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FUR MICROBIOMES OF CANIS SPS.

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ABSTRACT
Dogs are the most faithful domesticated animals and loved by all irrespective of their status as stray/pets. Depending on their habitat and surrounding environments they may harbour different types of microbes. The recent trend of dog lovers to cuddle and love the dogs is a common scenario. However, such practices can lead to the spread of many infectious agents which are transferred from the fur of the dog to the humans. The present study was therefore designed to identify and assess the different microbes associated on the fur of the stray dogs at selected locations in Mumbai. Fur samples collected from back, neck and head area of the dogs was represented by Seventeen different bacterial species of both Gram positive and Gram negative bacteria. An antimicrobial assay analysis of these microbes was carried out using four standard commercially available human consumed antibiotics indicated that these bacteria were resistant to three out of four standard antibiotics, except to that of Ciprofloxacin. The high MAR/MDR indices indicated that necessary precautions should be taken by dog lovers while managing their pets/ street dogs

KEY WORDS: Fur, microbes, antibiotics, resistant.

INTRODUCTION

Dogs are home to many different microorganisms, with a range of bacteria, fungi and archaea living on the dermal, intestinal parts of body. While many of these microorganisms are beneficial to their hosts, very little known about their impact on the environment. Dog microbiota or the dog microbiome, are of recent concern as effects of the exposure of the dog microbiota on humans is yet to be established (Aline *et al*., 2014). Research studies also proved that owners of these domesticated animals often share microbes with their own dogs as bacteria from a dog's fur and paws is easily transferred to the skin of humans living in the same space (Song *et al*., 2013). Stroking the dog by one person may leave bacteria behind, which will be picked up next person who pets the same dog. Studies indicated that mouths of 50 dogs harboured total of 353 different types of bacteria; of which 80% of bacteria didn't even have pre-existing names (Sujata, 2017). Normal flora found on the dog fur includes *Staphylococci, Streptococci, Escherichia coli*, members of *Bacillus spp, Pseudomonas spp, Lactobacillus spp, Neisseria spp, Alcaligenes spp, Pasteurella spp, Klebsiella spp,* and *Francisella spp.* (Sturgeon *et al*., 2012). The most abundant fungi present on canine skin, across all body sites and health statuses, are Alternaria and *Cladosporium*- two of the most common fungal allergens in human environmental allergies (Song *et al*., 2013). Gram-negative cocci, such as *Moraxella* or *Neisseria,* are not widely recognized as significant pathogens of dogs. However, they are commensals of the oral cavity of dogs and can cause bite wound infections in humans. Gram-negative rods are classified into Enterobacteriaceae and non-Enterobacteriaceae species. Enterobacteriaceae that are pathogenic and found on dogs include *Escherichia coli, Proteus, Salmonella, Enterobacter, Citrobacter, Serratia,* and *Klebsiella*. Non-Enterobacteriaceae species include the Pasteurellaceae *(Pasteurella multocida),* as well as *Pseudomonas aeruginosa* and *Acinetobacter*. Other gram-negative bacteria are coccobacilli or spiral-shaped organisms & include *Bartonella, Bordetella, Campylobacter, Francisella, Helicobacter,* and *Brucella*. (Courtney *et al*., 2015)

MATERIALS & METHODS

Sample collection

Samples were collected from 15 street dogs in Vile Parle. From head area, neck and back region using sterile culture swab applicator. Each swab applicator was rubbed on the skin 3 times, while rotating each swab by one quarter for every 10 strokes (Baron and Finegold, 1990). The swab was stored in a properly labelled tube containing 1ml of sterile saline solution and refrigerated at 4°C until further analysis.

Isolation and characterization

These swab samples were spread on 1% nutrient agar plate and incubated for 37 C for 24 hours and growth of different colonies were observed. The colonies were identified, characterized and analysed for resistance to the commercial antibiotics.

Different types of colonies were studied and their macroscopic characteristics were checked which included size, shape, surface, colour, elevation, margin and optical characteristics were noted. Gram nature of the colonies was carried out to check whether they were gram positive or gram negative. Gram negative colonies were chosen from the colonies obtained for antimicrobial sensitivity testing.

Preparation of antibiotic solutions

Four commercially available antibiotics were selected such as Amoxicillin, Ciprofloxacin, Chloramphenicol and Tetracycline for antibiotic resistance studies. Known weight of antibiotic powder was dissolved in sterile distilled water to obtain the stock solution of 0.5 g/10 ml. The stock solutions were diluted (10^4 times) at the time of disc preparation to obtain the working solution of 50µg /ml. (Bistner, 1980). 6mm diameter in Whatman filter paper sterilized discs were impregnated with the prepared antibiotic working solutions (Vineetha *et al*., 2015) and used for antibiotic studies.

Preparation of bacterial suspension

The bacterial colonies were subcultured on sterile nutrient agar slants and homogenized culture suspension prepared which matched for turbidity with 0.5 McFarland solutions (Carter and Cole, 1990)

Antibiotic sensitivity assays

Mueller Hinton Agar plates were streaked with bacterial suspension to obtain uniform growth (Carter and Cole, 1990). Antibiotic discs containing specific antibiotics were dispensed with sterile forceps onto the plate and ensured that the disc attached to the agar. The plates were incubated overnight at 37°C. Inhibition zone sizes (area of no growth around the disk) were measured in millimeters using a ruler or template. Interpret the results as Resistant, Intermediate or Sensitive for each antibiotic by comparing with the standard ranges listed on the Kirby-Bauer chart (Bauer *et al*., 1966).

Calculation of Multiple Antibiotic Resistance (MAR) index

Multiple antibiotic resistance (MAR) index was calculated as ***a/b*** where 'a' represents the number of antibiotics to which the isolates were resistant and 'b' represents the total number of antibiotics to which the isolate was exposed (Adenaike *et al*., 2016; Raminder *et al*., 2016).

RESULTS

Samples collected from 15 street dogs in Vile Parle, showed 17 different isolated bacterial species which belonged to both, Gram positive and Gram negative on Nutrient Agar. The Macroscopic colony characteristics and Gram nature of all 17 colony variants was determined and tabulated (Table -1).

TABLE 1. Macroscopic colony characteristics and gram nature of canine fur microbiome

Colony	Size	Shape	Suface	Colour	Elevation	Margin	Optical characteristic	Gram nature
Variant 1	5mm	circular	concentric	off white	Flat	serated	opaque	G -ve bacilli
Variant 2	7mm	circular	smooth	white	Flat	Undulated	opaque	G-ve bacilli
Variant 3	1mm	circular	smooth	orange	Flat	entire	opaque	G-ve cocci
Variant 4	1mm	Puncti-form	smooth	yellow	Flat	entire	opaque	G-ve cocci
Variant 5	1mm	Puncti-form	smooth	red	Convex	entire	opaque	G-ve cocci
Variant 6	2mm	Irregular	smooth	orange	Flat	Undulated	opaque	G-ve bacilli
Variant 7	2mm	circular	wrinkled	dark off white	flat	entire	translucent	G +ve bacilli
Variant 8	2mm	Irregular	smooth	pink	Convex	entire	translucent	G-ve bacilli
Variant 9	1mm	Puncti-form	smooth	no colour	Convex	entire	transparent	G +ve bacilli
Variant 10	2mm	circular	smooth	white	Convex	entire	opaque	G +ve cocci
Variant 11	3mm	circular	smooth	white	Raised	entire	translucent	G +ve bacilli
Variant 12	1mm	circular	smooth	white	Pulvinate	entire	translucent	G-ve bacilli
Variant 13	3mm	circular	smooth	white	Flat	entire	translucent	G +ve cocci
Variant 14	1mm	circular	smooth	yellow	Convex	entire	translucent	G +ve cocci
Variant 15	1mm	circular	smooth	white	Flat	entire	translucent	G-ve bacilli
Variant 16	2mm	circular	smooth	white	umbonate	entire	opaque	G +ve bacilli
Variant 17	2mm	circular	smooth	white	Flat	entire	opaque	G-ve cocci

The size of the colony variants ranged from 1mm to 7mm. While most of the variants were circular in shape a few of them were punctiform and irregular in shape. About 95% of variants had smooth surfaces and entire margins however; a variation was seen in the elevation, colour and optical characteristics of the variants. While flat and convex elevations were prominent, pulvinate, umbonate and raised elevations were also observed. The colour of the variants ranged from white and off white (variant: 1, 2, 7, 10, 11, 15, 16 and 17) to orange (variant: 3 and 6), yellow (variant: 4 and 14), red (variant: 5) and pink (variant: 8). About 50% of the variants were opaque while the remaining was translucent with only one transparent variant. From the 17 colony variants isolated only the Gram negative colony variants were further subcultured and subjected to antibiotic sensitivity testing. Out of 17 variants, 9 variants were Gram negative and exhibited two types of morphology, Gram negative bacilli (6 colonies) and Gram negative cocci (3 colonies).

Determination of susceptibility against antibiotics

The Kirby Bauer method of antibiotic sensitivity testing is based on the principle of diffusion and is the official method of the US FDA (Food and Drug Administration). In this method three grades of sensitivity are recognized namely: Sensitive, Intermediate and Resistant by comparing the diameter of the inhibition zone with the critical zone diameter in published tables provided by the National Clinical Control Laboratory Standards (National Committee for Clinical Laboratory Standards 2003).

Susceptibility testing of the isolated Gram negative bacteria to routine antibiotics (including: Amoxicillin, Chloramphenicol, Ciprofloxacin and Tetracycline) showed the results (Table 3) indicating that the relative potency of these antibiotic compounds were Ciprofloxacin > Amoxicillin > Tetracycline > Chloramphenicol. The results were obtained by taking the average of the observed values of zone of inhibition for each set (three sets were performed to ensure reproducibility). The results of the susceptibility testing for the Gram negative bacteria indicated that out of 9 Gram negative variants, 4 variants (*i.e.* variant 3, 6, 8 and 17) were found to be resistant to all the antibiotics included in the study. Variant 1 was found to be the most sensitive towards the antibiotics included in the study which was indicated by the observed zone of inhibitions (Tetracycline-7mm, Amoxicillin-9mm, and Ciprofloxacin-23 mm). Variant 2, 12 and 15 were found to be intermediately sensitive to Ciprofloxacin while variant 1 and 4 were highly sensitive to the same. No zone of inhibition was observer for Chloramphenicol indicating that all 9 variants were resistant to the drug. Thus the antibiotic susceptibility test results in this study revealed that the most effective antibiotic on the isolated Gram positive bacteria is Ciprofloxacin and the less effective antibiotics are Amoxicillin and Chloramphenicol.

TABLE 2. Zone size interpretative chart

Antibiotic (Antimicrobial Agent)	Disc Code	Resistant (< or = mm)	Intermediate (mm)	Sensitive (= or > mm)
Amoxicillin	AMC	<13	14-17	>18
Chloramphenicol	C	12	13-17	18
Ciprofloxacin	CIP	15	16-20	21
Tetracycline	T	14	15-17	18

TABLE 3. Canine microbiome - drug control by commercially available antibiotics

Colony	Amoxicillin	Tetracycline	Ciprofloxacin	Chloramphenicol	Gram nature
Variant 1	R	R	S	R	Gram +ve
Variant 2	R	R	I	R	Gram +ve
Variant 3	R	R	R	R	Gram +ve
Variant 4	R	R	S	R	Gram +ve
Variant 6	R	R	R	R	Gram +ve
Variant 8	R	R	R	R	Gram +ve
Variant 12	R	R	I	R	Gram +ve
Variant 15	R	R	I	R	Gram +ve
Variant 17	R	R	R	R	Gram +ve
Variant 5	R	R	I	R	Gram -ve
Variant 7	I	R	S	R	Gram -ve
Variant 9	R	R	I	R	Gram -ve
Variant 10	R	R	I	R	Gram -ve
Variant 11	R	R	R	R	Gram -ve
Variant 13	R	R	S	R	Gram -ve
Variant 14	R	R	R	R	Gram -ve
Variant 16	R	R	R	R	Gram -ve

(Key : R-resistant ; S: sensitive; I-Intermediate)

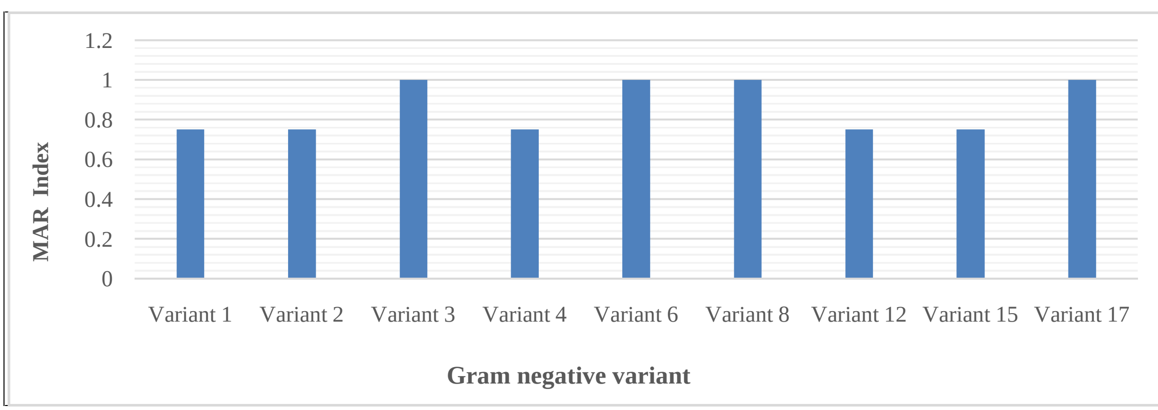

FIGURE 1: Multiple Antibiotic Resistance (MAR) index of Gram-ve variants

Multiple antibiotic resistance (MAR) index for the Gram negative variants was found 44.44% of the variants were resistant to all the four antibiotics included in the study (indicated by MAR index=1.00) while 55.56% of the variants were resistant to three out of four antibiotics (indicated by MAR index=0.75). Thus, indicating that the isolated Gram negative variants showed multidrug resistance (MDR) (Fig.1).

For the Gram positive bacteria out of 8 Gram positive variants, 3 variants (*i.e*. variant 11, 14, and 16) were found to be resistant to all the antibiotics in the study. Variant 7 was found to be the most sensitive towards the antibiotics included in the study which was indicated by the observed zone of inhibitions (Amoxicillin-14mm, Ciprofloxacin-21mm). Variant 5, 9, and 10 were found to be intermediately sensitive to Ciprofloxacin while variant 7 and 13 were highly sensitive to the same. No zone of inhibition was observed for Chloramphenicol and Tetracycline indicating that all 8 variants were resistant to these 2 drugs. Thus the antibiotic susceptibility test results in this study revealed that the most effective antibiotic on the isolated Gram positive bacteria is Ciprofloxacin and the less effective antibiotics are Amoxicillin, Tetracycline and Chloramphenicol. Multiple antibiotic resistance (MAR) index of Gram positive variants was found to beout 41.1% of variants were resistant to all 4 antibiotics

(MAR index=1.00) 52.9% of the variants were to 3 out of 4 antibiotics (MAR index=0.75) while 5.8% of the variants were resistant to 2 out of 4 antibiotics (MAR index =0.50) Thus, indicating that the isolated variants showed multidrug resistance (MDR) Fig. 2.

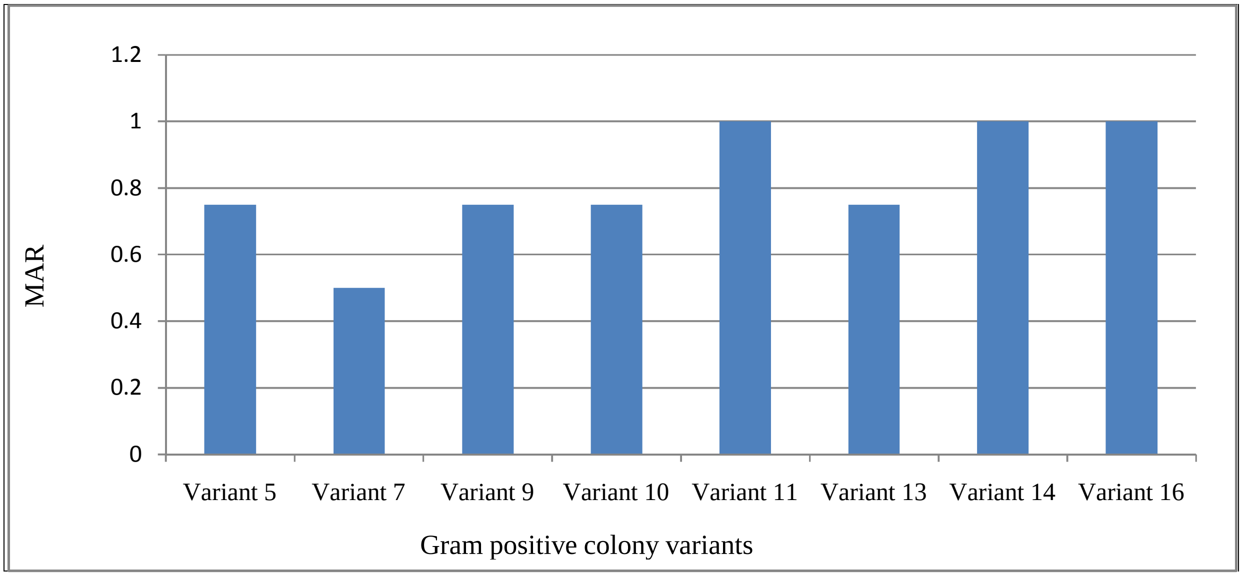

FIGURE 2: Multiple Antibiotic Resistance (MAR) index of Gram+ ve variants

DISCUSSION

Fur samples from various street dogs indicated that the dogs were carriers of various forms of microorganisms. Bacterial species belonged to both Gram positive and negative types indicated their resistance /sensitivity towards 4 commercially available antibiotics assessed by disc method using Kirby Bauer standards (concentration drug is 50 µg/ml). Amoxicillin being a beta lactam is not very effective in the inhibition of Gram negative microorganisms and helps to differentiate the Gram negative and Gram positive bacteria (Haghkhah *et al*., 2004). From the results it was seen that out of the 9 Gram negative organisms isolated from the dog fur, only 2 showed inhibition and sensitivity against the amoxicillin drug and the rest showed resistance it. Tetracycline being a broad spectrum antibiotic showed no effect on any of the 9 variants isolated except 1, showing that the microorganisms growing on the street dogs fur are highly resistant to tetracycline and it might indicate that the microorganisms found on the dog's fur have become resistant to the antibiotics and evolved over time. Ciprofloxacin was the most successful antibiotic out of all the 4 antibiotics used showing sensitivity for all 9 variants of isolated microorganisms and showing considerable amount of inhibition towards each of the type of microorganisms with as much as 23mm of inhibition in 1 type of microorganisms. Chloramphenicol showed no sensitivity for any of the strains of microorganism's isolated showing that all the strains were highly resistant to chloramphenicol. From the results obtained it can be interpreted that the best mode of action against the isolated microorganisms is by the method of inhibition of nucleic acid synthesis/ cell division synthesis as done by Ciprofloxacin while the method of inhibiting the protein synthesis was the least effect as can be interpreted due to no variants of microorganisms showing any sensitivity towards Tetracycline and Chloramphenicol which follows the method of inhibition of protein synthesis (Peiffer *et al*., 1984).

CONCLUSION

Research on oral flora of the dogs and diseases spread through them is well established as compared to the diseases spread through the skin of the dogs. The current study helps us to assess the importance of fur micro biome and spread awareness regarding bacteria on the skin of the dogs. Diseases spread by dogs can be serious whether through their skin or being bitten by them. Since fur microbes showed resistance to the antibiotics that are usually used by humans for bacterial infections, the results indicated their multidrug resistance (MDR) in the form of calculated multiple antibiotic resistance (MAR) index for the isolated Gram positive and negative organisms. Ciprofloxacin was the most effective drug while Tetracycline and Chloramphenicol were the least effective. This indicated that inhibition of nucleic acid synthesis/ cell division synthesis of the fur microbiomes was more effective way of controlling the organisms (Ciprofloxacin) than protein synthesis (Tetracycline and Chloramphenicol).

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