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Cultural and *in-vitro* antibacterial studies of canine bacterial dermatitis

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Abstract
In present investigation detailed cultural examination of all the 52 samples collected and processed from bacterial dermatitis dogs revealed positive for 72 isolates. Out of these 52/72 were *Staphylococci* spp. (72.22%) and among these 34 isolates were *S. intermedius* (65.38%), 12 were *S. aureus* (23.07%) and 6 were *S. epidermidis* (11.53%). Whereas, twenty of these samples also revealed *Klebsiella* spp. in 9 (12.5%), *Pseudomonas* spp. in 8 dogs (11.11%) and *E. coli* in 3 (4.17%) that were mixed with *Staphylococci*. The occurrence of single and mixed bacterial infection was 61.53 and 38.47 %, respectively. In this study 27 dogs (51.88%) with bacterial dermatitis were associated with other conditions like demodicosis (17.30%), *Malassezia dermatitis* (9.61%), tick infestation (7.69%), sarcoptic mange (5.76%), hypothyroidism, flea infestation and lice infestation (3.84%). *In-vitro* antibiotic sensitivity test was done on pure isolates by using conventional antibacterial agents and observed enrofloxacin had 100% efficacy against *S. intermedius*, *S. epidermidis*, *E. coli* and *Klebsiella*, while 83.3% and 75.00% sensitivity was recorded against *S. aureus* and *Pseudomonas* spp. respectively. Clindamycin had 100% efficacy against *S. intermedius*, 83.3% efficacy aginst *S. aureus* and *S. epidermidis*, *E. coli*, *Klebsiella*, *Pseudomonas* spp, are comparatively resistant

Keywords: Dogs, bacterial infection, isolates, antibacterial agents, dermatitis

Introduction
In the realm of small animal medicine, pyoderma is one of the most typical causes of dermatitis. Pyoderma is a term used to describe a bacterial infection of the skin that produces pus. Due to the distinct features of canine skin, including a thin stratum corneum, a lack of lipid plug in the hair follicles, and a high skin pH that increases the chance of bacterial invasion, subsequent colonization, and overgrowth, dogs are more susceptible to pyoderma. This could result in bacterial superficial folliculitis. Devriese *et al*. (2005) [8] and Takashi *et al*. (2007) [22] are two examples. Lesions can be quite superficial, affecting simply the epidermis, or they might involve deeper dermal or subcutaneous tissue structures. The majority of the *Staphylococcus* intermediusa coagulase positive microorganisms were recovered from an affected dog. *Pseudomonas* spp., *Actinomyces* spp., *Actinobacillus* spp., *Fusobacterium* spp., and Mycobacterium spp. are some of the additional causal organisms that can cause pyoderma (Scott *et al*., 1995; and Paradis *et al*., 2001) [18, 14].

Materials and methods
Sterile cotton swab method
Dry, sterile cork screw cotton swabs were used for collecting material from purullent lesions for isolation and identification of *bacterial* spp. and to perform the antibacteial test for whole cultures and individual organisms. Sterile cork screw cotton swabs moistened with sterile normal saline were used for collecting from dry lesions. The samples that were collected using sterile swab was then rolled on a clean glass slide and stained by using Gram's staining method. Further, swabs were also transferred to nutrient broth and incubated for 24 hrs at 370c. After 24 hrs of incubation the sample is streaked onto selective specific media i.e., MSA, EMB and Streptococcus broth and *Pseudomonas* broth.

Direct slide impression smear
Direct slide impression smears were collected from erythematic, papules and purulent lesions of the dogs suspected with bacterial dermatitis. The smear was stained with Gram's stain and examined under microscope to identify the different types of bacterial organisms.

Examination of skin scrapings
Skin scrapings were examined in all the dogs to confirm the possibility of presence of mites by collecting skin scrapings till blood oozed from active lesions of the dogs included in the study. Deep scrapings were performed for Demodex and multiple superficial scrapings for Sarcoptic mites. Skin was scraped in the direction of hair growth. Collected material was placed on a microscopic slide, a coverslip was applied and examined under low power (10x) of microscope. If the sample was found negative for mites by this method the remaining material was further processed as per the method of Scott *et al*. (1995) [18]. The sample thus collected was transferred into a test tube containing about 5 ml of 10% KOH and heated gently for 3-5 minutes. The sample was centrifuged at 1500 rpm for 3-5 minutes and supernatant was discarded. A drop of sediment was taken on a clean glass slide covered with a cover slip and examined under microscope (10x and 40x) for the presence of adult mites or their developmental stages which were identified as per their morphological characters as described by Soulsby (2005) [20].

Examination of hair pluck
The hair were collected using haemostat forceps, mounted on a glass slide with mineral oil, placed a coverslip and examined under low power of microscope.

Isolation of bacteria
Swabs collected from dogs affected with superficial pyoderma were inoculated into nutrient broth and incubated at 37oC for 24 hours and then a loopful of broth culture was streaked on nutrient agar plates. Based on morphology and Gram staining properties, cultures were inoculated into specific / selective media like Mannitol salt agar, Eosin Methylene Blue agar, MacConkey agar and Blood agar.
Primary identification of bacteria was done based on Gram's staining, colony morphology, type of hemolysis and pure cultures were identified up to genus level as per the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) [11]. The Gram negative rods were inoculated on MacConkey agar, EMB agar and their biochemical profile was analyzed.

Gram's staining
Bacterial smears were prepared by mixing 24 hour old cultures with sterile saline on a clean microscopic slide. The smear was then air dried, heat fixed over flame and then stained with Gram's staining kit and examined under oil immersion. The bacteria were studied and each isolate was recorded as Gram positive or negative, cocci, bacilli or coccobacilli.

Biochemical tests
The bacterial organisms that were isolated from the present clinical cases were subjected for the following biochemical tests to study their characters and further confirmations:

Catalase test
The catalase reaction was determined by application of a drop of hydrogen peroxide (10 vol.) onto a portion of a colony on a glass slide, or directly on colonies on the culture media. The production of gas bubbles indicated a positive reaction.

Urease test
The sterile urease medium slants were streaked with a single colony of pure culture under test an incubated at 37 °C for 24-48 hrs. After the completion of incubation period the color change in the medium due to pH variation were recorded.

Indole production test
5ml of peptone water was inoculated with a single colony of culture under test and incubated at 37 °C for 48 hrs. After incubation few drops of Kovac's indole reagent was added to the culture. A color change in the medium was recorded at the interface of the reagent and the culture. A positive result will have a red layer and a negative result will have a yellow or brown layer on the top.

Methyl Red test
5ml of glucose phosphate peptone water was inoculated with a single colony of test culture and incubated at 37 °C for 24-48 hrs. After incubation 5 drops of methyl red indicator was added to culture.
A color change in the medium due to pH variation was recorded. Presence of a distinct red or yellow layer at the top of the broth indicates positive or negative, respectively.

Voges Proskauer test
Inoculated one loop full of culture to 5ml of glucose phosphate peptone water and incubated at 37 °C for 48-72 hrs. After incubation 0.6ml of α-naphthol and 0.2 ml of 40% KOH was added to the culture. A color change in the medium due to pH variation was recorded. With a positive reaction the medium will change to pink or red indicating that acetoin is present. With a negative reaction the broth will not change its color or will be copper colored.

Citrate test
A single colony of the culture under test was streaked onto the Cimmon's citrate agar slant and incubated the slants at 37 °C for 24 hrs. After incubation the colour change in the medium due to the pH variation was recorded. A positive reaction is indicated by a slant with a Prussian blue color. A negative slant will have no growth of bacteria and will remain green.

Coagulase test
This test was done to identify the pathogenic strains of *Staphylococci*. A loopful of the Staphylococcal culture was emulsified in a drop of distilled water on a slide. A loopful of rabbit plasma was added and mixed well with the bacterial suspension. A positive reaction was indicated by clumping within one or two minutes when the slide was gently rocked (Quinn *et al*., 1999) [15].

Cytology
Tape impression smears were examined based on the technique used by Eluk *et al*. (2003) [10]. These were used to collect the samples from dry lesions. Hair present around the skin lesion was trimmed with a curved scissor. A piece of clear one sided cellophane acetate tape of 5.5 cm length and 2.5 cm width was taken from the roll and applied to the affected area, pressed twice firmly for two seconds. After removal, the strip was placed onto a pre cleaned microscopic glass slide with the sticky surface facing upward and fixed properly at both the ends. The prepared smears were stained with new methylene blue for one minute, the stain was removed, rinsed with distilled water and allowed to air dry. The stained slides were then examined under oil immersion

objective. Glass slide impression smears were used to collect the samples from wet lesions (Marsella *et al*., 2000) [12]. The hair around the lesions was clipped and cleaned with spirit. The adjacent skin was gently squeezed and the glass slide pressed over the lesion. The slide was air dried and stained with new methylene blue for one minute and examined under oil immersion.

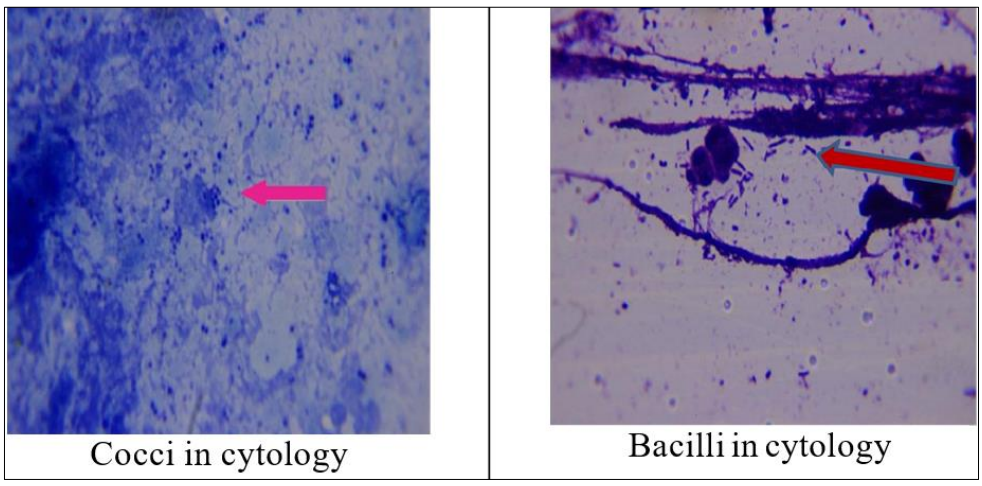
Antibacterial sensitivity testing

Swabs from dogs with suspected bacterial dermatitis were transferred on to Nutrient broth and incubated for 24 hrs at 37 °C in air supplemented with 5% CO_2. The inoculums were prepared in accordance with British Society for Antimicrobial Chemotherapy standards for susceptibility testing at a density equivalent to a 0.5 McFarland standard suspension and the turbidity was adjusted to yield 1 x 106 - 5 x 106 cells /ml i.e., 0.5 McFarland standard, so that following incubation, a semi-confluent lawn of growth was present. A stock 0.5 McFarland standard was obtained and kept at room temperature protected from light. The standard was mixed well using a vertex prior to use.

Suspensions of bacteria for susceptibility testing were prepared by the direct colony suspension method. In this, four distinct but morphologically similar colonies were touched with a sterile cotton swab and transferred into test tubes containing sterile deionized water. After homogenization of the bacterial suspension, comparisons were made with the 0.5 McFarland standard. A sterile cotton swab was dipped into the suspension and rotated several times. Excess fluid was removed from the swab was removed by pressing firmly against the inside wall above the fluid level. The cotton swab was then streaked on the dried surface of the agar according to the standard method described by CLSI. The plate may be left open for 3-5 minutes, allowing excess moisture to be absorbed. Using sterile forceps, the antibacterial discs (Enrofloxacin, Clindamycin, Lincomycin, Amoxicillin with clavulanic acid, Gentamicin, Cephalexin, Ceftriaxone, Ceftriaxone with sulbactam) were placed at the rate of six 0C and discs per plate with a 30 mm distance between the discs centers. The plates were incubated at 37 °C examine after 24 hrs of incubation. The diameter of zone showing complete inhibition of the growth of the organism under test was measured. The interpretation of results was made as per the inhibition zone size chart provided by the manufacturer.

Results and Discussions

The dogs with bacterial dermatitis (52) of the present study diagnosed primarily through cytological examination which were also further confirmed by the examination of Gram's stained smears of cultures obtained from the lesions. A total of 72 bacterial isolates were identified on cultural examination carried out on 52 samples. Bacterial isolation studies revealed *Staphylococci* spp. from all the 52 samples and *Staphylococci* spp. mixed with gram negative bacteria in 20 samples. Species identification of *Staphylococcus* was confirmed based on the cultural characteristics as well as the biochemical properties (Castellanos *et al*., 2011) [5]. Among 52 *Staphylococci* isolates, 34 were *S. intermedius* (65.38%), 12 were *S. aureus* (23.07%), and 6 were *S. epidermidis* (11.53%). The findings of the present study was in accordance with Bensignor and Germain (2004) [2], Cavalcanti *et al*. (2005) [6] and Wilkoek *et al*. (2006) [23] also identified *S. intermedius* as the major pathogen in dogs with pyoderma. *S. pseudintermedius* spp. along with *S. intermedius* and *S. delphini* are a part of *S. intermedius* group and was the main pathogenic species involved in a great variety of infections in dogs (Devriese *et al*., 2005 and Devriese and Coll, 2009). Muller *et al*. (1983) [8, 9] opined that *Staphylococcus pseudintermedius* is a part of normal skin microflora which breaks the cutaneous ecosystem and becomes pathogenic under certain favourable conditions. While, Bond and Loeffler (2011) [4] documented that every *Staphylococcus* intermedius was considered as part of *Staphylococcus pseudintermedius* species. Twenty-gram negative isolates of the present study included *Klebsiella* spp. (9/72), *Pseudomonas* spp. (8/72) and *E. coli* (3/72). Mixed infection of *Staphylococci* spp. with gram negative bacteria was previously reported by many scientists (Udaysree and Pillai, 2007; Reddy *et al*., 2011; Beigh *et al*., 2013 and Senapati *et al*., 2014) [21, 16, 1]. It was assumed that *S. intermedius* was the primary pathogen in mixed infections. Tissue invasion by *Klebsiella* spp, *Pseudomonas* spp, or *E. coli* was probably secondary to changes created by *S. intermedius* infection, as this organism created a tissue condition favorable to the replication of the other secondary bacterial invaders (Scott, 2003) [17]. From the findings of the present study it was evident that among 52 dogs with bacterial dermatitis the occurrence of single and mixed bacterial infection was 61.53% and 38.47%, respectively.



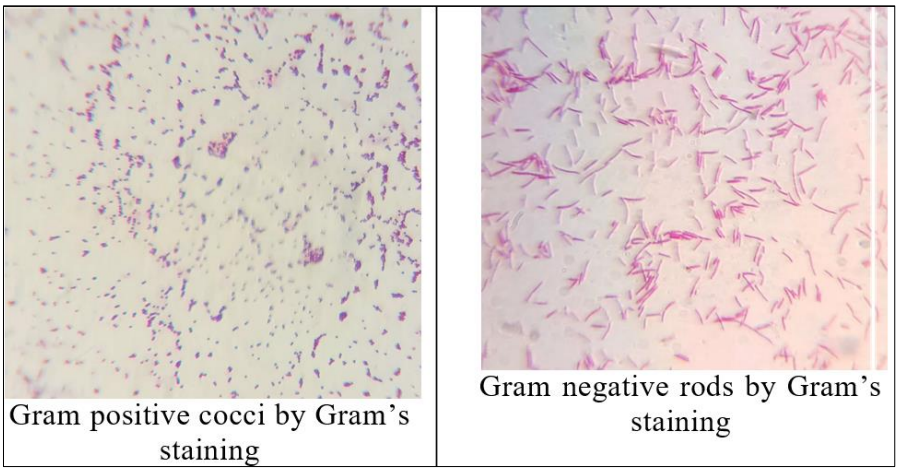


Fig 1: Cytology

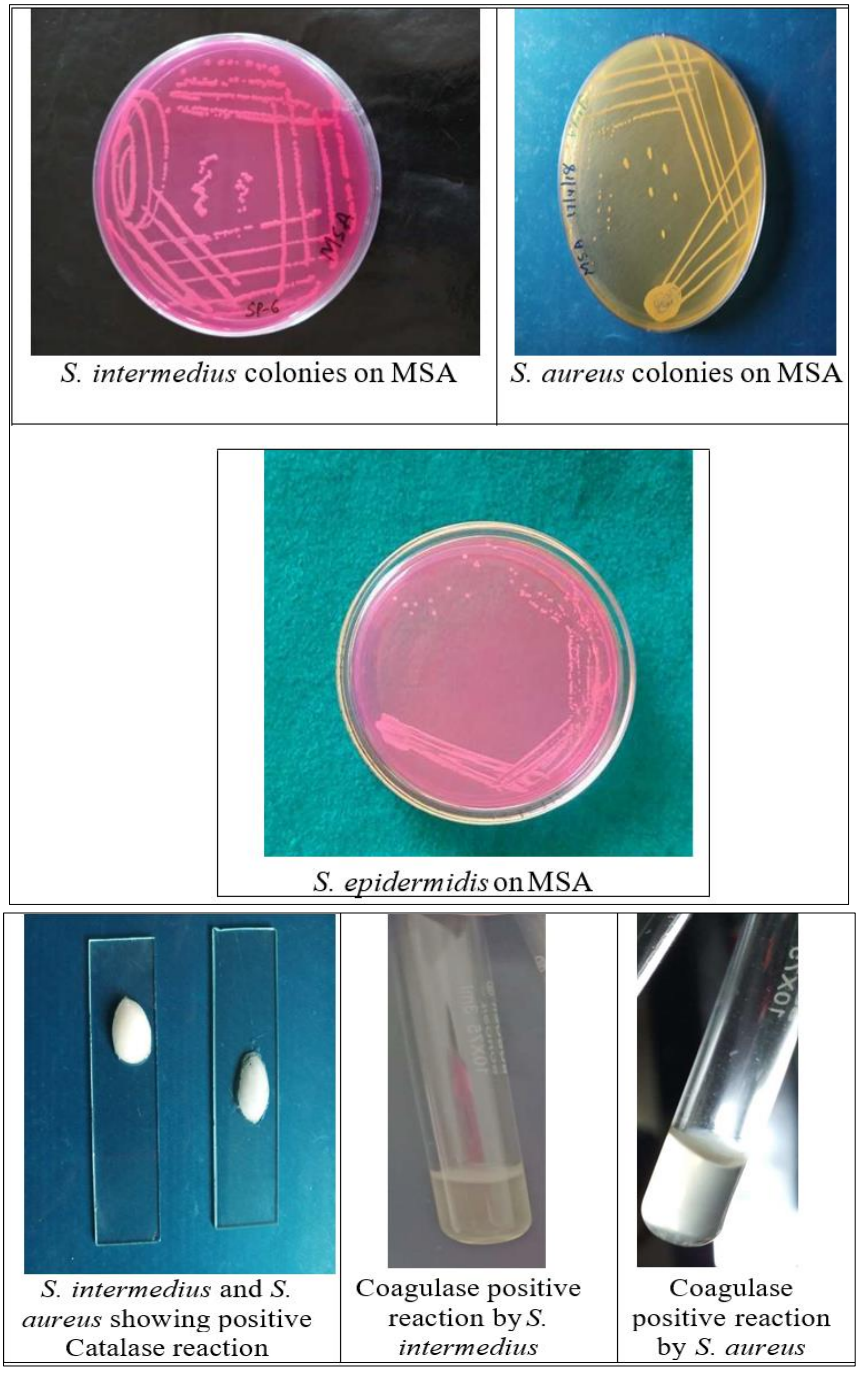


Fig 2: Culture of bacteria

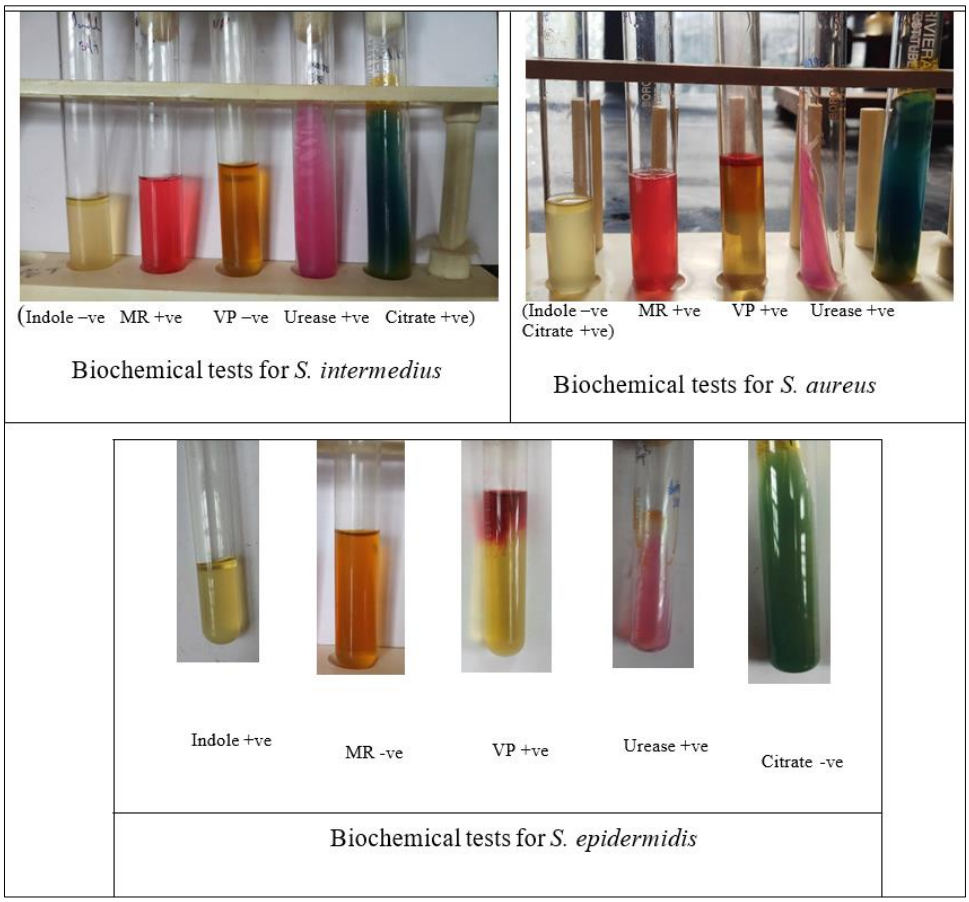


Fig 3: Biochemical tests for *Staphylococcus* species

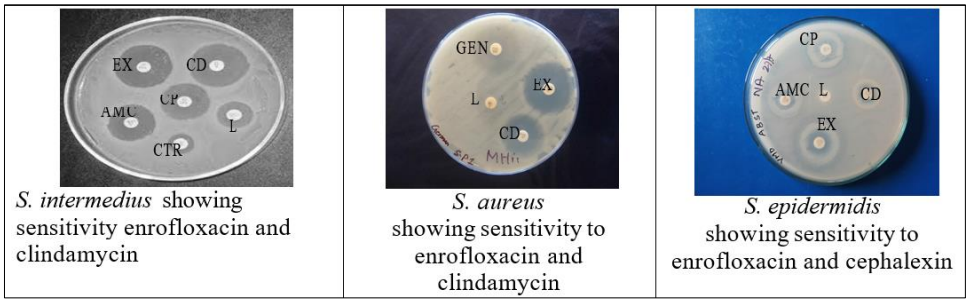


Fig 4: Antibiotic sensitivity pattern

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