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Research article

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# Isolation and characterisation of salivary microbiota of street dogs

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## Abstract

Diseases spreading from animals to humans have become a major issue as a result of the easy means of transmission through water, air, food and direct contact. It was found that, there are a lot of risks associated with dogs when humans come in contact with them. Many diseases are likely to spread from a common domestic animal, such as dogs. In the present study, the saliva samples of street dogs from Thiruvottiyur area, Chennai were collected. Colonies were isolated by spread plate technique and further subjected to morphological and biochemical characterisation to identify the bacteria. In order to evaluate its pathogenicity against humans it was tested against 7 commercial antibiotics by disc diffusion method. Out of ten isolates, one strain named as S2 had shown resistance to all antibiotics. All the isolates from the saliva of dogs were predicted to be human pathogens and the strain S2 was further subjected to 16S rRNA sequencing and identified as *Pasteurella multocida*. It appears like street dogs might be the mediators to spread the pathogenic microbes easily to the humans.

## Keywords

*Dog's saliva; Antibiotics; Pathogens; Microbes; Isolation*

## Introduction

Environments not only depend on the surrounding and climatic changes but also on the health and happiness of the people. Due to the present polluted environment, diseases gets transmitted through various factors, such as animals, water, air, food, etc. and the main cause of all this is the spread of various pathogenic microbes through the above mentioned factors. The change in the pattern of the present environment condition resulted in the outbreak of many diseases and health related issues concerned with humans. And the condition of the human population has become worse in the present era and likely to get even more worse.[1,2] Since 19th century, we have known that microbes cause infectious diseases. Near the end of the 20th century, researchers began to learn that microbes also contribute too many chronic diseases and conditions. Mounting scientific evidence strongly links microbes to some forms of cancer, coronary artery disease, diabetes, multiple sclerosis and chronic lung diseases.[3] Prevention of microbial infections needs more focus because the microbes can easily spread the diseases among everyone through several modes and create more diseases. Compared to all the other diseases that spread from various factors, the one that spread from animals to humans commonly termed as "zoonosis" is a very serious issue and dangerous too. According to the recent UN report, 45 diseases have been reported to get transmitted from animals to humans in the last two decades and are likely to increase in the present decade. A key factor for the issue is urbanization and globalization. People have started moving to places that have been untouched, and as a result of the interconnectivity between places, every disease that arises seems to result an epidemic.[4] Dogs are found to be the major source of a wide range of zoonotic infections that pose a significant threat to the human population. Every animal's mouth is the storehouse of microbes; similarly the dog's mouth also contains a number of microbes, such as bacteria, fungi and virus. They are commonly found in slums, streets and mainly in the waste areas. Since they get exposed to polluted water, faeces, waste materials and licking of the private parts, the saliva of the dogs normally contains germs and pathogens in it.[5]

There are proven studies that state immunosuppression as a factor for transmission of zoonosis from dogs to humans. A major population of human society is immune-deficient due to various reasons and moreover the elderly groups are being subjected to immunosuppressive agents.[6] People on immunosuppressive treatment, with alcoholism, with diabetes, and pregnant women are more susceptible to zoonosis as a result of their poor immune status.[7] Enteric pathogens, such as *Salmonella*, *Campylobacter* and *Cryptosporidium* species from canines are a frequent risk to the immunocompromised persons and they cause infections like brucellosis, campylobacteriosis and cryptosporidiosis. Hence, canines are of more concern to prevent transmittable zoonotic diseases. The main aim of this work was to determine the degree of pathogenicity, identify the diseases caused and analyse the antimicrobial sensitivity of microbes from canines. The study was performed to create awareness to people, confer the data for prevention and ensure the environment and social welfare.

## Material and methods

All the chemicals used in the study were laboratory grade purchased from Merck Chemicals Pvt Ltd, India, Loba Chemie Pvt Ltd, Mumbai, and the salivary samples of dogs were collected with the permission and help of Thiruvottiyur Municipal Corporation Office, Chennai, Tamil Nadu, India.

### Collection of dog's saliva sample

The street dog's saliva sample was collected from Thiruvottriyur Municipal Corporation in Chennai. Ten street dogs were caught with the help of professionals and their saliva was collected with the use of sterile oral swabs.

### Isolation of bacteria

The oral sterile swabs were well wiped in street dogs' mouth so that the saliva gets absorbed to them. The swabs were marked as S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10.

The saliva sample was cultured in nutrient agar medium through spread plate method in order to isolate the colonies. The plates were incubated at 37°C for 18 to 24 h. After incubation, the microbes were isolated and further subcultured on respective media in order to obtain the pure culture. The pure isolates were maintained at 4°C in refrigerator for further studies.

### Bacterial identification

The bacterial isolates were identified based on the morphological and biochemical characterisation. The colonies were identified based on the shape and size. Haemolytic test, motility test and Gram's staining was performed. Biochemical tests, such as Indole, MR-VP, catalase, oxidase, carbohydrate fermentation, etc. were performed to identify the isolates.

### Antibiotic sensitivity test

Antibiotic sensitivity test was followed based on the Kirby-Bauer's disc diffusion method. Muller Hinton agar was prepared and the isolates were uniformly spread over the plates and they were tested against antibiotics, such as Penicillin, Ampicillin, Ciprofloxacin, Erythromycin, Vancomycin, Chloramphenicol and Kanamycin. The plates were incubated at 35–37°C for 18–24 h.

### Molecular characterisation

Molecular characterisation of unknown isolate was performed by 16S rRNA gene sequencing. Experiments were carried with DNA isolation followed by primer designing. Universal primer was taken for the study in order to perform PCR amplification. The PCR reaction was performed using 50.0 ng (1.0 µl) of Template (DNA), 10 pmol (each 2 µl) of primer (forward and reverse separately), 10 µl of PCR Master mix (Amplicon) and 5.0 µl of double-distilled water to adjust the volume to 20.0 µl. The sample was amplified in the ratio 260:280 and the concentration 47.6. The forward primer of 16S rRNA was AGA GTT TGA TCC TGG CTC and the reverse primer was ACG GCT ACC TTG TTA CGA CTT. The PCR conditions were as following: initial denaturation for 2 min at 94°C followed by 1 min denaturation at 92°C; 30 s of primer annealing at 52°C followed by 30 s extension at 72°C and 10 min of final extension at 72°C. DNA sequencing was performed and its sequence was retrieved in FASTA format.

### Species identification

Basic local alignment search tool was used to identify the corresponding micro-organism of the available nucleotide sequence. Non-redundant database set was used to check the similarity in the database sequences. Similarity and identity were analysed based on the high similarity algorithm. The identified matched sequence was displayed in graphical and text format to find the maximum similarity species.

## Results and discussions

### Collection of saliva

Dog's saliva is found to be a good source to study about its microbiota and it can be a potent tool for collecting the DNA of the microbes.[8] Dog's saliva can be collected using a sterile oral swab and is predicted to be the store house of microbial pathogens. It was found that, several human pathogens were isolated from dogs; nearly 6 sub species of *Bartonella* have been isolated from them.[9] Similarly, DNA of pathogenic microbes can be extracted from dog's saliva and the organism can be identified.[10] In this study, nearly 10 street dogs were used for the collection of saliva to identify the microbiota and their pathogenicity (Fig. 1).

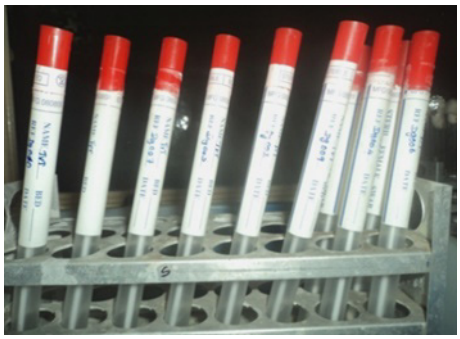


Fig.1. Oral swabs containing the dogs' saliva

### Salivary microbiota of dogs

Mouth is the indispensable anatomical part that is considered to be the store house of microbes. There has been a lot of research carried out in the microbiota of dogs and their pathogenicity against humans. Genera of Streptococcus, Actinomyces and Granulicatella are the most probable isolates of domestic dogs; nearly 28% of them match with oral microbiota of humans.[11]

Persistence of bacteria in the dogs of Nigerian and Makurdi regions was reported. Among them, *Escherichia coli* and *Staphylococcus aureus* were found in majority of the dogs followed by *Enterobacter aerogenes*, *Corynebacterium renale*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Streptococcus canis*, whereas, *Pasteurella multicoda* was found to be present in very few dogs.[12] Similar studies were reported about the root canal of the dogs and the most frequent isolates were *Prevotella* sp., *Fusobacterium* sp., *Peptostreptococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Clostridium* sp. and *Porphyromonas* sp.[13]

Nearly 5,958 clones were identified in oral canine microbiota under the phylum Firmicutes, Proteobacteria, Bacteroidetes, Spirochaetes, Synergistetes, Actinobacteria, Fusobacteria, TM7, Tenericutes, GN02, SR1, Chlorobi, Chloroflexi and WPS-2 were reported.[14]

In our study, nearly 10 different genera were identified by standard morphological and biochemical measures, and the results are tabulated (Table 1–3). From the results, microbial genera were identified to be *Haemophilus, Pasteurella, Staphylococcus, Neisseria, Capnocytophaga, Salmonella, Corynebacterium, Alkaligenes*, *Bacillus* and *Klebsiella* (Table 4). These organisms were present in the saliva of the street dogs from Thiruvottiyur area and they pretend to be human pathogens.

Table 1. Morphological result of Gram's staining and motility test

| Strains | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Gram staining | G +ve | G +ve | G +ve | G -ve | G -ve | G -ve | G +ve | G +ve | G -ve | G -ve |
| Motility | -ve | +ve | -ve | -ve | -ve | +ve | -ve | +ve | +ve | -ve |

G +ve - Gram positive bacteria; G-ve - Gram negative bacteria
+ve - Motile: -ve - Non-motile

Table 2. Biochemical test results of isolated bacteria

| Strains | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Catalase | +ve | -ve | +ve | +ve | +ve | +ve | +ve | +ve | +ve | +ve |
| Oxidase | -ve | -ve | -ve | +ve | +ve | -ve | -ve | +ve | -ve | -ve |
| Indole | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| Methyl red | -ve | +ve | -ve | -ve | +ve | +ve | +ve | -ve | -ve | +ve |
| Vogues Proskauer | -ve | -ve | -ve | +ve | +ve | -ve | -ve | -ve | -ve | +ve |
| Citrate | -ve | -ve | -ve | +ve | +ve | +ve | -ve | -ve | +ve | +ve |
| Urease | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| Triple sugar ion | K/A | -ve | K/- | A/AG | K/A/A | K/A | A/AG | -ve | K/A | K/A/AG |

+ve - Positive; -ve - Negative; A - Acid; K - Alkaline; AG - Gas; (-/-) - Slant/butt

Table 3. Carbohydrate fermentation and haemolysis test

| Strains | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Fructose | A | -ve | -ve | A/G | A | A | A | -ve | -ve | AG |
| Dextrose | A | -ve | -ve | A/G | A | A | A | -ve | A | AG |
| Lactose | A | -ve | -ve | A | A | -ve | A | -ve | A | AG |
| Sucrose | A | -ve | -ve | A/G | A | A | A | -ve | -ve | AG |
| Maltose | A | -ve | -ve | A/G | A | A | A | -ve | A | A |
| Haemolysis | Nil | Nil | ß | Nil | Nil | Nil | Nil | α | ß | α |

-ve - Negative; A - Acid; AG - Gas; ß - beta haemolysis; α - alpha haemolysis; Nil - No haemolysis

Table 4. List of micro-organisms isolated from dogs' saliva

| S. No. | Isolated bacteria |
|---|---|
| S1 | *Haemophilus* sp. |
| S2 | *Pasteurella* sp. |
| S3 | *Staphylococcus aureus* |
| S4 | *Neisseria* sp. |
| S5 | *Capnocytophaga* sp. |
| S6 | *Salmonella typhi* |
| S7 | *Corynebacterium* sp. |
| S8 | *Alkaligenes faecalis* |
| S9 | *Bacillus* sp. |
| S10 | *Klebsiella pneumoniae* |

**Pathogenicity of canine microbiota**

Prevalence of micro-organisms in dogs is common but the pathogenicity of their microbiota is problematic. Microbial existence in dogs infects the host and also spread various diseases among humans. Viral microbiota of canines is more prone to severe infections to its host from diseases, such as Distemper, Leptospirosis, Hepatitis, Kennel cough, Parvo, Corona and Rabies.[15] But bacterial microbiota is more pathogenic towards humans in comparison to viral microbiota. *Staphylococcus* is the most common pathogen carried even by the healthy dogs in low number in their skin.[16] *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* sp., *Escherichia coli*, *Corynebacterium* sp., etc. are commonly encountered pathogenic bacteria associated with canine myiasis that can spread disease to humans easily.[17] *Mycobacterium tuberculosis* identified from blood and faecal matter of dog may become a potent carrier of tuberculosis to humans.[18] Dogs are carriers of nontyphoidal *Salmonella,* especially the dogs which feed raw food contain *Salmonella* sp. in urine and faecal matter and they cause acute gastro-enteritis (salmonellosis) to 1.4 billion people of United states annually.[19] In our study, ten bacterial pathogens were identified in order to identify their pathogenicity among humans. The sensitivity pattern of isolated bacteria was studied among the commercial antibiotics, such as Penicillin, Ampicillin, Ciprofloxacin, Erythromycin, Vancomycin, Chloramphenicol and Kanamycin (Table 5). Each is at variance from the sensitivity pattern; among all the strains, *Pasteurella* sp. had shown resistance to all the antibiotics. All the other strains had also shown resistivity patterns among certain antibiotics. Antibiotic sensitivity study clearly indicates that, microbiota of dog's saliva is extremely prone to human infections and its resistance behaviour among antibiotics clearly implies the pathogenicity of the microbes. In order to identify the species, *Pasteurella* were subjected to molecular identification by 16S rRNA gene sequencing and the organism was identified to be *Pasteurella multocida*.

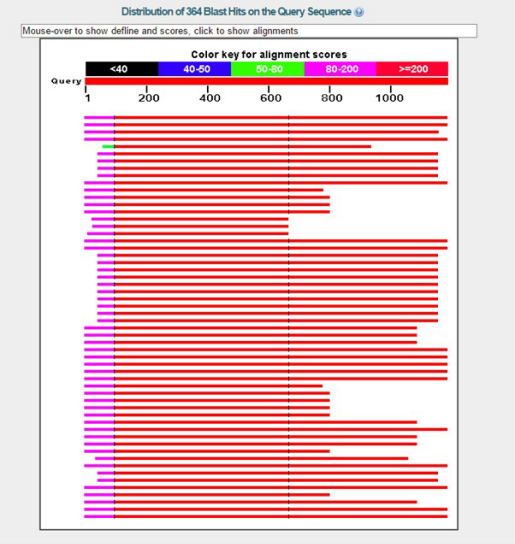
Table 5. Antimicrobial pattern of antibiotics against isolated strains

| S. No. | Drugs | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | P | R | R | R | R | R | R | R | 19 mm | 19 mm | 22 mm |
| 2 | E | R | R | 18 mm | R | R | R | R | 25 mm | R | R |
| 3 | V | R | R | 23 mm | R | R | R | R | 15 mm | 11 mm | R |
| 4 | C | 21 mm | R | 25 mm | 24 mm | 22 mm | R | 25 mm | 33 mm | 21 mm | 21 mm |
| 5 | A | R | R | R | R | 11 mm | R | 19 mm | 20 mm | 20 mm | 14 mm |
| 6 | K | 13 mm | R | 41 mm | 19 mm | 12 mm | R | 20 mm | 28 mm | 7 mm | 18 mm |
| 7 | Ch | R | R | 12 mm | 23 mm | 21 mm | 30 mm | 19 mm | 27 mm | 19 mm | 1 mm |

P - Penicillin; A - Ampicillin; E - Erythromycin; V - Vancomycin; Ch - Chloramphenicol; K - Kanamycin; C - Ciproflaxin; R - Resistance to antibiotics

**Sequence result:**

GATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAACGGTAGCAGGAAG
AAAGCTTGCTTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAAGGAAAGGGTGG
GACCTCTTGGCCACCTGCCATAAGATGAGCCCAAGTGGGATTAGGTAGTTGGTG
GGGTAAAGGCCTACCAAGCCTGCGATCTCTAGCTGGTCTGAGAGGATGACCAGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCGCAATGGGGGGAACCCTGACGCAGCCATGCCGCGTGAATGAAGAAGGCC
TTCGGGTTGTAAAGTTCTTTCGGTAATGAGGAAGGGATGTTATTTAATAGATGGC
ATCATTGACGTTAATTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCGAGCGTTAATCGGAATAACTGGGCGTAAAGGGCACGC
AGGCGGACTTTTAAGTGAGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATT
TCAGACTGGGAGTCTAGAGTACTTTAGGGAGGGGTAGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAATACCGAAGGCGAAGGCAGCCCCTTGGGA
ATGTACTGACGCTCAAGAACCTTACCTACTCTTGACATCCAGAGAAGCTTGCAGA
GATGCGAGTGTGCCTTCGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTCTTTG
TTGCCAGCGATTCGGTCGGGGAACTCAAAGGAGACTGCCAGTGACAAACTGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACG
TGCTACAATGGTGCATACAGAGGGCAGCGAGAGTGCGAGCTTGAGCGAATCTCA
GAAAGTGCATCTAAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
ATCCCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCATGGGAGTGGGTTGTTCCCGAAATAGTAGGCTTAACC
TTCGGGAGGGCGTTTACCACGGTATGATTCATGACTGGGGTG



There were nearly 360 retrieved similar sequences identified from the BLAST method. Mostly, species identified as highly similar sequence with our unknown sequence was given most priority in determining the species lineage. Highly similar sequence was validated based on the identity value, score value and E value.[20,21] Marginally, the gaps also influenced on validating the highly similar sequences. Based on the selection criteria, high number of matches to the unknown sequence tend to match with the species called *Pasteurella multocida.* The lineage tend to be identified as bacteria kingdom under the phylum proteobacteria with the class Gamma proteobacteria classified under the order Pasteurellales in Pasteurellaceae family.

They were Gram negative coccobacillus bacteria, which are mostly reported in dogs as highly responsible for infectious syndromes, such as bacteremia, meningitis, brain abscess, spontaneous bacterial peritonitis and intra-abdominal abscess. Dog infected with *Pasteurella multocida* is also reported to act as an opportunistic pathogen for other disorders in humans as well.[22]

## Conclusion:

Principal knowledge that is driven out of this present study has taught us that, more street dogs are still prone to the infectious pathogens that exist in their saliva. Extensive study performed at the molecular level is much more helpful to the biologist in determining its proper lineage. Hence, this study concludes that, among Indian population the prevalence of these pathogenic organisms could be controlled if proper measures are taken on the street dogs by the local governmental body to prevent the occurrence of the dangerous infectious syndromes among the people.

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