WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 10, Issue 4, 1972-1986. Research Article ISSN 2277– 7105

TO EVALUATE THE Anti-MICROBIAL EFFICIENCY OF CHLOROHEXIDINE, NISIN, CHITOSAN, AND IgY ANTIBODIES AGAINST *Enterococcus faecalis*, AND *Candida albicans*

Dr. Mahenthiran R.[1]*, Sri Alamelu K.V.[2] and Sornambiga Ravi[3]

[1]Assistant Professor, Department of Microbiology, Dr.N.G.P. Arts and Science College, Tamilnadu, India.

[2,3]PG Students, Post Graduate Research Department of Microbiology, Dr.N.G.P. Arts and Science College, Tamilnadu, India.

Article Received on
20 Feb. 2021,

Revised on 12 March 2021,
Accepted on 01 April 2021

DOI: 10.20959/wjpr20214-20237

***Corresponding Author**
Dr. Mahenthiran R.
Assistant Professor,
Department of Microbiology, Dr.N.G.P. Arts and Science College, Tamilnadu, India.

ABSTRACT

Intracanal medicament are used to treat root canal pathogens in endodontic treatment. Most common root canal pathogens are Enterococcus faecalis and Candida albicans Apart from this Streptococcus mitis, Streptococcus gordonii, Streptococcus anginosus, Streptococcus oralis, Lactobacillus paracasei, Lactobacillus acidophilus, Campylobacter rectus, Propionibacterium spp are few pathogens that causes infection in root canal. Enterococcus faecalis is a cocci Gram-positive facultative anaerobe, and it is the most prevalent species isolated from the root canals of previously root-filled teeth with chronic apical periodontitis, affected up to 77% of cases. This microorganism can survive extreme conditions, including exposure to different chemicals used in endodontic therapy by the starvation state. The occurrence of Candida albicans (C. albicans) reported in infected root canals with persistent cases of apical periodontitis varies between 7% and 18%, but yeasts can also be isolated in primary apical periodontitis. C. albicans is typically found with Gram- positive bacteria, such as streptococci, although it can be isolated in pure culture, which is an indication of its pathogenicity. A variety of virulence factors enable C. albicans to adhere to and penetrate into human dentine. Moreover, C. albicans can tolerate adverse environmental conditions, including high alkalinity.

KEYWORDS: *Enterococcus faecalis*, *Candida albicans*, ELISA, IgY.

1. INTRODUCTION

The main goal of endodontic treatment is the effective control of the root canal infection and the prevention of the entry of new microorganisms during and after treatment. However, root canal morphology is complex and contains numerous ramifications and anatomical irregularities that limit the chemo-mechanical cleaning and shaping procedures resulting in a unfavourable outcome. To maximize the disinfection of the root canal system in infected cases, especially in the retreatment of endodontically-treated teeth, the use of intracanal medicaments can help in reducing the remaining micro-organisms, and can provide an environment conducive to periapical tissue repair.

Calcium hydroxide (Ca [OH]2), as an intracanal medicament, plays an important role in dentinal disinfection through its moderate bactericidal activity, its cyclooxygenase unspecific inhibitor capacity, and its ability to induce hard tissue formation. Despite its wide use in endodontics, both *E. faecalis* and *C. albicans* are very resistant; furthermore, the dentin matrix has a buffer effect that might decrease its effectiveness in dentinal disinfection. If CA(OH)2 is mixed with another medicament, its effects might improve significantly, or not at all, depending on the substance added.

Chlorhexidine (CHX) is a synthetic cationic bisguanide used in endodontics as an irrigate and intracanal dressing. It has an immediate antimicrobial effect, a wide spectrum of action, substantivity, and relatively less toxicity, but it cannot dissolve organic debris and can stain the dentine when it comes into contact with sodium hypochlorite, which might interfere with the sealing of the root filling.

Chitin and chitosan have been investigated as an antimicrobial material against a wide range of target organisms like algae, bacteria, yeasts and fungi in experiments involving in vivo and in vitro interactions with chitosan in different forms (solutions, films and composites). Early research describing the antimicrobial potential of chitin, chitosan, and their derivatives dated from the 1980-1990s. Generally, in these studies the chitosan is considered to be a bactericidal (kills the live bacteria or some fraction therein) or bacteriostatic (hinders the growth of bacteria but does not imply whether or not bacteria are killed), often with no distinction between activities. Recent data in literature has the tendency to characterize chitosan as bacteriostatic rather than bactericidal, although the exact mechanism is not fully understood and several other factors may contribute to the antibacterial action.

Nisin is a naturally occurring antimicrobial peptide, produced by *Streptococcus lactis* sub species lactis. It has antimicrobial activity against a wide range of Gram-positive bacteria and their spores even against drug resistant *E. faecalis* isolates. Its use in dentistry has so far been limited. Therefore, many recent studies have focused on an alternative intracanal disinfection protocol which is able to eradicate such resistant microorganisms more rapidly. The use of IgY as a novel mode of immunotherapy using oral chicken immunoglobulin (IgY) to confer passive immunity has gained much interest as an inexpensive non-antibiotic alternative for the prophylaxis and treatment of a wide variety of infectious diseases. The stability of IgY in the oro-gastrointestinal tract and its safety profile has been well-documented. IgY has been used in the treatment or prevention of dental caries, periodontitis and gingivitis, gastritis and gastric ulcer, oral thrush and infant rotavirus diarrhea. A small amount of antigen in the milligram or microgram range usually elicits enough IgY response and the antibody titters persist over several weeks to several months.

2. OBJECTIVE

- Isolation of *Enterococcus faecalis, Candida albicans* and *Streptococcus salivarius* from clinical root canal and plaque specimen.
- Generation and characterization of chicken egg yolk antibodies against *Enterococcus faecalis* and *Candida albicans* to test its anti-microbial efficacy against biofilm formation.
- *In vitro* testing of anti-microbial effect of different medicaments used in root canal treatment.
- *Ex-situ* testing of anti-microbial effect of test composites and IgY against test pathogens in experimental tooth model.

3. MATERIALS AND METHODS

3.1 Clinical sample collection method and processing

Ten plaque samples were collected from buccal, mesial, distal, surface of either individual permanent first molar or pooled permanent molars using a sterile tooth pick and transferred to sterile saline**.** Plaque samples were sonicated for 20 seconds and inoculatedonto 2ml of sterile Brain Heart Infusion Broth (BHIB) and Sabouraud Dextrose Broth (SDB) tubes respectively for each plaque samples, followed by respective aerobic and microaerophilic incubation at 37ºC for 48 hours.

Plate 1a: Plaque Sample collection tubes

Figure 1: Separation of antigen.

3.2 Identification of microorganisms

The overnight grown inoculated SDB and BHIB tubes were further streaked onto Mitis Salivarius Agar (MSA) and Sabouraud Dextrose Agar (SDA) for the isolation of root canal pathogens (*Enterococcus faecalis & Candida* albicans). The inoculated MSA plates were incubated at 37ºC for 48 hours in microaerophilic condition and SDA plates were incubated at 37ºC for 24 hours.

3.2.1 Culture characterisation

The colony morphology of the bacterial strain was studied by growing them on MSA, Blood agar and MacConkey agar for *Enterococcus faecalis*, on BHI agar plate for *Streptococcus salivarius* and on SDA and Biggy Agar plates for *Candida albicans* and results was noted for the identification of the organism.

3.2.2 Microscopic studies

Gram stain helps to identify whether isolates are gram positive or gram-negative organism and also about the shape of respective organisms.

3.3 Generation of IgY against *Enterococcus faecalis* and *Candida albicans* whole cell antigen

3.3.1 Antigen Preparation

Enterococcus faecalis used as antigen was cultured for 24 hours in brain-heart infusion broth at 37ºC under microaerophilic condition. Similarly, *Candida albicans* was inoculated into the SD broth and incubated at 37 C for 24 hours. The culture was treated with 0.5% formalin for 24 hours and then the bacterial cells collected by centrifugation (6,000rpm,5min). The pellets were washed thrice with sterile saline containing 0.5% formalin and suspended in sterile

saline using a vortex mixer. This formalin killed whole cell antigen samples (Plate 1b) were in aliquots of 1ml in sterile cryovials and stored at 4˚C until required, after the concentration adjustment to 2× 109 CFU/ml for *Enterococcus faecalis* and 1× 105 CFU/ml for *Candida albicans.*

Plate 3: Characterization of Enterococcus faecalis

Plate 3a: E. faecalis on MS agar

Plate 3b: E.faecalis on BHI agar

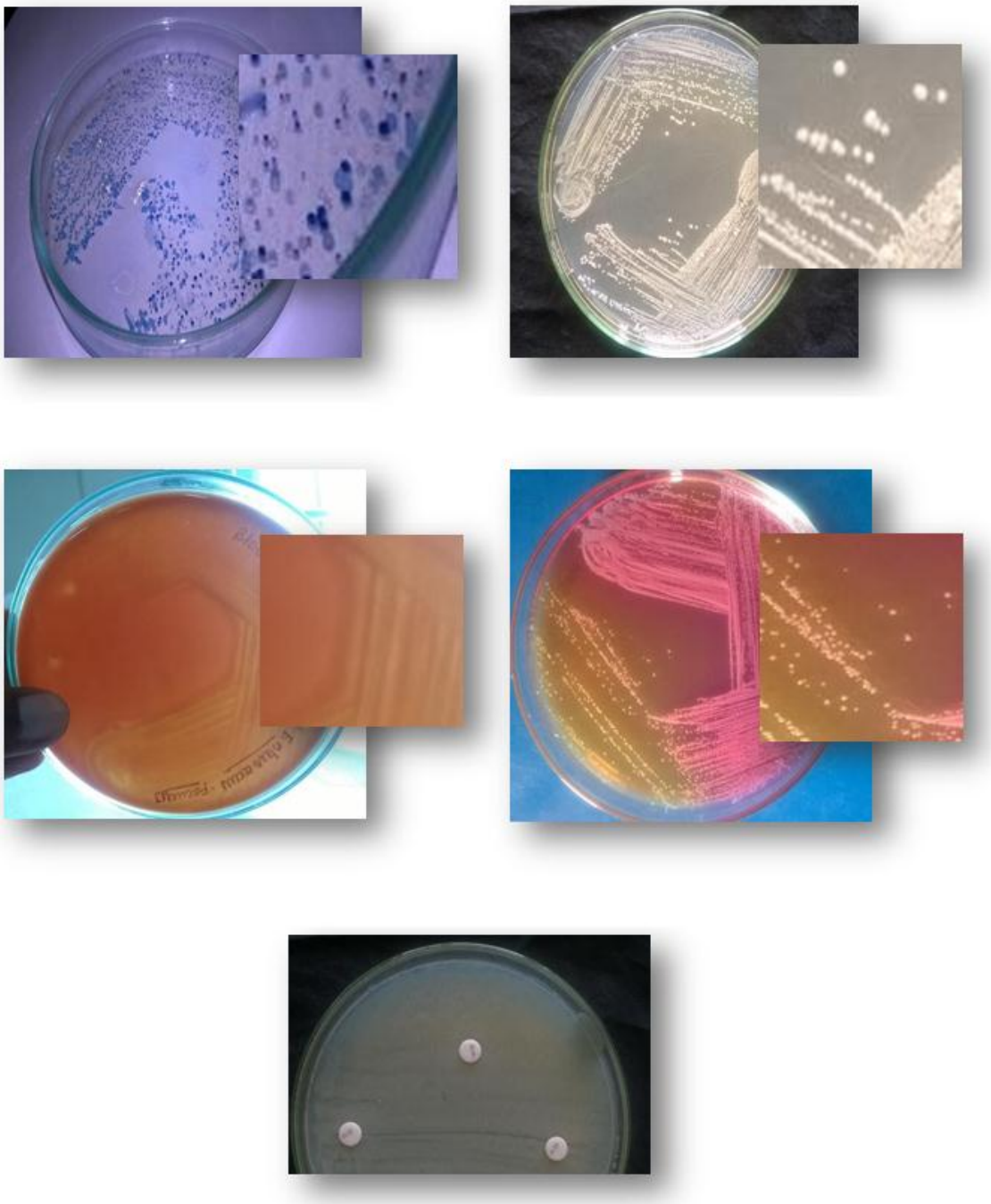
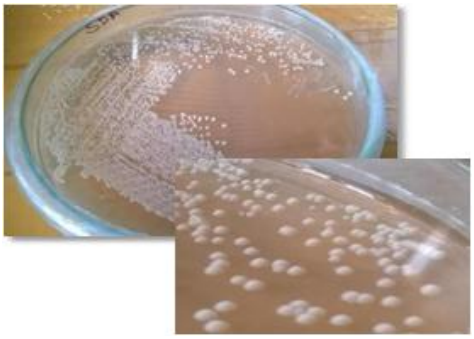
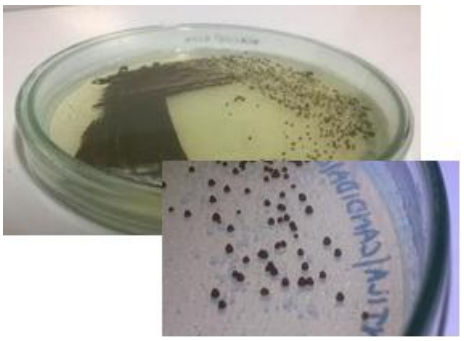


Figure 2: Characterization.

Plate 4a: Characterization of Candida albicans



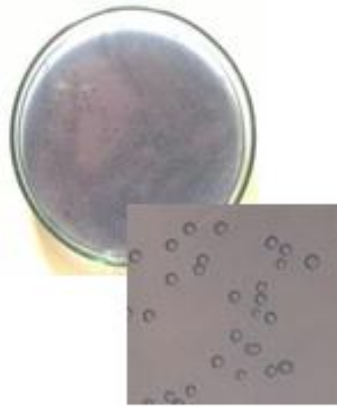
On SDA agar



On Biggy Agar

Plate 4b: Characterization of Streptococcus salivarius.

On BHI Agar



On MS Agar

Figure 3: Characterization of *S. salivarius* & *Candida albicans.*

3.3.1 Antigen sterility and purity

The prepared antigen was inoculated into the selective medium for *Enterococcus faecalis* on BHI ager plates and *Candida albicans* on SDA agar plates. The inoculated plates were incubated at 37ºC for 24 hours and observed for the absence of the growth which confirmed the sterility of formalin treated antigen. Performing the gram staining after formalin killing and presence of pure gram-positive cocci and budding yeast like morphology confirmed the purity of the antigen preparation.

3.3.2 Experimental Animal

Twenty-one weeks old egg laying white leghorn chicken were brought Chandran Poultry farm, Palladam with good health conditions. The white leghorn hens were kept in isolated cages at the animal house with good hygienic conditions and maintained with adequate food and water. It was used for the generation of egg yolk polyclonal antibodies (IgY) against

Enterococcus faecalis and *Candida albicans* (whole cell antigen).

3.3.3 Immunization of chickens

The 24 weeks old white leghorn chickens were intra muscularly injected at multiple sites of the breast muscles with prepared antigen. The whole cell *Enterococcus faecalis* and *Candida albicans* antigen was injected (1ml) with Booster doses given at two weeks interval.

Table 1: Immunization schedule Antigen.

Antigen	Dosage	Volume	Date of immunization					
			I Dose	I Booster	II Booster	III Booster	IV Booster	V Booster
Enterococcus faecalis	2× 109 CFU/ml	1ml	31st January 2021	7th February 2021	13th February 2021	20th February 2021	27th February 2021	04th March 2021
Candida albicans	1× 105 CFU/ml	1ml						

3.3.4 Collection and storage of eggs

The immunized eggs were collected, cleaned and labelled with the name of antigen used for immunization. The eggs were stored at 40C because yolk antibodies are stable in the refrigerator for at least 6 months. Pried to purification the eggs were kept at room temperature for 30 minutes.

3.3.5 Purification of egg yolk antibodies (Polson *et al*., 1980)

The egg yolk antibodies were purified according to the method described by (Polson *et al*., 1980.).

3.3.6 Desalting of IgY fraction by dialysis

3.3.6.a Preparation of dialysis tubing

The Dialysis membrane - 60 tubing was cut into pieces of required and convenient length and allowed to boil for 10 minutes in a large volume of 2% (w/v) sodium bicarbonate and 1mM of EDTA (pH 8.0). The tubing was rinsed thoroughly in distilled water. Again, it was allowed to boil in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C.

Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water.

3.3.6.b Dialysis of IgY extract

The IgY extract obtained from eggs yolk was transferred to a dialysis capsule after including some air (Plate 1h). The capsule containing IgY extract was dialysed overnight in 0.1% saline and gently stirred by means of magnetic stirrer. Next morning, the saline is replaced by 0.01M PBS and dialysed for another three hours. 2-4 drops of Nessler's reagent were added to check the completion of dialysis. Thereafter the IgY-extract was transferred to storage vials (Plate 1i).

3.4 Total Protein estimation of IgY extract (Lowry *et al*., 1951)

The total protein content of the immunoglobulin fraction of egg yolk was estimated by Lowry's method using Folin's reagent.

3.5 Protein Profile of IgY extract (Laemmli 1970)

Protein profile of IgY antibodies were analyzed by Sodium Dodecyl Sulphate Poly Acryl Amide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970).

3.6 Agglutination of whole cells of test organisms by IgY

Egg yolk antibodies to *E. faecalis* and *C. albicans* antigens were subjected to rapid slide agglutination for the qualitative assessment of antigen antibody specificity reaction. The cell suspension (20µl) was mixed with an equal volume of IgY extract (48th Day after immunization) and observed for clumping visually within 2-4 mins of reaction.

3.7 Minimum agglutination assay test

- 96-well micro titer plate labelled from 1 to 12 wells for (1:10 to1:2560) IgY dilutions, Neat (undiluted IgY), negative control (non-specific IgY), Blank (PBS), positive control.
- Add 100µl of formalin killed antigen (*Enterococcus faecalis and Candida albicans*)to each respective well.
- Add 90 µl PBS and 10µl antibodies (*Enterococcus faecalis and Candida albicans)* in the Eppendorf tubes and serially diluted into the 1:2560 dilution factors. The tubes were thoroughly mixed and transferred into the micro titer plates.
- The micro titer plates were incubated for 4ºc at 24 hours. The results were observed the next day for clumping. The antibody titer was expressed as the minimum concentration of IgY in the reaction mixture that gave positive agglutination.

3.8 Biofilm Inhibition Assay

The quantified biofilm production was made to 1 ml with sterile distilled water and OD read at 570nm.

3.9 Preparation of Teeth

Freshly extracted human teeth were sectioned and only roots with a type I root canal configuration and a round root canal at the cut surface were chosen. The roots are divided into 5 groups of 5 roots. The roots are rinsed in water for 30 min, then rinsed with EDTA (17% w/v) for 5 min in an ultrasonic bath to remove smear layer and rinsed in water for a further 30 mins. The teeth were stored in sterile water until used. Each root was dried, coated externally with clear nail varnish and autoclaved for further study.

3.10 Experimental Inoculation of Root Canal

Each root canal was inoculated with cultured bacterial solution of *Enterococcus faecalis, Streptococcus salivarius* and *Candida albicans* up to the canal entrance using a sterile syringe/pipette. All samples were incubated in a closed Eppendorf at 37ºC for 14 days. The canals are re inoculated with fresh bacterial samples at every 3 days interval. The inoculum establishment of *Enterococcus faecalis, Streptococcus salivarius* and *Candida albicans* inside the root canal was confirmed by streak plate using tooth picks onto sterile BHI agar and SD Agar plates.

3.10.1 Testing the potential of Root canal Medicaments against the oral microbes

After 14days, the canal contents were rinsed with 5 ml saline and dried. The specimens are then divided into 5 groups under each organism and subjected to intra canal medicament treatment with Calcium Hydroxide (since it is the currently used medicament in dental procedures), chlorohexidine, Nisin, Chitosan and Saline (Negative Control). The canals were then sealed with dental wax and all samples were incubated at 37ºC for 7 days. At the end of incubation for 7 days, the wax seal was then removed from each of the canals. Sterile paper points/sterile toothpicks were inserted into root canals. After adsorption of the canal contents for 1min, the points were dipped into sterile broth medium, incubated overnight under appropriate conditions. After incubation, they were plated onto sterile BHI agar and SD Agar for count of *E. faecalis, C. albicans* and *Streptococcus salivarius* in termsof CFU/ml.

3.11 Testing the potential of IgY to inhibit colonization of oral pathogens in root canal

The IgY was tested for its ability to inhibit the colonization of root canal with the test

inoculum of *Enterococcus faecalis* and *Candida albicans.*

4. RESULT AND DISCUSSION

4.1 Gram staining

The isolated root canal pathogens like *Enterococcus faecalis* was gram positive cocci observed singly, in pairs, or in short chains *& Candida albicans showed* gram positive yeast like budding cells and *Streptococcus salivarius was observed as* gram positive cocci in chains under the oil immersion objective were confirmed.

4.1.1 Cultural Characterization

*Enterococcus faecalis***:** It was confirmed that the isolate was gram positive cocci observed singly, in pairs, or in short chains. It was negative for Catalase production with hydrogen peroxide and incapable of producing Oxidase, incapable of hydrolysing urea to ammonia by producing Urease, capable of fermenting glucose, lactose, sucrose with acid production, and showed high resistance to bacitracin. Their colony morphology in various plates was recorded.

4.1.2 Characterization of *Candida albicans*

It was confirmed that the isolate was gram positive yeast like budding cells.

C. albicans was present as short, slender, tube-like structures (germ tube) when observed under the microscope. It was capable of hydrolysing urea to ammonia by producing Urease. Their colony morphology on non-selective and selective media were recorded.

4.1.3 Characterization of *Streptococcus salivarius*

It was confirmed to be gram positive cocci in chains under the oil immersion objective.

4.2 Purification and characterization of anti-*Enterococcus faecalis* IgY and anti-*Candida albicans* IgY

Twenty-four weeks old white leghorn chicken were immunized with the antigens intramuscularly. Egg yolk antibodies were purified from immunized eggs by PEG extraction method prescribed by Polson *et al.*, (1980). IgY extract was then further purified by dialysis. The purity of chicken egg yolk antibodies and its molecular weight were determined by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 10% gel according to the method of Laemmli (1970). IgY migrates as two protein bands, 27kDa and 68kDa that correspond to light and heavy chains, respectively in a reduced gel.

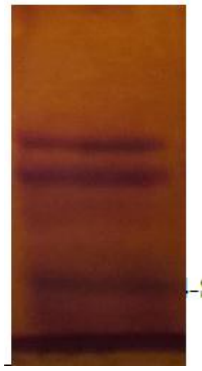


Figure 4.

4.3 Total Protein Estimation of IgY fractions

Protein content of the IgY fraction obtained by Polyethylene Glycol method was estimated by method described by Lowry *et al*., 1951 using Folin-Ciocalteu reagent. The protein concentration in egg yolk was increased during the immunization period and reached maximum of 45.21 mg/ml for anti-*E. faecalis* IgY and 46.2 mg/ml for anti-*C. albicans* IgY at the 49th day after immunization. Estimation of Protein concentration and Total IgY concentration of purified chicken egg yolk Antibody fractions.

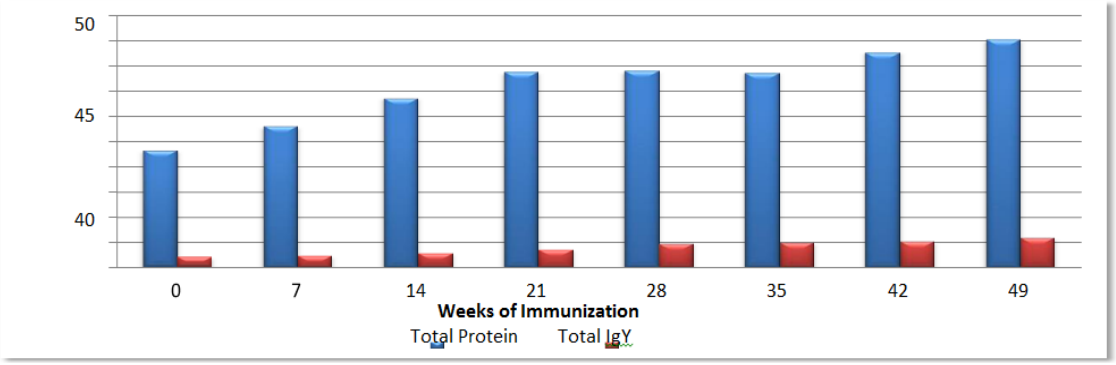


Figure 5: Protein estimation by ELISA.

Table 2: Total protein concentration.

Egg collection (Days)	Total Protein Concentration(mg/ml)	Total IgY(mg/ml)
Pre-immune egg yolk	23.1	1.89
Day 7	28.0	2.30
Day 14	33.6	2.84
Day 21	38.7	3.55
Day 28	39.0	4.73
Day 35	38.64	4.82
Day 42	42.7	5.05
Day 49	45.21	5.84

4.4 Total IgY estimation from egg yolk of immunized chicken

The total IgY content of the IgY purified from the egg yolk was measured spectrophotometrically and the values were recorded. The total IgY concentration of egg yolk was ~1.89 mg/ml of egg yolk during the 7th day of immunization after which there was a steady increase in the concentration to reach a maximum of 5.84 mg/ml for anti-*E. faecalis* IgY and 4.68 mg/ml for anti-*C. albicans* IgY respectively at 49th Day of immunization.

4.5 Determination of IgY titre by RSA Test

The qualitative assessment of the specificity of IgY against whole cell of *E. faecalis* and *C. albicans* was confirmed by positive clumping within 2mins in the slide agglutination assay. The titre of anti-*E. faecalis*-IgY and anti-*C. albicans*-IgY obtained from the eggs of laying hens were also determined by Minimum Agglutination titre in microtiter plate. The binding pattern of IgY was visualized by agglutination. The minimum agglutination concentration of anti-*Enterococcus faecalis* IgY against whole cell antigens was found to be 1:1280 and minimum agglutination concentration of anti-*Candida albicans* IgY against whole cell antigens was found to be 1:640 (Table 4). Similarly, when non-specific IgY (control) were used no agglutination were observed.

4.6 Testing the potential of Root canal Medicaments against the oral microbes

Freshly extracted human teeth were sectioned, divided into 5 groups of 5 roots, coated externally with clear nail varnish and autoclaved. Each root canal was inoculated with cultured bacterial solution of *Enterococcus faecalis, Streptococcus salivarius* and *Candida albicans* up to the canal entrance using a sterile syringe/pipette. All samples were incubated in a closed Eppendorf at 37ºC for 14 days. The inoculum establishment of *Enterococcus faecalis, Streptococcus salivarius* and *Candida albicans* inside the root canal was confirmed by streak plate using tooth picks onto sterile BHI agar and SD Agar plates. And further confirmed using microscopic observation of the grown colonies. After 14days, the canal was subjected to intra canal medicament treatment with Calcium Hydroxide (3:1), Nisin (400mg/ml) and chitosan (400mg/ml), chlorohexidine (2%), Saline (0.85% NaCl).

At the end of incubation for 7 days, sterile paper points/sterile toothpicks were inserted into root canals. After adsorption of the canal contents for 1min, the points were dipped into sterile broth medium, incubated overnight under appropriate conditions. After incubation, they were plated onto sterile BHI agar and SD Agar for count of *E. faecalis*, *C. albicans* and *Streptococcus salivarius* in terms of CFU/ml. In summary, Nisin, Chitosan and Calcium

hydroxide had significant effect on *Streptococcus salivarius* and not the other test pathogens *Candida albicans* and *Enterococcus faecalis*. Chlorohexidine had moderate effect on *Enterococcus faecalis* and significant effect on *Streptococcus salivarius*, whereas it was not effective against *Candida albicans.*

4.7 Testing the potential of IgY to inhibit Biofilm formation

The biofilm inhibition assay using IgY against the test pathogens was carried out as described. To quantify biofilm production the wells were washed with 200µl of 33% acetic acid and their OD read at 570nm. The undiluted IgY purified from Day 49 egg yolk of hyperimmunized chicken showed effective inhibition of biofilm formation in the case of both *Enterococcus faecalis* and *Candida albicans.* The IgY antibodies of Day 49 egg (1:1000) were able to significantly inhibit the progressive multiplication of the *Enterococcus faecalis* and *Candida albicans.* Slight inhibition was observed at 1:100000 dilution of IgY.

4.8 Studying the ability of IgY to inhibit colonization of oral pathogens in root canal

Since the medicaments did not show effect inhibition of colonization of test pathogens in the root canal, the IgY was tested preliminarily for its ability to inhibit the colonization of root canal with the test inoculum of *Enterococcus faecalis* and *Candida albicans.*

5. CONCLUSION

The study shows that IgY was incubated with the test pathogens individually and in combination with chlorohexidine and then loaded into the root canal of individual test teeth. The teeth were then sealed with dental wax and incubated for 7 days under appropriate conditions. At the end of incubation for 7 days, the bacterial CFU for the determination of inhibition of colonization showed, IgY recorded 57.3% reduction of *Enterococcus faecalis* and 55.6% reduction of *Candida albicans.* Therefore, with further testing specific IgY may be considered as a antimicrobial component or possibly an adjunctive tool to complement the disinfection of the root canal system effectively.

6. ACKNOWLEDGMENT

The authors are thankful and gratefully acknowledge to our college funding department DBT-Star scheme, DST-FIST scheme, and to the management of Dr. N.G.P. Arts and Science college, Coimbatore, our college Principal, Deans of Dr. N.G.P. Arts and Science college, Coimbatore as well as all faculty members and our guide, Department of Microbiology, Dr. NGP. Arts and science college, Coimbatore for providing constant support

for this entire work.

7. REFERENCES

1. Abdulkader A, Duguid R, Saunders EM. The antimicrobial activity of endodontic sealers to anaerobic bacteria. International Endodontic Journal, 1996; 29: 280.
2. Akita, E.M and Nakai S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. – J Immunol Methods, 1993; 160(2): 207-214.
3. Ando N, Hoshino E, Arendorf TM, Walker DM. The prevalence and intraoral distribution of Candida albicans in man. Archives of Oral Biology, 1980; 25: 1–10.
4. Arendorf TM, Walker DM. Oral candidal populations in health and disease. Br Dent J., 1979; 147: 267-272.
5. Arendorf TM, Walker DM. The prevalence andintra-oral distribution of Candida albicans in man. Arch Oral Biol., 1980; 25: 1-10.
6. Atila-Pektas B, Yurdakul P, Gu¨lmez D, G€orduysus O. Antimicrobial effects of root canal medicaments against Enterococcus faecalis and Streptococcus mutans. Int Endod J., 2012; 46: 413-8.
7. Baltoh JL Endo Distel JW, Hatton JF, Gillespie MJ. Biofilm Formation in Medicated Root Canals, 2002; 28(10): 689–93.
8. Balvedi RP, Versiani MA, Manna FF, Biffi JC. A comparison of two techniques for the removal of calcium hydroxide from root canals. International Endodontic Journal, 2010; 43: 763–8.
9. Basson NJ, Tait CM. Effectiveness of three root canal medicaments to eliminate Actinomyces israelii from infected dentinal tubules in vitro. South Afric Dent J., 2001; 6: 499e-01.
10. Bowden GH. Microbiology of root surface caries in humans. J Dent Res., 1990; 69: 1205–10.
11. Calt S, Serper A, Ozc¸ elik B, Dalat MD. pH changes and calcium ion diffusion from calcium hydroxide dressing materials through root dentine. Journal ofEndodontics, 1999; 25: 329–31.
12. Cate, A.R. Ten. Oral Histology 2013, Nancy, Elsevier, 1998; 122.
13. Evans M, Davies JK, Sundquist G, Figdor D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J., 2002; 35: 221-228.
14. Fabricius L, Dahlén G, Öhman AE, Möller AJ. Predominant indigenous oral bacteria

isolated from infected root canals after varied times of closure. Scand J Dent Res., 1982; 90: 134–44.

15. Kishen A, George S, Kumar R. Enterococcus faecalis-mediated biomineralized biofilm formation on root canal dentine in vitro. J Biomed Mater Res A., 2006; 77: 406. Kishen A, Haapasalo M., 2010.
16. Biofilm models and methods of biofilm assessment. Endod Topics, 2008; 22: 58–78. Kishen A, Shi Z, Shrestha A, Neoh KG.
17. An investigation on the antibacterial and antibiofilm efficacy of cationic nanoparticulate for root canal disinfection. J Endod, 2008; 34: 1515-1520. Kishen A, Sum CP, Mathew S, Lim CT.
18. Influence of irrigation regimens on the adherence of Enterococcus faecalis to root canal dentin. J Endod, 1893; 34: 850-854. Klemperer F.
19. Komorowska R, Grad H, Wu XY, Friedman S., 2000.
20. Antimicrobial substantivity of chlorhexidine treated bovine root dentine. J Endod, 26: 315-7.
21. Kovacs-Nolan J, Phillips M, Mine Y. Advances in the value of eggs and egg components for human health. J Agric Food Chem., 2005; 53: 8421-31.
22. Gaikwad B., Banga K.S., Thakore A.J. Effect of calcium hydroxide as an intracanal dressing on apical seal – An invitro study. Journal of Endodontology, 2000; 12: 7- 12.
23. Gomes BPFA, Ferraz CCR, Vianna ME, Rosalen PL, Zaia AA, Teixeira FB, 2001. In vitro antimicrobial activity of calcium hydroxide pastes and their vehicles against selected microorganisms.
24. Braz Dent J., 2005; 3: 155-61. Haapasalo M, Endal U, Zandi H, Coil J. Eradication of endodontic infection by instrumentation and irrigation solutions.
25. Endod Top., 2003; 10: 71-102. Haenni S, Schmidlin PR, Mueller B, Sener B, Zehnder M. Chemical and antimicrobial properties of calcium hydroxide mixed with irrigating solutions.