that is frequently involved as a secondary/perpetuating factor in canine otitis externa, along with numerous species of bacteria. As a result, otitis is generally treated with topical therapies using combinations of antifungal, antimicrobial and anti-inflammatory agents. The evaluation of the combined effect of drugs included in commercially available products is therefore important. *In vitro* synergy of polymyxin B and miconazole has been demonstrated against one strain type of *M. pachydermatis*. This study was aimed at investigating the *in vitro* interactions of these two agents against five clinical strains of this yeast. A checkerboard broth microdilution method was employed. We observed a synergistic action against all strains tested (fractional inhibitory concentration index comprised between 0.2 and 0.4). Our results indicate a strong therapeutic value for the association of these antimicrobial agents in the treatment of canine otitis externa caused by *M. pachydermatis*.

Keywords: Dog, *Malassezia pachydermatis*, miconazole, otitis externa, polymyxin B, synergy.

Canine otitis externa represents one of the most common diseases of multifactorial aetiology in veterinary practice [1]. Micro-organisms, including both bacteria and yeasts, are considered common perpetuating factors that can worsen and prevent the resolution of otitis [2]. *Malassezia pachydermatis* is the yeast isolated with the highest frequency from affected ears [3]. This organism is part of the commensal microflora of canine skin and ears, but it may become an opportunistic pathogen under favourable conditions, e.g. allergic, parasitic and immune-mediated diseases [1]. Topical therapy is the most popular and convenient way of treating canine otitis externa. Treatment usually involves the use of antibiotics, antifungals and anti-inflammatory agents, often included in a single formulation. A polyvalent product is indicated in first line treatment because bacteria and yeast are simultaneously present in a large number of cases [4]. In-depth evaluation of the combined effect of drugs included in marketed products is therefore important. Among the possible combinations available, a commonly used product in clinical practice contains polymyxin B (PMX) as an antibiotic and miconazole nitrate (MCZ) as an antifungal. This combination has proven to be effective for the treatment of dogs with otitis externa complicated by a number of bacterial and yeast species [5, 6]. Such a positive response stems from the fact that PMX is effective against a broad variety of Gram-negative and, to a lesser extent, Gram-positive bacteria, as well as having antifungal properties [7, 8], while MCZ is active against several pathogenic fungi,

such as *Candida albicans* and *M. pachydermatis* and can also act upon certain Gram-positive bacteria [9, 10]. Moreover, a synergistic interaction *in vitro* between PMX and MCZ has been demonstrated against different species of bacteria frequently involved in canine otitis externa [11-13]. Synergy, in this context, can be defined as the increased effectiveness of multiple drugs that, in combination, are more efficient than the cumulative effects of the individual components [14]. Regarding *M. pachydermatis*, synergy has so far only been demonstrated against one type strain (*M. pachydermatis* CBS 1879) [12]. The aim of the present study was to evaluate the *in vitro* antifungal activity of the combination of PMX and MCZ against clinical strains of *M. pachydermatis* associated with canine otitis externa.

Five strains of *M. pachydermatis* from dogs with otitis externa, randomly selected from different regions of Italy, were investigated. Ears were sampled through the use of sterile cotton swabs, and yeasts were isolated on Sabouraud Dextrose Agar with chloramphenicol and gentamicine. Identification of these clinical isolates was based on macroscopic and microscopic characteristics [15]. All strains were examined for their *in vitro* susceptibility to PMX and MCZ as single substances and in combination. A modified CLSI (Clinical and Laboratory Standards Institute) M27-A3 method [16] was employed. Christensen's urea broth medium with Tween 80 and Tween 40 was used, instead of the RPMI 1640 medium suggested by the CLSI [16, 17], in order to meet the growth requirements of *M. pachydermatis*. Uniform yeast suspensions were prepared from 4-day-old colonies subcultured at 37°C on Sabouraud Dextrose Agar. Inoculum suspensions were adjusted by spectrophotometer, and the final yeast cell concentration was approximately $1-5 \times 10^5$ cells/ml. Stock suspensions of MCZ (Sigma-

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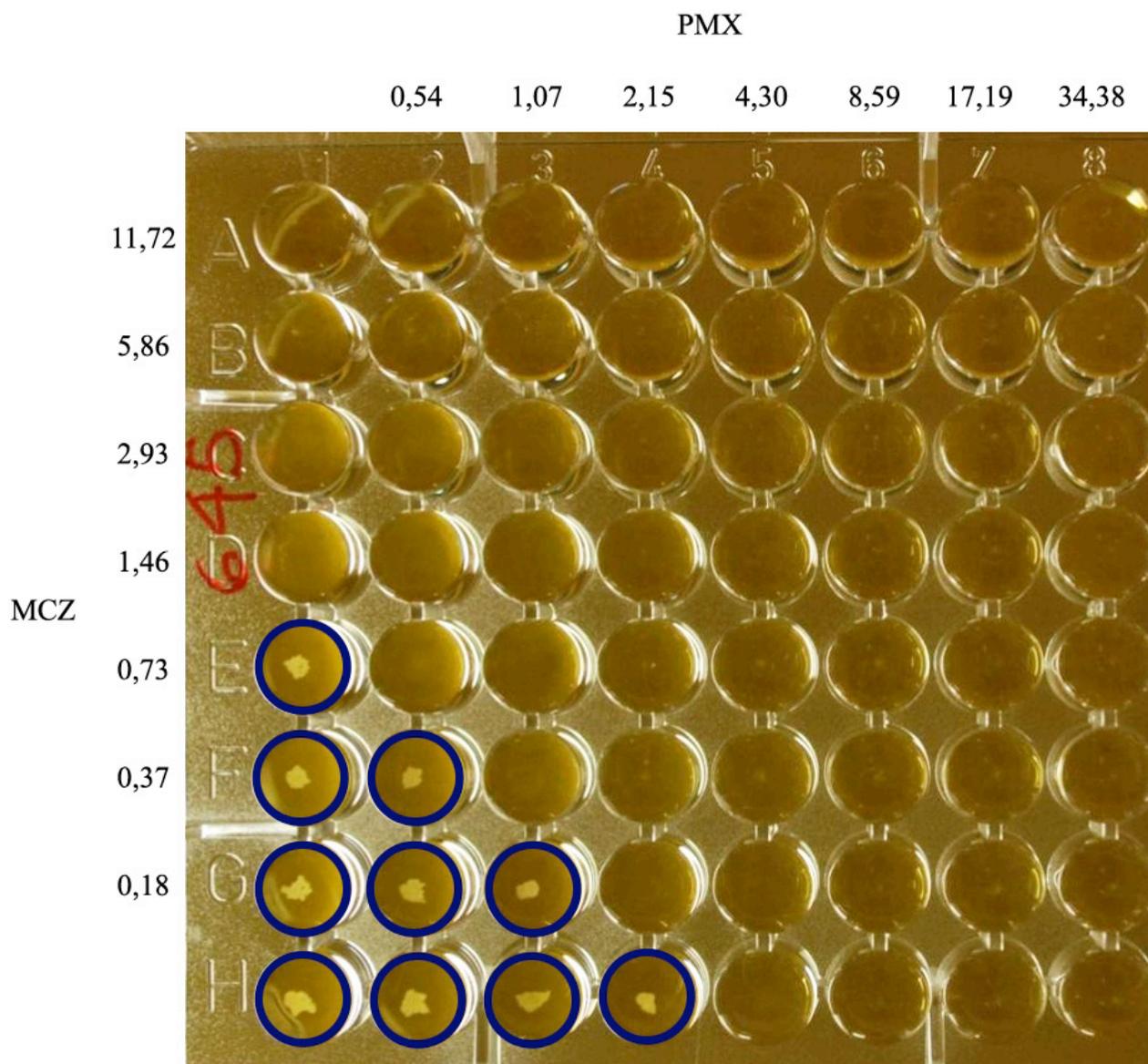


Fig. (1). Test showing synergy between miconazole (MCZ) and polymyxin B (PMX) in the strain of *M. pachydermatis* VMT 645 – Concentration varied from 34.38 mg/l PMX combined with 11.72 mg/l MCZ to 0.54 mg/l PMX combined with 0.18 mg/l MCZ- Wells with yeast growth are highlighted (blue circles).

Aldrich, Taufkirchen, Germany) and PMX (Sigma-Aldrich) were prepared in dimethyl sulfoxide. Synergy trials were performed as checkerboard interactions in 96-well plates. Yeasts were added to a twofold serial dilution of a single antibiotic agent or in combination with an identical dilution of the other antibiotic agent. Plates were incubated at 37°C and analysed at 48 h after inoculation. Concentrations tested in combination consisted of equal molar concentrations of PMX and MCZ [13]. They varied from 34.38 mg/l PMX combined with 11.72 mg/l MCZ (corresponding to 24.81×10^{-6} mol/l of each drug) to 0.54 mg/l PMX combined with 0.18 mg/l MCZ (corresponding to 0.39×10^{-6} mol/l of each drug). The minimum inhibitory concentration (MIC) was read as the lowest concentration of antimicrobial substance which inhibited visible growth, as assessed by using a spectrophotometer (630 nm filter). Each strain was tested on three separate occasions to verify the reproducibility of the results obtained [18]. The fractional inhibitory concentration

index (FIC_i) was calculated as follows: FIC of drug A (MIC of drug A in combination/ MIC of drug A alone) + FIC of drug B (MIC of drug B in combination/MIC of drug B alone). FIC_i was interpreted as follows: ≤ 0.5 = synergy; $> 0.5 - 4$ = no interaction; > 4 = antagonism [19].

MICs of MCZ for *M. pachydermatis* isolates ranged from 1.46–5.86 mg/l; MIC of PMX for all strains tested was 4.3 mg/l. If both antimicrobial agents were used in combination, the susceptibility of *M. pachydermatis* was altered to an MIC of 0.18 mg/l (MCZ) and 0.54 (PMX) mg/l, with corresponding FIC_i < 0.50, which indicates a synergistic response, as shown in Fig. (1). Results of the susceptibility tests are summarized in Table 1.

As mentioned above, synergy is said to occur when the combination of drugs results in a more efficient response than the cumulative effects of the individual components [14]. In the case of the combination of PMX and MCZ,

Table 1. The *In Vitro* Susceptibility of *M. pachydermatis* Isolates to Miconazole (MCZ) and Polymyxin B (PMX) as Single Substances and in Combination

Yeast Strain	MCZ MIC*	PMX MIC	FIC _i *
VMT 645	1,46	4,3	0,4
VMT 622	5,86	4,3	0,2
VMT 620	5,86	4,3	0,2
VMT 601	1,46	4,3	0,4
VMT 605	1,46	4,3	0,4

*MIC, minimum inhibitory concentration (mg/l); FIC_i, fractional inhibitory concentration index

synergy occurs because PMX is able to increase the efficacy of hydrophobic antibiotics, such as MCZ, by facilitating penetration so that MCZ can more readily reach the intracellular space in order to carry out its action [20]. MCZ is known to inhibit the synthesis of ergosterol, a bioregulator of membrane integrity in fungal cells [21]. MCZ can also function in its antifungal role by inducing reactive oxygen species which results in fungal cell death [22, 23].

Up until now, synergy between PMX and MCZ has been demonstrated against different bacterial species often involved in canine otitis externa, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* [11, 12], but in most cases experiments have been conducted on reference strains. Moreover, Pietschmann *et al.* (2013) have recently shown an *in vitro* synergy of PMX and MCZ against field strains (i.e. strains sampled from dogs with otitis externa) of *E. coli* and *P. aeruginosa*. Regarding fungi, synergy of PMX and MCZ has also been demonstrated against *Candida albicans* [24] and *M. pachydermatis*. However, with reference to *M. pachydermatis*, data has only been collected for one strain, which was, furthermore, a reference strain coming from a fungal collection (*M. pachydermatis* CBS 1879) [12]. Our data confirm that a synergistic interaction of PMX and MCZ exists against field strains of *M. pachydermatis*. Although further studies on a larger number of strains are necessary for confirmation, these findings support the rationale for applying PMX and MCZ in combination for the treatment of canine otitis externa caused by *M. pachydermatis*. In addition, the fact that these two drugs display a synergistic effect may enable a reduction of their dosage and of the length of treatment, which, in turn, may reduce the risk of the development of drug resistance.

CONFLICT OF INTEREST

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