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Research Article

ISOLATION AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCUS SCIURI FROM ROOT CANAL INFECTION

Sri Ramkumar S.R[1]., Mano Christaine Angelo J[2] and Sivakumar Natesan*[3]

[1]Department of Microbiology, Bharathiyar University, Coimbatore – 641 046, Tamil Nadu, India
[2]Department of conservative Dentistry, Sri mookambika institute of Dental Science, Kulasekharam - 629 161, Tamil Nadu, India
[3]Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai - 625 021, Tamil Nadu, India

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ABSTRACT

Aim: The aim of this study was the molecular detection of multi-drug resistant dental pathogens in association with the infection of root canal system.
Methods and Material: Study was conducted during the period from March 2013 to September 2013. Totally 94 samples were collected aseptically and the yielded isolates were biochemically characterised and screened through antibiogram study. Standard disc diffusion method was used for antibiotic sensitivity study of Staphylococcal isolates. 16S rDNA sequence was analyzed for the confirmation of multidrug-resistant isolates.
Results: The isolates *Enterococcus* sp., *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* sp. and *Candida* sp. were found and reported more in females (n=55) than males (n=39). Among the 19 Staphylococcus isolates 41.7% were *S.sciuri*, which show a higher percentage of antibiotic resistant (21.05%). In root canal system, this is the first report of isolation of multidrug-resistant *S.sciuri*. 16S rDNA sequencing results confirmed the multidrug resistant Staphylococcus strains and were 99% similar to *S.sciuri.*
Conclusions: Study showed the prevalence of *S.sciuri* in root canal system. The level of antibiotic susceptibility of the *S.sciuri* strains clearly indicated the emergence of multidrug-resistant pathogens. This information alarms the clinicians to concentrate on MDR pathogen while treating root canal infection.

INTRODUCTION

Root canal refers to the central space containing soft tissue of the tooth. Root canal infection is being seriously considered to be a major oral health problem in all age group of human beings. The anatomical structure of tooth especially root canal with necrotic pulp tissue is a complex ecologic nich, which seems to be a suitable habitat for the microbes to be grown inside (Figdor *et al.* 2003). This endogenous infection is opportunistic, develops due to the growth of the oral microorganism (Haffajee *et al.* 2004). However, penetration of microbes into root canal is also depending on the status of host resistance mechanism. Moreover, studies proved that microbes such as *Enterococus faecalis, E. faecium, Fusobacterium* sp., *Propianobacterium* sp., *Streptococcus mutans, Streptococcus* sps., *Staphylococcus* sp. and *Candida albicans* are responsible for causing this infection (Roca's *et al.* 2004).

In endodontic therapy, the infected pulp of a tooth is removed and protected from future microbial invasion (Kamberi *et al.* 2011). Failure may happen due to some procedural errors (Siqueria *et al.* 2008), which may enhance the growth of *Staphylococcus* sps. and *Enterococcus* sp. However infection with *Staphylococcus* sps. is common in root canal (Soares de Lima Guimarães *et al.* 2012). In the present context, the new finding is the presence of *S.sciuri* in the root canal. The molecular method is being employed specifically for identification of organism since two decades. Hence, the present study was to investigate the prevalence of endodontic infections in patients of Kanyakumari District, and evaluated the antibiotic susceptibility of the isolated *S.sciuri* strains.

MATERIALS AND METHODS

Sample collection

Around 94 samples of infected root canal were collected from the patients with different age groups attending dental OPD

*Corresponding author: **Sivakumar Natesan***
Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai - 625 021, Tamil Nadu, India

(Orthodontics and Pediatric Dentistry) at local Dental Hospitals, Kanyakumari District, Tamil Nadu for the period of six months [from March 2013 to September 2013] after getting the ethical clearance from Kanyakumari Medical College Ethical Committee (Ref No:13118/ME2/2013). Prior to taking the sample, the infected tooth was examined by radiographic imaging. After the crown's surgical access, root canal was profusely irrigated with 0.9% saline (Alliance, 533-JF7633P), following a sterile paper point with size compatible with root canal's anatomic diameter (AD) was inserted for 30 seconds to collect the lesion material from root canal(6) and then samples were aseptically drawn. Thereafter it was transferred to the laboratory by Amies transport media (Himedia, MS684) within 2 hours. Individual patient's record was also maintained for each collection.

Bacterial isolation and characterization

In this, the following culture media were used: Nutrient agar (Himedia, M001), Brain Heart Infusion (BHI) agar (Himedia, M211), MacConkey agar (Himedia, M081B) and Bile esculin azide agar (Himedia, M493). After inoculating the sample on the Nutrient agar and BHI agar, plates were incubated at 37^0C for 48 hours. The morphologically distinct bacterial colonies were examined by stereoscope microscope and then subcultured on BHI medium for further characterization. Gram's reaction, motility test, catalase test oxidase test, indole test, Methyl red test VP test, citrate utilization test and coagulase test were done for genus characterization.

Antibiogram study of the isolates

In disc diffusion method, Mueller hinton agar was used to determine the antibiotic sensitivity pattern of the isolates. Bacterial inoculums were prepared in 5 ml BHI broth with a single pure colony of each strain. The inoculum was incubated at 37°C for 16 hours to get sample approximately close to 0.5% Mc Ferland standard (1.5×10^8 cfu/ml equal to that of 0.5 MC Ferland) for susceptibility testing (NCCLS 1993). Antibiotic disc such as Vancomycin (30µg), Gentamycin (50µg) Tetracycline (30µg) and Cephalaxin (30µg), Co-trimoxazole (10µg) and Erythromycin (15µg) were used. Test inoculum was swabbed over MHA and antibiotic discs were placed on that medium thereby plates were incubated at 37^0C for 24 hours. Zone of inhibition were observed.

Molecular Identification of the isolates

Multi-drug resistant bacterial isolates were further characterized by 16S rDNA sequencing. Bacterial culture was multiplied in Luria Bertani (LB; Himedia, M1151) broth and genomic DNA was extracted by manual phenol - chloroform method (Sambrook *et al*. 1989), and the quality was checked by nanodrop ND-1000 UV-vis spectrophotometer. 10 ng of genomic DNA was amplified with universal 16S rRNA genes primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R primer (5′-TACCTTGTTACGACTT) and the amplified fragments were visualized on a 1% agarose gel stained with ethydium bromide under UV light to confirm the presence of a ~1,450 bp band. 16S rDNA sequencing was performed using an ABI PRISM BigDye reaction kit (Applied Biosystems 3500; genetic analyser) as recommended by the manufacturer. Sequences were aligned by using CLUSTAL W (Thompson *et al*. 1994), using Phylogeny Reconstruction Analysis with Maximum Likelihood Method phylogentic tree was constructed. The stability of relationships was assessed by 1000 Replications Bootstrap method contained within the Mega 5.1 program. The aligned sequence were deposited in gene bank and got the accession numbers.

RESULTS

Table 1 shows the analyses of patients with three different stages of root canal infection like acute pulpitis, chronic pulpits and chronic with periapical inflammation. A total of 94 samples at four different age groups were characterized. 41.49% of male samples (n=39) and 58.51% female samples (n=55) were analysed. Amongst the 94 samples, 70.21% of samples (n=66) were reported from the age group 21- 40. Maximum samples were reported under the acute stage of infection in both male (18.09%) and female (43.62%) cases. None of the samples reported in female above 60 and male below 20 age groups.

Table 1 Percentage distribution infection of root canal samples (n=94)

Age group	Male % (n=39)			Female % (n=55)		
	Acute pulpitis	Chronic pulpitis	Chronic with Periapical	Acute pulpitis	Chronic pulpitis	Chronic with Periapical
<20	0	0	0	3.19	0	0
21-40	18.09	3.19	1.06	43.62	4.25	0
41-60	5.32	6.38	3.19	6.38	0	1.06
60<	1.06	1.06	2.13	0	0	0
Total	24.47	10.64	6.38	53.19	4.25	1.06

Table 2 clearly illustrated the percentage of possible bacterial and fungal growth on respective isolation and selective media. Among them, 84.62% of male (n=39) and 89.09% of female samples (n=55) samples were reported to be microbial positive. The age group 41-60 was the dominant group for bacterial positive in both male (92.86%) and female (100%) samples.

Among the total 94 samples, genus like *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus, Candida* etc. were identified. *Enterococcus* was the dominant bacterial genera reported in both male and female in all age groups (Table-3 &4; Figure -1), but other unidentified bacterial genera also reported significantly. Percentage of Staphylococcus was high in female (21.2%) samples than male (19.95%) samples (Figure-1). However, species of Staphylococcus and its variations have been reported in Figure -2, unidentified species of Staphylococcus (41.7%) and *S.sciuri* (26.32%) were the dominating groups.

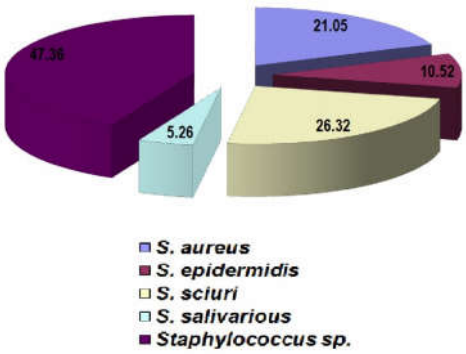

Figure1 Species variations of Staphylococcus isolated from Root canal samples (n=19).

Table 2 Percentage positive growth of bacteria & candida on different media

Age group	Male % (n=39)					Female % (n=55)				
	Samples	NA	BHI	BEAA	HCA	Samples	NA	BHI	BEAA	HCA
<20	0	0	0	0	0	3	100	66.67	33.33	66.67
21-40	21	80.95	52.38	38.09	28.57	45	86.67	71.11	48.89	40.00
41-60	14	92.86	78.51	42.85	92.86	7	100	57.14	28.57	71.42
60<	4	75.0	75.0	25.0	50.0	0	0	0	0	0
Total	39	84.62	64.10	38.46	53.85	55	89.09	69.09	45.45	45.45

NA = nutrient agar; BHI = Brine heart infusion agar; BEAA = Bile Esculin Azide Agar; HCA- Hi-Chrome Candida Agar

Table 3 Bacterial abundance in root canal samples age group wise (male; n=39)

Age group	*Enterococcus* sp.	*Staphylococcus* sp.	*Streptococcus* sp.	*Bacillus* sp.	Others	*Candida* sp.
<20	0	0	0	0	0	0
21-40	25.64	10.25	7.69	7.69	25.64	15.38
41-60	25.64	7.69	5.13	5.13	23.07	30.76
60<	7.69	0	2.56	2.56	5.13	7.69

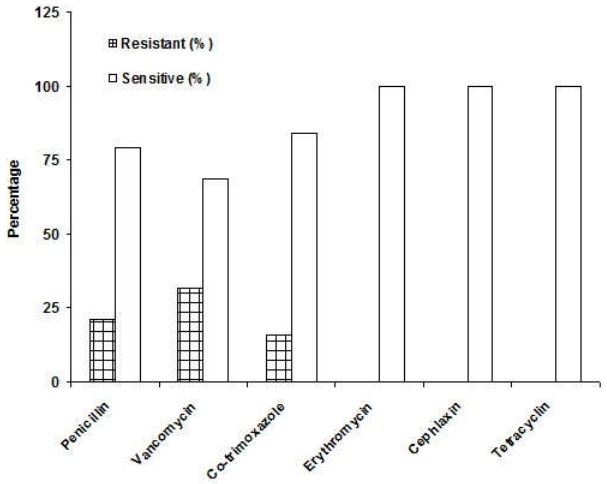

Figure- 2 Antibiotic resistant profile of Staphylococcus isolated from Root canal samples (n=19)

Antibiogram of the 19 Staphylococcus isolates was summarized in Figure-3. The isolates were highly resistant to Vancomycin (31.58%) and Penicillin (21.05%), but none of the strains showed resistant against Erythromycin, Cephlaxin, and Tetracyclin (Figure - 3). Based on the results, three bacterial strains showed resistance against both Vancomycin and Penicillin, and two strains showed Penicillin and Co-trimoxazole. Amongst four *S.sciuri* isolates, two isolates had single drug resistant and two isolates were more than two drugs resistant (multi drug resistant).

Table 4 Bacterial abundance in root canal samples age group wise (female; n=55)

Age group	Enterococcus sp.	Staphylococcus sp.	Streptococcus sp.	Bacillus sp.	Others	Candida sp
<20	3.64	0	0	1.8	3.64	3.64
21-40	52.73	18.18	9.09	12.73	18.1	36.36
41-60	5.45	3.64	3.64	1.8	5.45	9.09
60<	0	0	0	0	0	0

Analysis of the 16S rRNA gene sequences of the MDR *S.sciuri* isolates were given in Figure-4. Sequence data analysis for the 16S rRNA gene of MDR *S.sciuri* SS11 and *S.sciuri* SS18 were the length of 1468bp and 1430bp respectively. Multiple alignments of 16S rRNA gene sequences of MDR *S.sciuri* were obtained using ClustalW programme. Sequence similarity of 16S rRNA gene among 2 different isolates ranged from 98% of query cover with a mean identity of 99%. The pairwise comparisons among different reference Staphylococcal species showed a wide variability. The relationships among the MDR isolates were confirmed by phylogenetic analysis based on the 16S rRNA gene sequencing, and the topology of the tree was evaluated by 1000 bootstrap values. The 16S rRNA gene sequences were deposited in Genebank and its accession numbers are KX364716 (for isolate SS 11) and KX364717 (for isolate SS 18).

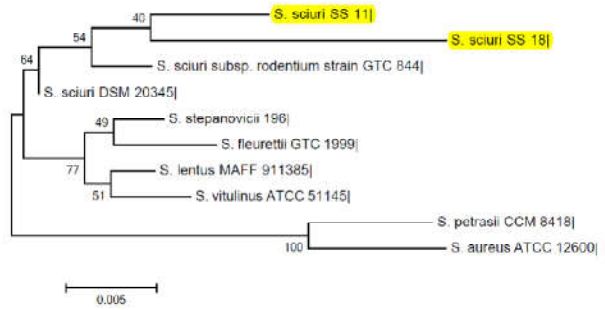

Figure-4 Neighbor-joining tree showing the phylogenetic relationships among *Staphylococci sciuri* based on a comparison of 16 S r RNA gene sequences. Bootstrap values based on 1,000 replications are given at the branching points when they are above 50%.

Table-5 Resistant profile of *Staphylococcus* spp.

Antibiotics	Number of isolates
Penicillin & Vancomycin	3
Penicillin & Co-trimoxazole	2
Co-trimoazole & Vancomycin	2
Penicillin & Vancomycin & Co-trimoxazole	2

DISCUSSION

In this study, Gram-positive cocci were isolated and identified. Especially the *Staphylococcus* and its antibiotic susceptibility have been evaluated. Among the total 94 samples, 58.51% of the samples were from female patients. This report is slightly matched with the previous studies (Lupi-pegurier *et al*. 2002). Interestingly, the endodontic cases with the stage of acute pulpitis were maximum in the age group of 21 to 40 age 20, since this group is concentrating on oral health. Cases treated with root canal are reportedly more common in the elder population (Mukhaimer *et al*. 2012).

In endodontic infection, both anaerobic and facultative anaerobic microorganisms are living in the form of monomicrobial and polymicrobial communities and the same is reported in our study also. As the samples from the age group 40-60 have yielded 100% microbial growth, it seems to be more significant. More than 85% of reported samples were showing culture positive in different culture media. Similar study confirmed the frequency of apical periodontitis (AP) increase with age (Jimenez-Pinzon *et al*. 2004).

In this study, genus includes *Enterococcus, Staphylococcus, Streptococcus, Bacillus,* and *Candida* etc. were reported. As *Enterococcus* and *Streptococcus* are resistant to antiseptic agents, they can easily penetrate into the root canal and flourish well in incomplete treatment (Endo *et al*. 2013; El-Din Mohamed Saber and El-Hady 2012). *Enterococcus* sps. (60.64%) was the dominant bacteria identified in all age group of samples in both male and female. This may be due to the fact that, Enterococci are able to survive for a long time in this microenvironment with a minimum amount of nutrients (Figdor *et al*. 2003). Although a number of previous studies reported that the *E.faecalis* is the dominant group in affecting the root canal (Cogulu *et al*. 2007). The isolates of staphylococci (20.21%), including the species like *S.sciuri* (26.32%), *S.aureus* (21.05%), *S.epidermidis* (10.52%), and *S.salivarius* (5.26%) have been also reported from the patients. Similar results of *S.aureus* associated endodontic infections have been observed in previous studies (You *et al*. 1999; Kloos *et al*. 1997). According to *S.sciuri* no data has been reported so far in association with endodontic infections, interestingly, 26.32 % of the *Staphylococcus* isolates were *S.sciuri* (Fignure-3). *S.sciuri* is one of the facultative coagulase negative human pathogen causing several infections for human being (Couto *et al*. 2000) surviving in mouth and nasal of dogs (Stepanović *et al*. 2001), cats also cause caries in human (Anitha Rani *et al*. 2016). Inhalation of contaminated dust and shared medical devices containing *S.sciuri* could be the route for dispersal of this bacterium (Dakic *et al*. 2005). However, *S.sciuri* does not require an organic source of nitrogen and is capable of a free-living existence (Kloos *et al*., 1980). Our finding suggests that the *S.sciuri* could have been penetrated into root canal through blood stream under the conditions of bacteremia resulted from urinary tract infection. This finding also enunciates that the *S.sciuri* might be reported from the root canal cases with urinary tract infections. However, the way in which the penetration of organism to root canal remains unclear. Similar, studies for *S.sciuri* associated urinary tract infection have been reported by the researcher (Stepanovic *et al*. 2003).

Previous studies reported that antibiotic resistance bacteria are found in endodontic infections (Baumgartner and Xia 2003; Baumgartner 2001). The coagulase negative *Staphylococci* with a high frequency of antimicrobial resistance also accounted by Petersen (2008). In this present study, the antimicrobial susceptibility test was performed by using known concentration of usual antibiotics and identified the resistant of *Staphylococcus* species to penicillin (21.05%), vancomycin (31.58%) and co-trimoxazole (15.79%). The related study of the high incidence of tetracycline and penicillin (66%) resistant *Staphylococcus* species were reported from different animals (Al-Thani and Al-Ali 2012). Findings of this study revealed that two of the isolates (50%) namely *S.sciuri* were resistant to against more than two antibiotics, which are classified under MDR *S.sciuri* (Tsakris *et al*. 2002). Similar findings with the 70% of the drug-resistant *S.sciuri* strains isolated from hospital environment were reported in previous studies (Dakic *et al*. 2005). This species is potentially resistant due to genes encoding resistance to antibiotics (Wu *et al*. 2001).

CONCLUSION

Although various isolates have been reported in association with root canal infection, the results highlighted that the *S.sciuri* (50%) were represented with multidrug resistant. The report of *S.sciuri* associated root canal infection is nascent finding, as it is reported for the first time. Besides, the known pathological potential of *S.sciuri* isolates and the increasing multi-drug-resistance strains attract the need for microbial diagnoses of root canal infections. Further investigation should be needed for the molecular importance of drug-resistant isolates and the prevention of the spread of the pathogen in root canal system.

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