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Use of Vital Stains in Determination of the Viability of Enterococcus Faecalis in Dentinal Tubules

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USE OF VITAL STAINS IN DETERMINATION OF THE VIABILITY OF *Enterococcus faecalis* IN DENTINAL TUBULES

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USE OF VITAL STAINS IN DETERMINATION

OF THE VIABILITY OF *Enterococcus faecalis*

IN DENTINAL TUBULES

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INTRODUCTION

Pulpal infection has been shown to be the fundamental etiologic factor in periapical inflammation and osteolysis (Kakehashi et al 1965, Bergenholts 1974, Möller et al 1981, Sundqvist 1976, Sundqvist 1994). The principle goal of root canal therapy is to eliminate bacteria from the root canal system. Microorganisms penetrate into the dentinal tubules (Chirnside 1958, Shovelton 1964, Nair et al 1990), and it is shown that chemomechanical debridement does not predictably result in a bacteria-free root canal system (Akpata 1976, Byström et al 1981, Byström et al 1985). The elimination of residual microorganisms in infected root canals traditionally requires inter-appointment antimicrobial medication. The necessity of inter-appointment medication has been questioned by advocates of single-appointment endodontic treatment (Soltanoff 1978, Oliet 1983, & Wahl 1996). The fate of microorganisms entombed after root canal obturation is not known.

The presence of residual bacteria, in dentinal tubules after obturation, has been suggested as a possible cause of persistent periapical inflammation (Haapasalo et al 1987, Sjögren et al 1997). Microorganisms remaining in the root canal and dentinal tubules can be observed with light and electron microscopic methods. These methods, however, do not

allow determination of viability of the microorganisms at the time of specimen fixation.

In previous studies, culturing has been the conventional method of determining bacteria viability. However, culturing methods are lengthy, difficult, and prone to error. Möller (1966) recommended two weeks incubation time for cultures to grow before determination of the results. Sundqvist (1976) recommended a minimum of 1 week incubation time for recovery of microorganisms from root canals. Procedural errors can occur even in the hands of professionals. If contamination occurs, false positives, and overgrowth of bacteria can occur (Myers et al 1969, Molven et al 1991).

Technology has improved our ability to use more specific techniques for identifying microorganisms and understanding their metabolic functions. With the development of highly specific nucleic acid probes and stains, very accurate information at the molecular level can be obtained (Lauer et al 1981, Jones et al 1985, Schupp et al 1987, Byrd et al 1991).

Nucleic acid dyes have been used in experimental biology and medicine (Terzieva et al 1996). Molecular Probes (4849 Pitchford Avenue, Eugene, OR) manufactures a bacterial viability assay kit that uses two nucleic acid dyes (propiduium iodide and SYTO 9) that have been

shown to rapidly differentiate between live from dead cells (FEMS Microbiol Lett 133, 1, 1995). The basis of the dual stain method in evaluating vitality is that cells with intact membranes stain with SYTO 9, and cells with broken membranes stain with PI and SYTO 9. The exclusion of propidium iodide from cells with intact membranes allows live and dead cells to be distinguished from each other in mixed populations (Jones et al 1985, Schupp et al 1987). SYTO 9 emits a different wavelength than PI making differentiation easy. Propidium iodide (PI) has previously been used to evaluate the disinfection of dental plaque (Rundegren et al 1992). SYTO 9 is a cyanide dye that is membrane permeable. Once these dyes are bound to nucleic acids, their fluorescence is enhanced 20-to-30 fold making stained cells easy to distinguish from the background dye (Arndt-Jovin et al 1989). What is unknown is how these stains behave under *in-vitro* conditions with bacteria and dentin.

The use of nucleic acid dyes to evaluate the efficiency of root canal disinfecting agents has never been investigated. This method has the potential to quantitatively evaluate the presence of viable bacteria inside the dentinal tubules of human teeth. If this can be established, the effect of viable bacteria inside of dentinal tubules can be further evaluated for their suggested role in persistent periapical inflammation.

It has been shown that periapical disease does not occur in the absence of inflammation (Kakehashi et al 1965). The presence of bacteria in dentinal tubules after obturation is a probable cause of persistent periapical inflammation (Haapasalo et al 1987, Sjögren 1990). Teeth with a negative culture at the time of obturation have shown an increase in prognosis (Sjögren et al1997). Myers et al (1969) showed that culture reversals occur in 25.9% of samples after 1 negative culture. Nucleic dyes have been shown to be a more reliable method for identifying microorganisms, and also have the ability to differentiate live from dead cells (Lauer et al 1981, Byrd et al 1990, Pedersen et al 1993). Increased knowledge of the fate of bacteria in dentinal tubules may lead to treatment modalities that will lead to a better prognosis for endodontically treated teeth.

REVIEW OF THE LITERATURE

The role of microorganisms in pulpal and periapical inflammation was first illustrated by S. Kakehashi, H. R. Stanley, and R. J. Fitzgerald in 1965. Conventional and Germ-free Fisher rats were used. The influence of viable microorganisms on the fate of surgically exposed dental pulps was investigated from a histological approach. Without exception, all of the conventional rats showed complete pulpal necrosis with chronic inflammatory tissue accompanied by abscess formations in the apical areas after eight days. In no instance did any of the injured pulps show signs of repair, matrix formation, or dentinal bridging. In contrast, pulpal inflammation was minimal in every specimen obtained from germ free rats, and no apical abscess were found. The presence of wound healing was evident as shown by of matrix formation and dentinal bridging. Bergenholts (1974) recovered microorganisms from the pulps of intact traumatized teeth. In his study, a significant relation was found between periapical destruction and the recovery of microorganisms from the root canal (Fig. 1).

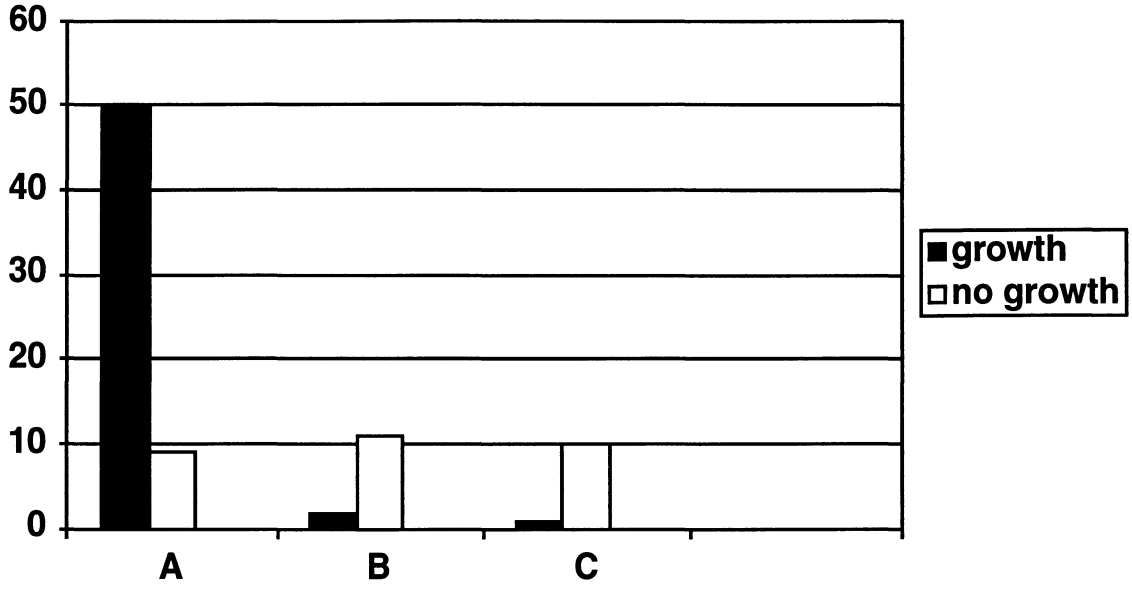


Fig. 1. Distribution of specimens with and without growth of micro-organisms among teeth with different appearances of periapical area. A. Teeth with periapical destruction. B. Teeth with widening of the periapical space. C. Teeth with intact periapical space. The Y axis represents the number of specimens analyzed. Bergenholts (1974).

A significant dependence between root resorption and growth of bacteria was also observed. These findings are in agreement with Sundqvist 1976. In a study of the bacteriology of necrotic pulp of human teeth devitalized by trauma, it was shown that no bacteria could be isolated from teeth without apical periodontitis (Table 1).

Table 1. Bacteriological and clinical findings. Sundqvist 1976.

Tooth (1) Code	Tooth (2) designation	Age of patient	Periapical area rarefaction present	size	Time lapse between trauma and sampling	Bacteria present	Number bacterial strains isolated	Remarks
B	22	23	+	2	6 months	+	1	
C	31	25	+	10	unknown	+	6	
D	41	24	+	2	8 months	+	6	4
E	11	15	+	2	7 months	+	1	
F	21	23	-		6 months	-		
G	32	25	+	5	10 months	+	7	5
H	31	32	+	6	5 years	+	6	5
I	11	14	-		3 months	-		
K	31	24	-		8 months	-		
L	11	12	-		4 weeks	-		
M	32	21	+	2	2 months	+	1	
N	41	21	-		3 months	-		
O	11	16	-		10 months	-		
P	31	25	+	8	4 years	+	10	4
R	21	14	+	2	2 months	-		
S	11	15	-		10 months	-		
T	21	28	-		6 years	-		
U	42	47	+	6	15 years	+	1	
V	22	28	-		6 years	-		

X	32	47	+	2	15 years	+	2	
Y	41	20	-		18 months	-		
Z	21	21	-		12 months	-		
AB	41	56	+	5	6 months	+	12	4
AC	31	60	+	2	3 months	+	4	
BA	32	20	+	6	unknown	+	8	
IN	21	19	+	2	3 months	+	1	
BS	11	41	-		unknown	-		
UJA	31	16	+	4	5 years	+	2	
UJB	41	16	+	10	5 years	+	9	5
JH	11	15	-		3 months	-		
BN	31	23	+	8	8 years	+	10	5
EL	31	29	+	2	unknown	+	1	

1) In five patients two teeth were sampled. Teeth B and F, D and K, T and V, U and X, UJA and UJB were from these patients.
2) Two-digit system (Federation Dentaire Internationale)
3) Diameter in millimeter.
4) Pian tenderness swelling after treatment
5) Pain tenderness swelling and exudation on treatment

Möller et al (1981) using a monkey model, found that periapical inflammation was significantly increased due to the presence of microorganisms in the root canal as compared to teeth with sterile necrotic pulps (Table 2). Fabricius et al (1982) also showed that the presence of multiple synergistic microorganisms in the root canal system

produced a heavier inflammatory response in the periapical tissues as revealed by radiographic interpretation.

Table 2. Möller et al (1981).

Infectious status of the necrotic pulp	Clinical examination	Radiographic examination	Histological examination
Noninfected	0/26	0/26	2/24
Infected	12/52	47/52	10/10

Inflammatory reactions after 6-7 months in periapical region of 26 noninfected and 52 infected teeth with necrotic pulps. Number of teeth with inflammatory reactions in relation to total number of teeth.

Möller (1966), in a series of controlled studies, demonstrated that the improvement in detection of root canal microorganisms which may have diverse nutritional requirements required that the following conditions be achieved: a) the sampling fluid be nontoxic to bacteria, b) the sampling vehicle (e.g., paper cone) be effective for successful microbial retrieval, c) the transport medium be effective for sustaining viability until cultivation, and d) the culture medium be supportive of the fastidious nutritional needs of the microorganisms in the sample. With advancements in culturing anaerobic bacteria, the recovery and identification of many more pathogens from the root canal system have become possible (Kantz & Henry 1974, Wittgow & Sabiston 1975,

Sundqvist 1976, Zavistoski et al 1980, Sundqvist et al 1989, Ando et al 1990, Baumgartner et al 1991).

With the role of microorganisms in periapical inflammation established; eliminating them from the root canal system has become the principal goal of endodontic treatment.

In 1958, Chirnside histologically evaluated 50 non-vital teeth with pulp canals open to the oral cavity before time of extraction. He found that bacterial penetration varied between tubules in infected areas, and that the dentinal tubules of 31 of the 50 teeth were heavily infected by gram positive cocci.

The presence of microorganisms in the dentinal tubules of chronically infected teeth has been shown by Shovelton, 1964. In his study, 97 non-vital teeth were investigated. Immediately after extraction, teeth were subjected to histological examination. The bacterial invasion of the dentinal tubules was evaluated in two ways. First, the degree of invasion was recorded according to the numbers of tubules containing bacteria. Sections containing bacteria in 1 to 20 tubules/section, were classified as mild. Sections with 21 to 50 contaminated tubules were classified as moderate, and sections contaminated with more than 50 tubules were classified as heavy. Also, the depth of penetration into the dentinal tubules surrounding the root canal was recorded. Results showed

that teeth with granulomas or periapical bone destruction were accompanied by an increase in tubular infection (Table 3).

Table 3. Relationship between the status of teeth and occurrence of invasion in dentin. Shovelton, 1964.

	A	B	C	D	E
	Total number of teeth	Teeth with bacteria Present in pulp or root canal	Teeth with no organisms present	Teeth with invasion of dentin	Teeth with no invasion of dentin
All teeth	97	79 (81.4 percent)	18 (18.6 percent)	61 (77.2 percent)	18 (22.8 percent)
Teeth With acute periapical inflammation	17	17 (100 percent)	Nil (0 percent)	10 (58.8 percent)	7 (41.2 percent
Teeth with granulom or periapical bone destruction	47	35 (74.5 percent)	12 (25.5 percent)	26 (74.3 percent)	9 (25.7 percent)

Note; Percentages shown in columns B and C are percentages of the total number of teeth shown in column A. Percentages in columns D and E are percentages of the infected teeth whose totals are shown in column B.

Akpata (1976) and Byström et al (1981), showed that chemomechanical debridement does not predictably produce a bacteria-free root canal system. Akpata compared the effect of instrumentation with sterile saline alone or in combination with 35% camphorated parachlorophenol. Root samples were crushed and then cultured for up to 65 days. Results indicated that 35% camphorated parachlorophenol significantly reduced the contamination of infected specimens. Despite treatment, 10% of samples remained contaminated. Byström et al (1981) studied 15 single rooted teeth with periapical lesions. Bacteria were found in all initial samples. The root canals were irrigated with physiological saline solution during instrumentation. Mechanical debridement reduced the amount of bacteria considerably (Table 4).

Table 4; Bacterial species isolated during the treatment of 15 teeth with periapical destruction. Byström et al (1981).

Bacterial species	Total number of isolates	No. of strains Eliminated by treatment*	Persisting strains at the 5th appointment
Streptococcus sp.	3	1	2
S. mitior	4	4	
S. mutans	2	1	1
S. sanguis	3	1	2
S. morbillorum	1		1
Peptostreptococcus sp.	3	2	1
P. anaerobius	5	2	3
P. micros	6	3	3
Eubacterium sp. Group 1**	1	1	
Eubacterium sp. Group 4**	2	1	1
E. alactolyticum	5	3	2
E. lentum	2		2
Actinomyces sp.***	2	2	
A. israelii	1	1	
Arachnia propionica	1	1	
Lactobacillus sp. Group 1 and 3**	7	4	3
Fusobacterium sp.	5	4	1
F. nucleatum	6	4	2
Bacteroides sp.	9	6	3

B. asaccharolyticus	1	1	
B. oralis	5	3	2
B. mel. ssp. intermedius	5	5	
Capnocytophaga ochracea#	2	2	
Selenomonas sputigena	3	3	
Anaerobic Vibrio sp.##	2	2	
Enterobacter agglomerans###	1		1
Eikenella corrodens	1	1	
Veillonella parvula	1	1	

* The treatment was given on five occasions
** These strains do not fit into recognized species and were classified according to numerical taxonomy (28).
*** Actinomyces sp. Group 2 in Borssen & Sundqvist (4).
\# Classified according to Socransky et al. (26).
\## Classified according to Tanner et al. (30).
\### Classified according to Ewing & Fife (13).

Specimens obtained at the beginning of each appointment usually contained 10^4 - 10^6 bacterial cells. At the end of treatment, contamination was reduced by 10^3. Bacteria were eliminated from the root canals of 8 teeth during treatment, but in 7 root canals, bacteria persisted despite treatment on five successive occasions. Teeth in which the infection persisted, were those that initially had high numbers of bacteria. It was concluded that the supporting action of disinfectants are necessary for predictable elimination of viable bacteria from the root canal system.

Byström et al (1985) tested the antimicrobial effects of 0.5 percent, 5.0 percent sodium hypochlorite, and 5 percent sodium hypochlorite in conjunction with 15 percent ethylene diamine tetra-acetic acid (EDTA) on 60 single rooted teeth in which necrotic pulps and periapical bone destruction was noted. Bacteria were recovered from all initial samples. Quantitative analysis of bacteria in teeth treated with 0.5 percent sodium hypochlorite prior to treatment was $1.6x10^5$ (range=$8x10^2$ - $2.5x10^6$). Corresponding figure for teeth treated with 5.0 percent sodium hypochlorite solution was $3x10^5$ (range=$6x10^5$ - $2x10^7$) and for canals treated with 5 percent sodium hypochlorite and EDTA $3x10^5$ (range=$1x10^3$ - $1.6x10^7$). After three appointments, bacteria were recovered from 8, 6, and 3 of the root canals in their respective groups (Fig. 2)

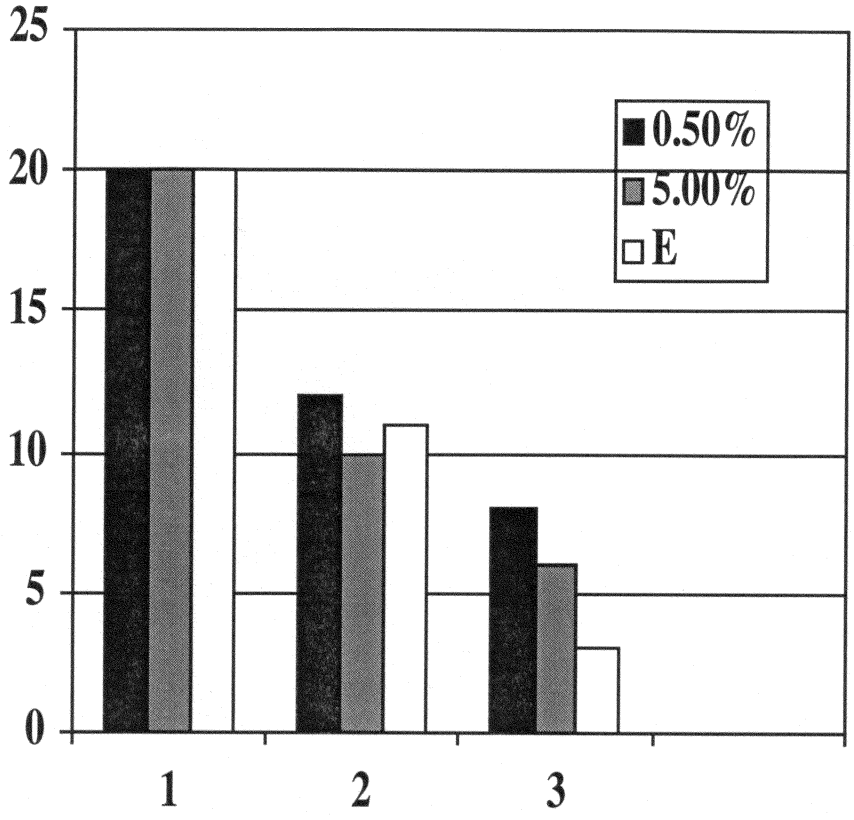


Fig. 2. Sample of 20 root canals. Recovery of bacteria from samples taken at the beginning of each of the three appointments. The colored part of the bars represent the number of root canals from which bacteria was recovered. 0.5 and 5.0 percent sodium hypochlorite solution, respectively. E: canals irrigated with 5 percent sodium hypochlorite and EDTA solutions. The Y axis represents the number of contaminated root canals. Byström et al (1985).

The median number of bacterial cells present at the third appointment was $2x10^5$ (range=$8x10^2$ -$2.5x10^6$) in specimens from root canals treated with 0.5 percent sodium hypochlorite, $1x10^6$ (range=$1.4x10^4$ - $2x10^7$) for teeth treated with 5 percent sodium hypochlorite, and $8x10^5$ (range=$1x10^4$ - $2x10^6$) for canals treated with 5

percent sodium hypochlorite and EDTA. These studies indicate that during the interappointment time, the amount of bacteria in the root canal system increased. The need for an interappointment antimicrobial dressing was, thus, established.

Teeth yielding a negative culture at the time of obturation show a significant increase in healing over the teeth that yield a positive culture (Zeldow & Ingle 1963, Engström et al. 1964, Oliet & Sorin 1969). However, these studies were limited because the culturing techniques did not allow recovery of obligate anaerobic bacteria that were probably present in the root canal system at the time of sampling. When advanced techniques are used in recovering bacteria from the root canal system, obligate anaerobes form a high proportion of the total flora in infected root canals (Kantz & Henry 1974, Wittgow & Sabiston 1975, Sundqvist 1976, Zavistoski et al 1980, Sundqvist et al 1989, Ando et al 1990, Baumgartner et al 1991). It is probable that the teeth that yielded negative cultures at the time of obturation in previous studies harbored anaerobic bacteria that may have affected the outcome of some teeth. Sjögren et al (1997) treated 55 single-rooted teeth, all of which had intact pulp chamber walls, necrotic pulps and radiographic evidence of periapical bone destruction. Initial bacteria samples were taken, all canals were instrumented, irrigated with 0.5 percent sodium hypochlorite, inactivated

with 5.0 percent sodium thiosulfate solution (Möller 1966), post-instrumentation sample taken, obturated with chloroform fitted master cones with lateral condensation. The teeth were followed for up to 5 years after treatment. The results showed that all teeth had recoverable bacteria at initial sampling. At the completion of instrumentation, 22 root canals (40%) still contained recoverable bacteria. The number of species in root canals with persistent infection ranged from 1 to 6, and 93 percent of these strains were anaerobes. Two teeth were lost for follow-up leaving 53 teeth. Fourty-four of the lesions healed completely and 9 cases, all of which were symptomless, were judged to be failures. Seven of the failures were among the 22 teeth that had yielded a positive culture before obturation. Thus, the success rate for the teeth with a positive culture at the time of obturation was 68 percent. Twenty-nine of the 31 teeth with a negative bacterial culture at the time of obturation healed; a success rate of 94 percent. With the use of advanced endodontic anaerobic techniques for recovering bacteria from the root canal system, a clearer view of the role of microorganisms in periapical inflammation is obtained. These findings emphasize that complete elimination of viable bacteria from the root canal system before obturation is essential for long term prognosis. They conclude that this objective cannot be reliably achieved in a single-visit treatment since it is not possible to reliably eradicate all infection

from the root canal system without the support of an interappointment antimicrobial dressing.

Despite the presence of scientific evidence supporting the use of interappointment antimicrobial medication in infected root canal systems, some clinicians believe that teeth with infected dentin can be treated in a single-visit with a high success rate (Soltanoff 1978, Oliet 1983, Wahl 1996). These studies which are in support of single-visit treatment for chronically infected teeth lack a scientific approach. Critical data regarding microbial infection at the time of obturation, and the criteria for success is not reported. The reader is left to take the authors' word as scientific fact. However, the fact remains that the periradicular area of some teeth with infected root canal systems heal. The fate of residual microorganisms in the root canal system at the time of obturation remains known.

The prolonged viability of microorganisms in dentinal tubules has been a concern in dentistry for many years. In coronal dentine studies, microorganisms were shown to remain in dentinal tubules of teeth after the removal of all soft dentin (Whitehead et al 1960), and to survive under sealed restorations for extended periods of time (Schoubue et al 1962, Fisher 1966). It is believed that the source of nutrients for the microorganisms came from leaky restorations or the pulp itself via the

dentinal tubules (Fisher 1966). Residual bacteria in the dentinal tubules after obturation have been suggested as a possible cause of persistent periapical inflammation (Happasalo et al 1987, Sjögren et al 1997). Microorganisms remaining in the root canal and dentinal tubules have been observed by light and electron microscopic methods. These methods, however, do not allow determination of viability of the microorganisms at the time of specimen fixation. In disinfection studies, artificially infected dentinal tubules have been exposed to various anti-microbial agents *in-vitro* (Haapasalo et al 1987, Ørstavik et al 1990, Safavi et al 1990). Microbial viability was evaluated by placing dentin shavings, or the entire sample, in culture medium monitoring geowth by increase in turbidity. *In-vivo*, however, bacterial sampling of the root canal system is a complex process that is prone to procedural errors (Möller 1966, Sundqvist 1976). Also, conventional light or electron microscope methods do not allow determination of viability of the microorganisms at the time of fixation, only their presence.

The presence of microorganisms in the root canal system has been shown by light and electron microscopy. Ando et al (1990) showed *in-vivo* that bacteria can penetrate as far as 2mm into the root canal dentin. Armitage et al (1983) showed bacterial penetration half way to the cementum. Gutierrez et al (1990) showed *in-vivo* that bacterial

penetration to a depth of 250μm. Sen et al (1995) showed bacterial penetration of 150μm into dentin. The degree of bacterial penetration into the dentin of the root canal *in-vivo* varies greatly. The presence of the smear layer over the dentinal tubules at the time of bacterial infection in these studies are not clear, and may be the reason for inconsistencies of microorganism penetration *in-vivo*. Studies have shown that the smear layer significantly reduces the depth of bacteria penetration *in-vitro*. Also, the presence of cementum hinders the migration of microorganisms through the tubules. Akpata et al (1982) using a model consisting of freshly extracted human single canal teeth showed that with the smear layer intact, bacteria could penetrate half way through the sample after a three week infection time. Safavi et al (1990) removed the smear layer and cementum from test samples, and incubated them in infected broth for 27 days. They found bacterial penetration up to 300μm into the dentin. Perez et al (1993) showed bacterial penetration into dentinal tubules of 792μm after infecting samples for 28 days without the smear layer. On the other hand, Meryon et al (1990) did not detect an effect of smear layer on bacterial penetration. Both of their groups had a depth of bacteria penetration of approximately 500μm after 72 hours of infection time. These results indicate that microorganisms can penetrate into dentinal tubules over a relatively short period of time.

Akpata et al (1982) showed that the degree of bacterial penetration into the dentinal tubules is time-dependent and related to the growth rate of the microorganism *in vitro*. In this study, the penetration of two obligate anaerobes, *Bacteroides melaninogenicus* ss. melaninogenicus (ATCC 25847) and *Peptococcus asaccharolyicus* (ATCC 14963), - and two facultative anaerobic bacteria *Streptococcus faecalis* (ATCC 19433) and *Streptococcus sanguis* (ATCC 10556), were studied. Bacteria were sealed inside sterile extracted human teeth for 1, 2, and 3 weeks. The degree of bacterial penetration was evaluated by light microscopy on histological sections stained by Brown and Brenn technique. No evidence supporting penetration of obligate anaerobes was obtained. The facultative anaerobes penetrated the dentinal tubules from the cervical area first, and then progressed to the apical area of the root samples. *S. sanguis* was first to penetrate, but *S. faecalis* showed the heaviest infection after the three week period (Table 5). The obligate anaerobes are known to grow slowly, as compared to the *Streptococci*, which have a relative rapid growth rate. The differences of penetration may be attributed to the growth rate, cell adhesions, or other cellular factors. These findings are in agreement with Perez et al (1993). In their study, two anaerobes and one aerobic bacterium were used to infect bovine dentin for periods of 10-28 days. *Actinomyces naeslundii* and

Prevotella intermedia showed no tubule penetration when examined by Scanning Electron Microscope (SEM) or light microscopy. *Streptococcus sanguis* was observed at a depth of 792 μm. In a recent study by Siqueira et al (1996), penetration of 5 obligate anaerobes and 1 facultative anaerobic bacteria were studied by SEM. Bovine samples were infected for 21 days. Results showed that *E. faecalis*, *Propionibacterium acnes*, and *Actinomyces israelii* heavily infected the root canal walls and dentinal tubules. *Porphyromonas gingivalis* infected few tubules, but penetrated to great depths. *Porphyromonas endodontalis* penetrated a small percentage of dentinal tubules and remained restricted to just within the circumpulpal dentin. *Fusobacterium nucleatum* heavily colonized the root canal walls, but further cell migration was apparently prevented because of their spindle-shaped cells.This study suggest that obligate anaerobes can penetrate into dentinal tubules. The fate of these microorganisms is unknown. The vitality of cells is lost during fixation procedures.

Table 5. Bacterial invasion of tubules in dentin surrounding the dentinal pulp. Akpata (1982)

Incubation Period	Microorganisms	Bacterial Invasion		
		Cervical 1/3	Middle 1/3	Apical 1/3
1 WK	*B. melaninogenicus*	-	-	-
	P. asaccharolyticus	-	-	-
	S. sanguis	+	-	-
	S.faecalis	-	-	-
2 WK	*B. melaninogenicus*	-	-	-
	P. asaccharolyticus	-	-	-
	S. sanguis	+	+	-
	S.faecalis	++	++	+
3 WK	*B. melaninogenicus*	-	-	-
	P. asaccharolyticus	-	-	-
	S. sanguis	+	+	+
	S.faecalis	+++	+++	+

Most studies have used culturing techniques to evaluate bacteria viability. Although endodontic culturing methods are a valuable tool, they have considerable flaws. Many investigators have identified problems with culturing bacteria of different growth characteristics. Some obligate anaerobic bacteria are slow growing, and can easily be overgrown or die due to lack of nutrition (Möller 1966). Also, many bacteria in the root

canal system are known to require synergistic relationships with other bacterial species, and will not survive as an isolate (Sundqvist 1992). It is shown that the microflora of the root canal system consists of more than 90 percent obligate anaerobes (Sundqvist et at 1989). Unless fastidious efforts are used, recovery and transport techniques decrease the chances of culturing these microorganisms.

Culturing techniques have been shown to render false negatives in the endodontic literature. Myers et al (1969), investigated culture reversals. In this study, 108 teeth were scheduled for obturation after the canal produced a negative culture, and 106 teeth were scheduled for obturation after 2 consecutive cultures were achieved. Between 10 and 14 day after the last negative culture was taken, the teeth were obturated. At that time, a new culture was taken. They found that in the group with 1 negative culture, 25.9% of the canals were found to harbor bacteria. The second group had a reversal of 13.9%. Molven et al in 1991, used SEM to evaluate the apical 2mm of infected roots. They found that 83% of the samples were infected, but it was very difficult to culture the microorganisms.

The literature on disinfection of the root canal system is lacking a reliable direct method for determining the viability of microorganisms in dentinal tubules.

The use of nucleic acid dyes to assessing bacterial viability has been used in experimental biology, water treatment, and medicine (Lauer, et al 1981, Schupp, et al 1987, Byrd et al 1991, Rundegren et al 1992, Terzieva et al 1996). Propidium iodide (PI) has been used to evaluate the disinfection of dental plaque. Rundegren et al (1992) found that propidium iodide gives a more intense stain than ethidium bromide, a similar nucleic acid, thus making the distinction between live and dead bacteria cells easy. PI is more water-soluble and less membrane permanent than ethidium bromide (Haugland 1996). The efficiency of PI and fluorescein diacetate (FDA) in distinguishing between live and dead *G. muris* cysts was investigated *in-vivo* by Schupp et al (1987). FDA has similar characteristics as SYTO 9. Using a mouse model for giardiasis, they inoculated FDA or PI stained cysts into neonatal mice. Feces were examined at days 3, 5, 8, and 11 postinoculation for the presence of cysts. Using 1,000 FDA-stained cysts as the inoculum, they detected cysts at days 5, 8, and 11 postinoculation in 19 of 19 mice, whereas a 50-fold greater dose of cysts produced infection in 27 of 27 mice at day 3 as well as at days 5, 8, and 11 postinoculation. Inoculation of mice with either 5,000 or 50,000 PI-stained *G. muris* cysts did not produce infection in any of the animals. Necropsy of mice infected with FDA-stained cysts showed trophozoites within the intestines. No trophozoites were detected within

animals inoculated with PI-stained cysts. Their results demonstrated that FDA-positive cysts are viable, as determined by infectivity, while PI-positive cysts are nonviable and incapable of producing *G. muris* infections *in-vivo* (Table 6).

Table 6. *G. muris* cyst viability: correlation between fluorogenic dye incorporation and infectivity in neonatal mice. Schupp et al (1987).

No. of cysts inoculated per animal (no. of animals)	Fluorescent substrate and appearance [a]	Method of cyst isolation	*G. muris* cyst appearance on indicated day postinoculation			
			3	**5**	**8**	**1**
Control: 0 (n=8)	none	none	0	0	0	0
Experimental						
1,000 (n=9)	FDA positive	Sucrose density gradient	0	9	9	9
50,000 (n=27)	FDA positive	Sucrose density gradient	27	27	27	27
50,000 (n=40)	PI positive (induced)	Sucrose density gradient	0	0	0	0
1,000 (n=10)	FDA positive	FACS IV	0	10	10	10
5,000 (n=10)	PI positive (induced)	FACS IV	0	0	0	0

[a] As determined by fluorescence

Lauer et al (1981), Showed that fluorochrome staining was more sensitive and reliable than gram staining alone (Table 7).

Table 7. *Comparison of gram and AO stains in the direct microscopic examination of clinical specimens* [a]

Stain results (%)						
Test	CSF		Other specimens		Total specimens	
	Gram	AO	Gram	AO	Gram	AO
Sensitivity	76.7	82.2	45.1	48.6	55.8	59.9
Specificity	100	100	98.8	100	99.6	100
Predictive value (+)	100	100	98.5	100	99.2	100
Predictive value (-)	88.9	91.3	64.4	66	74.4	76.3

[a] A total of 209 cerebrospinal fluids (CSF) and other body fluids, tissues, and exudates were examined. AO= Acridine Orange.

Direct viable counts (DVC) have been shown to produce quicker more reliable results than culturing techniques (Byrd et al, 1991). *Klebsiella pneumoniae, Enterobacter aerogenes, Agrobacterium tumefaciens, Streptococcus faecalis, Micrococcus flavus, Bacillus subtilis,* and *Pseudomonas* strains L2 and 719 were tested for the ability to grow and maintain viability in drinking water. Growth was evaluated in this study by plate counts, acridine orange direct counts (AODC), and DVC. As shown in tables 8a and 8b, *K. pneumonia, E. aerogenes, A. tumefaciens, S. faecalis,* and *M. flavus* would go undetected if only plate counts were used to determine viability.

Table 8a. Survival of *S. faecalis, M. flavus, and B. subtilis* in sterile drinking water at 25° C (Byrd et al 1991).

Day	Log cells/ml [a]								
	S faecalis			M. flavus			B. subtilis		
	AODC	KSA	TSA	AODC	NA	TSA	AODC	NA	TSA
0	6.5	6.4	6.4	6.1	5.5	5.3	6.1	4.2	4.7
1	6.2	5.5	5.7	6.0	5.3	5.1	5.8	1.6	1.5
2	6.2	2.5	2.9	5.9	2.7	2.4	5.8	1.8	1.5
3	6.2	0.2	0.7	6.0	1.9	1.6	ND	ND	ND
4	6.1	0 [b]	0.2	5.9	1.7	0.3	ND	ND	ND
5	6.2	0	0	5.9	0.5	0.5	5.7	1.6	1.7
7	ND	ND	ND	5.9	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	5.7	1.7	1.7
38	ND	ND	ND	ND	ND	ND	ND	1.2	ND

[a] ND. Not determined; [b] Limit of detection; AODC=acridine orange direct counts; TSA=Tryptic soy agar; KSA=KF Streptococcus agar; NA=nutrient agar.

Table 8b; Survival of *A. tumefaciens*, *K. pneumoniae*, and *E. aerogenes* in streile drinking water at 25°C (Byrd et al 1991).

Day	Log cells/ml[a]										
	A. tumefaciens			*K. pneumoniae*				*E. aerogenes*			
	ADOC	DVC	SYC	AODC	DVC	TSA	MAC	AODC	DVC	TSA	MAC
0	6.3	6.2	3.2	6.2	6.2	5.2	6.4	6.3	6.0	3.6	3.9
1	6.3	6.1	2.0	6.0	6.2	ND	ND	5.7	5.6	ND	ND
2	6.2	6.2	0[b]	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	5.8	5.4	ND	ND	5.7	5.1	ND	ND
4	6.2	6.1	0	ND	ND	0	0	ND	ND	0	0.7
7	6.2	6.1	0	5.7	5.1	ND	ND	5.9	5.2	ND	ND
10	ND	ND	ND	5.8	ND	0	0	5.1	5.1	0	0

[a] ND, Not determined. [b] Limit of detection. AODC=acridine orange direct counts; TSA=Tryptic soy agar; DVC=direct viable counts; MAC=MacConkey agar.

Molecular Probes (4849 Pitchford Avenue, Eugene, OR) manufactures a bacterial viability assay kit that uses two nucleic acid dyes (propidium iodide and SYTO 9) that can rapidly differentiate between live and dead bacterial cells (FEMS Microbiol Lett 133, 1, 1995). Once bound to nucleic acids, the fluorescence of these dyes is enhanced 20-to-30 fold (Arndt-Jovin et al 1989). SYTO 9 is a cyanide dye that is membrane permeable. Cells with intact membranes stain with SYTO 9 and appear green, however, cells with broken membranes stain with PI and SYTO 9 and appear red to orange. The exclusion of propidium iodide from cells with intact membranes allows live and dead cells to be distinguished from each other in mixed populations. SYTO 9 emits a different wavelength than PI making differentiation possible. It is unknown how these stains behave under *in-vitro* conditions with bacteria and dentin. If this assay is able to differentiate between live from dead bacteria cells in dentinal tubules, this approach would prove to be a more reliable and quicker method for understanding the fate of microorganisms in the root canal system than traditional culturing techniques.

OBJECTIVES OF THIS STUDY

The main objective of this study is to determine if nucleic acid stains (SYTO 9 and PI) can effectively differentiate between dead and killed bacteria in dentin. First, reliable controls will be established using *Entrococcus faecalis.*

Enterococcus faecalis was selected as a test microorganism because: its' ability to survive exposure to oxygen, it has been recovered from infected root canals (Winkler et al 1959, Sundqvist in 1998), it has been used in previous infection and disinfection studies (Olgart et al 1974, Akpata et al 1982, Haapasalo et al 1987, Ørstavik et al 1990, Safavi et al 1990, Vahdaty et al 1993, Tanriverdi et al 1997), and ease of technical manipulation.

Specific Objectives

1) To test the ability of the fluorescence dual stain method in differentiation of live and killed preparations of *E. faecalis* and *Escherichia coli.* For this purpose, it will be necessary to establish a standard assay protocol in which acceptable working ratios of SYTO 9: Propidium iodide: Bacterium are defined.

a) Identification or the relationship between bacterial viable count and optical density in exponentially growing cultures of the model test organisms.

b) Evaluation of standard methods for producing killed bacteria cells.

c) Fine-tuning of SYTO 9: Propidium iodide: Bacterium ratios to establish the parameters which result in acceptable differentiation of live and killed bacterial cells in a population.

2) To evaluate the ability of the fluorescence dual stain method to differentiate live and killed *E. faecalis* cells in the presence of dentinal shavings. Evaluate the degree of background staining of dentin and adjustment of the parameters of the standard assay method if necessary.

3) To evaluate the ability of the fluorescence dual stain method to differentiate live and killed *E. faecalis* cells in dentinal slurries recovered from artificially infected dentinal root sections. For this purpose, if will be necessary to develop an *in-vitro* root model in which dentinal tubules may be easily infected and subsequently examined.

4) To evaluate the effects of standard endodontic disinfection treatments on the parameters of the fluorescence dual stain method in dentinal shavings and artificially infected dentinal root sections.

MATERIALS AND METHODS

Establishing the Growth Curve

Experiment a: Selection of Growth Medium

Enterococcus faecalis 9790 (obtained from the Microbiology Department, at the University of Connecticut Health Center, Farmington Connecticut) was inoculated in 5ml of Luria Bertani (LB) broth, 5ml of LB broth containing 0.02% glucose, and 5ml of LB broth containing 0.02% glucose and 0.02% caso amino acids. All broths were incubated over night in a shaking water bath at 37° C to obtain exponential growth phase. Next, using an Optical Spectronic 20, (Spectronic Instruments), set at a wavelength of 600nm and calibrated with deionized water, the turbidity of the broth was measured by optical density.

Experiment b: Establishing a Growth Curve

E-coli C-600, obtained from the Microbiology Department, University of Connecticut, Farmington Connecticut, was used as a control microorganism. *E-coli* was inoculated into 5ml of modified LB broth and incubated over night at 37° C in a shaking water bath. Next, using the spectrophotometer set at a wavelength of 600nm and calibrated with deionized water, an optical index was taken of the turbidity of the broth.

Optical density of 0.05 was calculated and established. The new density of microorganisms were again placed into the shaker bath and a plot was made using optical density readings every 30 minutes until a density of 0.40 was achieved. A growth curve was plotted on semi-log paper with time on the x-axis and optical density on the y-axis. *Enterococcus faecalis* was grown in 5ml of modified Luria Bertani broth to log phase in an overnight shaker water bath at 37° C. Optical density was adjusted to 0.05. Optical density readings were taken every half-hour until a reading of 0.40 was reached. A growth curve was made from plotting the data on semi-log paper as described above.

Experiment c: Calibrating Optical Density with Cell Population

Enterococcus faecalis was grown over night in modified LB broth using a shaking water bath at 37° C as described above. The optical density was adjusted to 0.40 and serial dilutions were made. Modified LB agar plates were inoculated with the diluted broth. Plates were placed into the CO_2 incubator at 37° C overnight. The colony forming units (cfu) were then counted the next morning.

Establishing the Negative Control

The killing efficiency of three known methods 70% isopropyl alcohol (group 1), Ultraviolet (UV) light (group 2), were tested on *Enterococcus faecalis*.

Experiment d: UV Killing Curve

Enterococcus faecalis was placed on modified LB agar plates at a density of 300 cells/plate, and exposed to different doses of UV. For the UV experiment, three plates were divided into three sections. A lead shield was used to isolate different areas of the agar dish. A Minerlight lamp model UVG-11 by UVP Inc. San Gabriel, CA., was used for UV disinfection. The UV source with a 254nm wavelength at 40 watts was placed three inches from plates. Plate 1 was exposed at 30, 60, and 90 seconds. Plate 2 was exposed at 120, 300, and 600 seconds. Plate 3 was exposed for 900 and 1200 seconds.

Experiment e: Establishing the UV Critical Dose

A total of 27 modified LB agar plates were seeded with *Enterococcus faecalis* at a density of 300 cfu. Eight different UV dose regimen were tested in triplicate. Three plates served as controls. UV source was moved to 10 inches away from plates. Exposure times were, 5,

10, 15, 20, 30, 45, 60, and 90 seconds. All plates were incubated at 37 ^{o} C overnight and observed for growth the next day.

Experiment f: Evaluation of UV Killing in Broth Cultures

An overnight broth of *Enterococcus faecalis* was prepared. Cells in exponential growth phase at a concentration of 1 x 10^7 cells/ml were established as described previously. Five different UV exposure doses were evaluated in triplicate. The 40 watt, 254 wave length source was placed 10 inches from microwells containing *Enterococcus faecalis* at a concentration of 1x10^7 cells/ml. Wells were placed on a shaker at a setting of 7. Exposure times are 30, 60, 90, 150, & 210 seconds. Three wells served as controls and received no UV. The contents of the wells were placed on LB agar plates, incubated at 37 ^{o} C overnight, and then evaluated for growth.

Experiment g: UV verses 70 percent Isopropyl Alcohol

An overnight broth of *Enterococcus faecalis* was prepared and grown to exponential phase at an optical density of 0.40, as described above. At that time, a 12ml of suspension was divided into 3 groups of 4ml each. Group 1 served as the control, and was washed by 3 centrifugations in saline. Two serial dilutions were plated on modified LB

agar. Group 2 was placed into the lid of an agar dish and exposed to UV as described in the previous experiment, but for 3 minutes. Serial dilutions of 1:4, and 1:10 were made, and 100µl from each cell suspension were used for evaluation of the nucleic acid stains. The remaining sample was plated on modified LB agar plates. Group 3 was washed by 3 centrifugations in saline, and suspended in 4ml 70% isopropyl alcohol. After a two hour period, the suspension was washed by centrifugation and suspended in saline of equal volume. A 100µl of cell suspension was used to stain with nucleic acid dyes, and the remainder was used for plating on modified LB agar. All plates were incubated at 37° C overnight and evaluated for growth the next day. The manufacture recommends that dyes be mixed together in equal concentrations, or to modify the stains concentrations to bacteria.

Calibration of the Dyes to the Microorganism

Positive and negative control cells were suspended in saline at $3.2x10^9$ cells/ml, stained with different ratios of SYTO 9 and PI (1:1, 1:2, 1:10, 1:20, 1:40), and viewed by fluorescence microscopy. Ratios were based on volume of dye from their original concentration as bottled by the manufacturer. Both controls were mixed in different ratios (0:100, 10:90, 50:50, 90:10, 100:0) and stained SYTO 9 and PI to determine the

correct ratio of dye needed to obtain optimum fluorescence, and evaluate the dyes ability to differentiate live and dead bacterial cells in a population.

Establishing the Protocol for In-Vitro Study

Evaluation of Nucleic Dyes on Dentin

Mixtures of positive and negative bacterial cells, were optimally stained as descibed above, and placed with dentin shavings which had been obtained from sterile bovine teeth using a sterile endodontic file. Non-stained mixtures of bacterial cells were placed with dentin shavings and then stained. Both were viewed by fluorescence microscopy and compared. This correlated with previous tests, established reliable controls, and ruled out dentin as a confounder with background stain.

To Evaluate the Dyes Ability to Effectively Stain *Enterococcus faecalis* in Dentinal Tubules.

Fresh extracted anterior bovine teeth were used in this study. The crowns and 5mm of the apex were removed and discarded. The cementum was removed from the samples using a diamond bur with a highspeed handpiece. Bovine dentinal samples were approximately 5mm tall, 4mm in diameter, and had a lumen approximately 3.1mm in diameter. The

smear layer was removed from both sides with 35% phosphoric acid for 60 seconds. Samples were rinsed for 30 minutes with tap water, and then sterilized in an autoclave. Samples were divided into two groups. Group 1 consisted of 2 samples that were infected with *Enterococcus faecalis*. Group 2 consisted of 2 samples and served as the negative control with no bacterial infection. Both groups were surrounded by modified LB agar, and incubated at 37° C for 9 days. Group 1 was replenished with log phase bacteria in the canal lumen every 12 hrs. Group 2 was incubated with sterile LB broth in the lumen. After 9 days, the samples were rinsed with 5ml of saline. A sterile endodontic file was used to remove dentin shavings from one wall of the inner lumen from each group. Dentinal shavings were suspended in saline and stained with nucleic acid dyes as described above. Slides were viewed by an Olympus BHS microscope with an integrating digital camera. The remaining samples were fixed, and gram stain to verify bacterial penetration into dentinal tubules.

Evaluation of Treatment and Dye Interactions

Overnight bacterial suspensions of *E. faecalis* were centrifuged and rinsed three times with sterile saline. Bacteria were suspended in treatment solutions at a concentration of 3.2×10^9 cells/ml. Group 1 was suspended in 2ml of 1% buffered NaOCl. Group 2 was suspended in 2ml

of 2% IKI. Group 3 was suspended into 2ml of aqueous $Ca(OH)_2$. Samples were challenged for 5 minutes, centrifuged, rinsed three times, and stained with the dual nucleic acid stains. Slides were made using 10µl of suspension and viewed by fluorescent microscopy.

In-Vitro Disinfection Evaluation

Twenty four standardized dentinal samples made from freshly extracted anterior bovine teeth were randomly divided into five groups. All bovine samples had the crowns and 5mm of the apex removed and discarded. The cementum was removed with a high speed diamond bur. The smear layer on the inner and outer surface of the samples were removed with 37% phosphoric acid. All samples were rinsed for 30 minutes and sterilized by autoclaving. The test groups each contained 6 samples. Control groups contained 2 each. Samples were embedded in modified LB agar and the lumen filled with broth containing *E. faecalis* in exponential growth phase, or sterile broth for the negative control. Test groups were infected with *Enterococcus faecalis* for 9 days with the broth replenished every 12 hours. After 9 days, samples were rinsed with saline. Group III was then irrigated with 5ml of 1% buffered NaOCl. One ml of NaOCl was introduced in 1 minute intervals over a period of 5 minutes. Group IV was treated with 2% IKI for 5 minutes, and Group V

received $Ca(OH)_2$ treatment for 7 days. The positive controls were infected for 9 days and did not receive any chemical treatment, and two more samples were not infected, but incubated for the 9 day duration (negative controls). All samples had dentinal shavings removed in a serial method similar to Haapasalo and Ørstavik 1987. The initial lumen size was 3.1mm. ISO bur sizes of 033, 035, 037, 040, 042, 045, 047, and 050 were used to remove dentinal shavings sequentially. Dentinal shavings were stained, and random fields viewed by fluorescence microscopy. Two random samples from each group were sectioned medially, one half being fixed and stained for bacterial penetration, and the other used for fluorescent staining. Fixed and decalcified samples were stained with a simple gram stain for verification of bacterial penetration.

Each slide examined by fluorescent microscopy was given two scores. One score for the level of bacterial infection, and the other for percentage of cells on that slide that emitted green or red light. Bacterial infection was scored on a scale of 0-4. A rating of 0 meant no bacterial cells were found during inspection of slide. The rating of 1 equaled 1-19 cells; 2 equaled 20- 39 cells; 3 equaled 40-50 cells; and 51 cells and over was given a 4. Viability was given a percentage for green and red cells viewed.

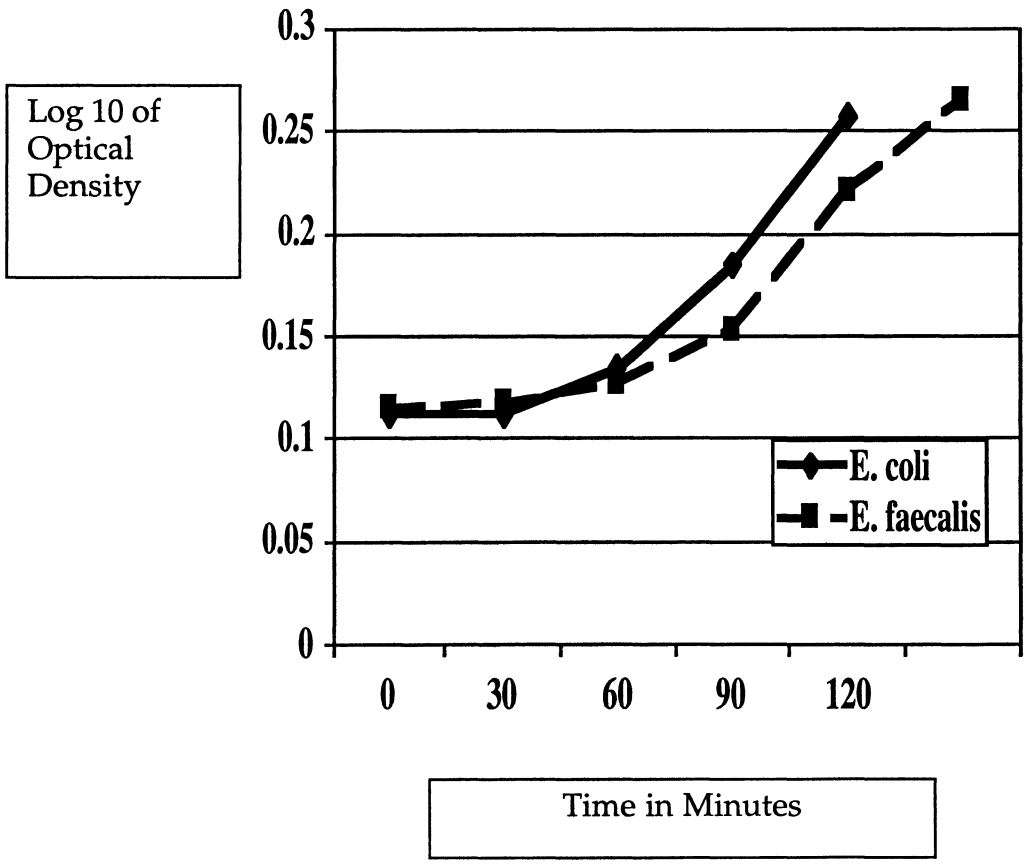
Image Colorization Method

Images were recorded by an Olympus BHS microscope equipped with a Integrating Charged Coupled Device (CCD) camera. The camera records 8 bit images. A rhodamine filter was used to record wavelengths above 635nm and a fluorecein filter (FITC) was used to record the wavelengths above 500nm. Two identical micrographs were recorded of each image. The images were combined and colorized using Adobe PhotoShop software. The eight bit images were split into four channels to allow for colorization. A color camera can eliminate this process. To overcome the problem created by the black and white camera, the FITC image was subtracted from the rhodamine image in PhotoShop. This created a new image. The images were then opened in RGB, and the channels were split. Four channels exist, green, red, blue and RGB. The blue channel was eliminated since that channel served no purpose. The new image was then copied, and pasted onto the red channel. The colorized channels were then merged. Since the killed cells stained with both SYTO 9 and propidium iodide, a range of emissions from yellow to red were seen.

RESULTS

In order to determine the specific ratios of dye to bacterial concentration it was necessary to establish the relationship between the number of cells and optical density. The growth rates of *E. coli*, control microorganism, and *E. faecalis* was established by plotting optical densities verses time (Fig. 3).

Figure 3, Growth curve of *E. coli* and *E. faecallis*



Cells per ml were measured by serially diluting the suspension, and plating with modified LB agar. Plates were incubated overnight at 37 ^{o}C and counted the next morning. The remaining fields were calculated. In figure 3, the vertical axis represents the log 10 of optical density. The growth curves of both test and control microorganisms allowed cells to be plated on modified LB agar plates in predictable counts. The data is shown in table 9,10.

Table 9; Optical density vs. time of *E. coli.*

TIME	OPTICAL DENSITY	CELLS/ml
9:00	0.050	4.34×10^{7}
9:30	0.087	7.56×10^{7}
10:00	0.130	1.13×10^{8}
10:30	0.268	2.33×10^{8}
11:00	0.410	3.56×10^{8}

Table 10; Optical density vs. time of *E. faecalis.*

TIME	OPTICAL DENSITY	CELLS/ml
9:35	0.063	8.31×10^{7}
10:00	0.073	9.63×10^{7}
10:30	0.104	1.37×10^{8}
11:00	0.187	2.47×10^{8}
11:30	0.346	4.56×10^{8}
11:45	0.423	5.58×10^{8}

Two killing methods were evaluated. The results of UV on *E. faecalis* placed on agar plates at a density of 300 cells per plate are shown in table 11.

Table 11; UV exposures @ 10 inches at various times on *E. faecalis*

TIME	PLATE 1	PLATE 2	PLATE 3	AVERAGE
0	297	263	218	260
5	187	143	165	165
10	237	208	133	196
15	157	229	130	172
20	233	209	231	224
30	183	149	167	166
45	88	96	70	85
60	6	20	5	10
90	0	0	0	0

The results of Table 11 indicate that at a distance of 10 inches, a 90 second dose from a UV light of 40 watts effectively kills *E. faecalis* in a cell density of 300. The efficiency of UV to kill *E. faecalis* in a broth suspension at a cell density of $1x10^7$cells/ml is shown in table 12.

Table 12; UV exposure on *E. faecalis* in modified LB broth. TTC= too many to count.

EXPOSURE	WELL 1	WELL 2	WELL 3	AVERAGE
0	TTC	TTC	TTC	TTC
30	TTC	TTC	TTC	TTC
60	TTC	TTC	TTC	TTC
90	461	160	353	324
150	0	2	0	0.67
210	0	0	4	1.33

The results of table 12 indicate that UV is effective in killing *E. faecalis* in modified LB broth in a cell density on $1x10^7$ cells/ml.

The efficiency of 70% isopropyl alcohol and UV light to kill *E. faecalis* was investigated. The test organism was suspended at a cell density of $3.2x10^9$ cells/ml. The results are shown in table 13.

GROUP 1	GROUP 2	GROUP 3
34	26	TTC

Table 13; Colony counts of *E. faecalis* after UV and 70% isopropyl alcohol challenge. Group 1= UV and group 2=70% isopropyl alcohol Group 3=control.

70% isopropyl alcohol was determined to offer less margin of error, and became the killing method of choice for these experiments.

Calibration of the nucleic acid dyes to the test microorganism was initially investigated using different dye concentrations with the

concentration of 1 dye held constant. The test microorganism was suspended in a cell density of $3.2x10^9$ cells/ml. The results are shown in Table 14, 15, and Figures 4, and 5.

Table 14; Dilution ratios of Propidium Iodide with *E. faecalis* at a density of $3.2x10^9$cells/ml.

Dilution	Aliquot	Stain in µmol	Actual Ratio	Results
1:49	5µl	0.008	1:10	Intense
1:99	5µl	0.004	1:20	Strong
1:199	5µl	0.002	1:40	Excellent

Table 15; Dilution ratios of SYTO 9 with *E. faecalis* at a cell density of $3.2x10^9$ cells/ml.

Dilution	Aliquot	Stain in µmol	Actual Ratio	Results
1:49	5µl	0.25	1:10	Excellent
1:9	5µl	0.12	1:2	Strong
1:1	1µl	0.06	1:1	Intense

The data above indicates that a 0.25µmol of SYTO 9, and 0.002µmol for propidium iodide will result in optimal fluorescence of *E. faecalis* in a cell population of 3.2×10^9 cells/ml.

Differentiation of live and killed *E. faecalis* was investigated using populations of live and killed cells mixed in the indicated ratios. The stain concentrations were 0.25µmol for SYTO 9, and 0.002µmol for propidium iodide. The results are shown in table 16, and Figure 6.

Table 16: Results of *E. faecalis* stained in mixed population ratios with a nucleic acid stain concentration of 0.25µmol for SYTO 9 and 0.002µmol for propidium iodide.

Live Cells	Killed Cells	Results live/killed	Color
100%	0%	100/0	Green 100%
90%	10%	90/10	Green/Red
50%	50%	50/50	Green/Red
10%	90%	10/90	Green/Red
0%	100%	0/100	Red 100%

The data recorded above indicates that live and killed *E. faecalis* cells in a density of $3.2x10^9$ cells/ml can be differentiated in a mixed population using stain concentrations of 0.25µmol for SYTO 9 and 0.002µmol for propidium iodide.

Background fluorescence was evaluated by prestaining *E. faecalis* cells, and placing them with sterile bovine dentin shavings. *E. faecalis* was also placed with sterile bovine shavings and then stained with the nucleic acid dyes. Both prestained and poststained samples were examined by fluorescence microscopy. The results are seen in figures 7-9. Damaged cells were stained with both SYTO 9 and propidium iodide. Their appearance ranged between orange and red. Cells with intact membranes were stained with SYTO 9, and these cells appeared green. SYTO 9 and propidium iodide efficiently and easily differentiated live and killed cells of *E. faecalis* in a mixed population with dentinal shavings. The dentin

retained SYTO 9, but failed to intensify the signal when stimulated by a mercury lamp. The bacterial cells were easily recognized, by intense staining. This also suggested that it would be possible to recognize bacteria that were located deep within the dentin. SYTO 9 and propidium iodide both had intensified signals of green and red respectively when bound to live and killed cells.

Sterile bovine root dentin was used in this investigation. Samples were approximately 5mm tall, 4mm in diameter, and had a lumen the size of an ISO 031 round bur. The cementum and smear layer was removed as described above. The infection time was 9 days. Group 1(non-infected) and group 2(infected) were examined by fluorescence microscopy as describe above. The results are shown in figures 10 and 11. Bacterial viability and penetration into the dentinal tubules is seen on fluorescence micrographs of samples taken from the infected group. The noninfected group failed to show the presence of live or dead bacteria in the dentinal tubules. Gram staining (Figures 12 and 13) was in agreement with fluorescence findings for both groups. The dentin root model proved to be reliable for evaluation of bacterial penetration of dentinal tubules. Inconsistencies in the depth of penetration and degree of infection were noted. This was shown by deep penetration, and dense populations of bacteria in some areas while in other areas shallow penetration and light

bacterial populations were seen. SYTO 9 and propidium iodide effectively differentiated between live and killed bacteria in dentinal tubules of infected root canal dentin. A few dead bacteria could be seen in heavy populated areas. The method was fast and easy.

The second part of the investigation failed to show a correlation between histological samples and the fluorescence microscopy. The slide sections were limited in number and contained many artifacts (see fig. 14 and 15). Heavy infection was noted very deep into the root canal dentin of some samples. A gradient of infection was the trend seen beginning with a heavy infection at the sample lumen and usually terminating at a depth of approximately 500µm.

The results of 1% NaOCl, 2% IKI, and $Ca(OH)_2$ interactions with SYTO 9 and propidium iodide are shown in table 17.

Table 17: Interaction between nucleic acids and chemotheraputic agents. X1, 2,3, & 4 refers to the concentration multipling factor.

Agent	Stain Ratio X 1	Stain Ratio X 2	Stain Ratio X 3	Stain Ratio X4	Result
NaOCl	Weak	Weak	Weak	Good	Good
IKI	Excellent	X	X	X	Excellent
$Ca(OH_{)2}$	Excellent	X	X	X	Excellent

Evaluation of the interactions of chemical agents and the vital stains indicated the need to modify the concentration of the stains needed to identify the test microorganism. 2% IKI and $Ca(OH)_2$ required no change in stain concentration. The 1% buffered NaOCl produced a residual bleaching effect on the stains and required an increase in the amount of stain. The stain concentrations were increased 4 fold to overcome the bleaching action and allow identification of cells.

No samples were lost during infection. One sample from group II did break during the removal of dentinal shavings. The breakage occurred while using ISO bur 050, and the data was adjusted accordingly.

Group V was dropped from data analysis. Fluorescent evaluation was rendered non-conclusive due to the lack of visual interpretation of stain. In group V, dentinal tubules were filled with a elongated substance that was morphologically non consistent with bacteria. Penetration in group V samples were consistent with the positive control and group III. The presence of bacteria was noted in 1 of the 6 samples. The matter contained in the other samples was not determined.

This protocol provided infection of the dentinal tubules to a depth ranging from 450 micrometers to 950 micrometers or more as shown by fluorescent microscopy. The evaluation of bacterial penetration was limited to the largest bur in the armamentarium for fluorescence staining, and by the few histological sections for gram stain evaluation. All infected samples were identified as having bacterial penetration.

Evaluation of the negative control by both gram stain and vital staining showed no evidence bacterial penetration into dentinal shavings (Fig. 19-21).

The infection level and viability of the positive control are shown in figure 16. At 100μm, both samples were heavily infected and all cells appeared green (live). Between 100-200μm, infection was moderate, and again all cells appeared green. At a depth of 200-300μm, infection was low and all cells appeared green. Between 300-450μm, infection was low and all cells appeared green. In samples removed from depths between 450-950μm, bacteria were not detected (Fig. 22-24).

In group III (NaOCl), the penetration of bacteria into dentinal tubules was similar to the positive control. The results of bacterial infection, penetration, and viability are shown in figure 17. At 100μm,

the infection was rated heavy in 5/6 samples and moderate in 1/6. Viability was 0% red in 5/6 samples as shown by the absence of green stain, and 5% red in 1. In samples removed from depths between 100-200μm, infection was moderate in 4/6 samples and mild in 2/6 samples. Viability was 50% in 4/6 samples and 100% green in 2/6 samples. At depths between 200-300μm, infection was low in all samples, and viability was 100% in 5/6 samples and 90% in 1/6 samples. At a depth between 300-450μm, infection was low in 5/6 samples, and no infection was seen in 1. Viability was 100% in all samples. Between 450-550, only one sample contained 1 cell that was vital. None of the remaining samples showed bacterial penetration beyond 450μm (Fig. 25-27).

Group IV (IKI) showed a much deeper penetration of bacteria than the other groups. The reason in not clear. The results of bacterial infection, penetration, and viability are shown in figure 18. At 100μm, infection was heavy in 4/6 samples and moderate in 2/6 samples. Viability was 0% in all samples. Between 100-200μm, 3/6 samples were moderately infected, 2/6 were mildly infected, and 1/6 samples was low. Viability was 0% in 2/6 samples, 10% red in 2/6 samples, 30% in 1/6 samples, and 50% in the last sample. At depths between 200-300μm, infection was mild in 2/6 and low in 4/6 samples.

Viability was 0% red in 1/6, and 50% in 5/6 samples. At depths between 300-450μm, infection was low in all samples, and viability was 0% in 1/6, and 50% in 5/6 samples. At depths between 450-550μm, infection was low in all samples. Viability was 10% in 1/6, and 50% in 5/6 samples. At depths between 550-700μm, infection was low in all samples except one failed to show any infection. Viability was 10% in 1/6 samples, and 50% in 4/6. Depths between 700-800μm, infection was low in 4/6 samples. Two samples failed to show bacterial infection. Viability was 0% red in 1/6, and 50% red in 3/6. At depths between 800-950μm, infection was low in 3/6 samples, two samples failed to show bacterial infection and one sample broke. Viability was 0% in one, 10% in one, and 50% in one.

Two-way analysis was used to compare groups 3 and 4 at levels between 100-450μm. A significant difference was found at $p<0.001$. This suggest that 2% IKI had a more significant killing effect on *E. faecalis* at these depths than 1% buffered NaOCL.

Only in the IKI group did bacterial penetration extend beyond 450 micrometers. The infection level was mild to none between 300 micrometers and 950 micrometers for all infected groups (Fig. 20-21).

The data recorded during testing of groups 1, 3, and 4 are listed in tables 18-20.

Table 18; Data recorded from positive control

Sample	1			2		
Depth	infection	% Green	% Red	infection	% Green	% Red
100µm	4	100	0	4	100	0
200µm	3	100	0	3	100	0
300µm	1	100	0	1	100	0
450µm	1	100	0	1	100	0
550µm	0	N/A	N/A	0	N/A	N/A
700µm	0	N/A	N/A	0	N/A	N/A
800µm	0	N/A	N/A	0	N/A	N/A
950µm	0	N/A	N/A	0	N/A	N/A

Table 19; Data recorded from group III (NaOCl).

Penetration in µm	Sample	Infection	% Green	% Red
100	1	4	100	0
100	2	4	100	0
100	3	4	100	0
100	4	4	100	0
100	5	4	100	0
100	6	3	100	0
200	1	3	50	50
200	2	3	50	50
200	3	2	50	50
200	4	3	100	0
200	5	3	100	0
200	6	2	50	50
300	1	1	100	0

300	2	1	100	0
300	3	1	90	10
300	4	1	100	0
300	5	1	100	0
300	6	1	100	0
450	1	1	100	0
450	2	1	100	0
450	3	1	100	0
450	4	1	100	0
450	5	1	100	0
450	6	0	N/A	N/A
550	1	0	N/A	N/A
550	2	0	N/A	N/A
550	3	0	N/A	N/A
550	4	0	N/A	N/A
550	5	0	N/A	N/A
550	6	1	100	0
700	1	0	N/A	N/A
700	2	0	N/A	N/A
700	3	0	N/A	N/A
700	4	0	N/A	N/A
700	5	0	N/A	N/A
700	6	0	N/A	N/A
800	1	0	N/A	N/A
800	2	0	N/A	N/A

800	3	0	N/A	N/A
800	4	0	N/A	N/A
800	5	0	N/A	N/A
800	6	0	N/A	N/A
950	1	0	N/A	N/A
950	2	0	N/A	N/A
950	3	0	N/A	N/A
950	4	0	N/A	N/A
950	5	0	N/A	N/A
950	6	0	N/A	N/A

Table 20; Data recorded for group IV (IKI).

Penetration in μm	Sample	Infection	% Green	% Red
100	1	4	0	100
100	2	3	0	100
100	3	3	0	100
100	4	4	0	100
100	5	4	0	100
100	6	4	0	100
200	1	3	30	70
200	2	1	90	10
200	3	2	0	100
200	4	2	50	50
200	5	3	0	100

200	6	3	10	90
300	1	2	50	50
300	2	1	50	50
300	3	1	0	100
300	4	1	50	50
300	5	2	50	50
300	6	1	50	50
450	1	1	50	50
450	2	1	50	50
450	3	1	0	100
450	4	1	50	50
450	5	1	50	50
450	6	1	50	50
550	1	1	50	50
550	2	1	50	50
550	3	1	10	90
550	4	1	50	50
550	5	1	50	50
550	6	1	50	50
700	1	1	50	50
700	2	1	50	50
700	3	1	10	90
700	4	1	50	50
700	5	1	50	50
700	6	0	N/A	N/A

800	1	1	50	50
800	2	0	N/A	N/A
800	3	1	0	100
800	4	1	50	50
800	5	1	50	50
800	6	0	N/A	N/A
950	1	1	50	50
950	2	0	N/A	N/A
950	3	1	10	90
950	4	1	0	100
950	5	N/A	N/A	N/A
950	6	0	N/A	N/A

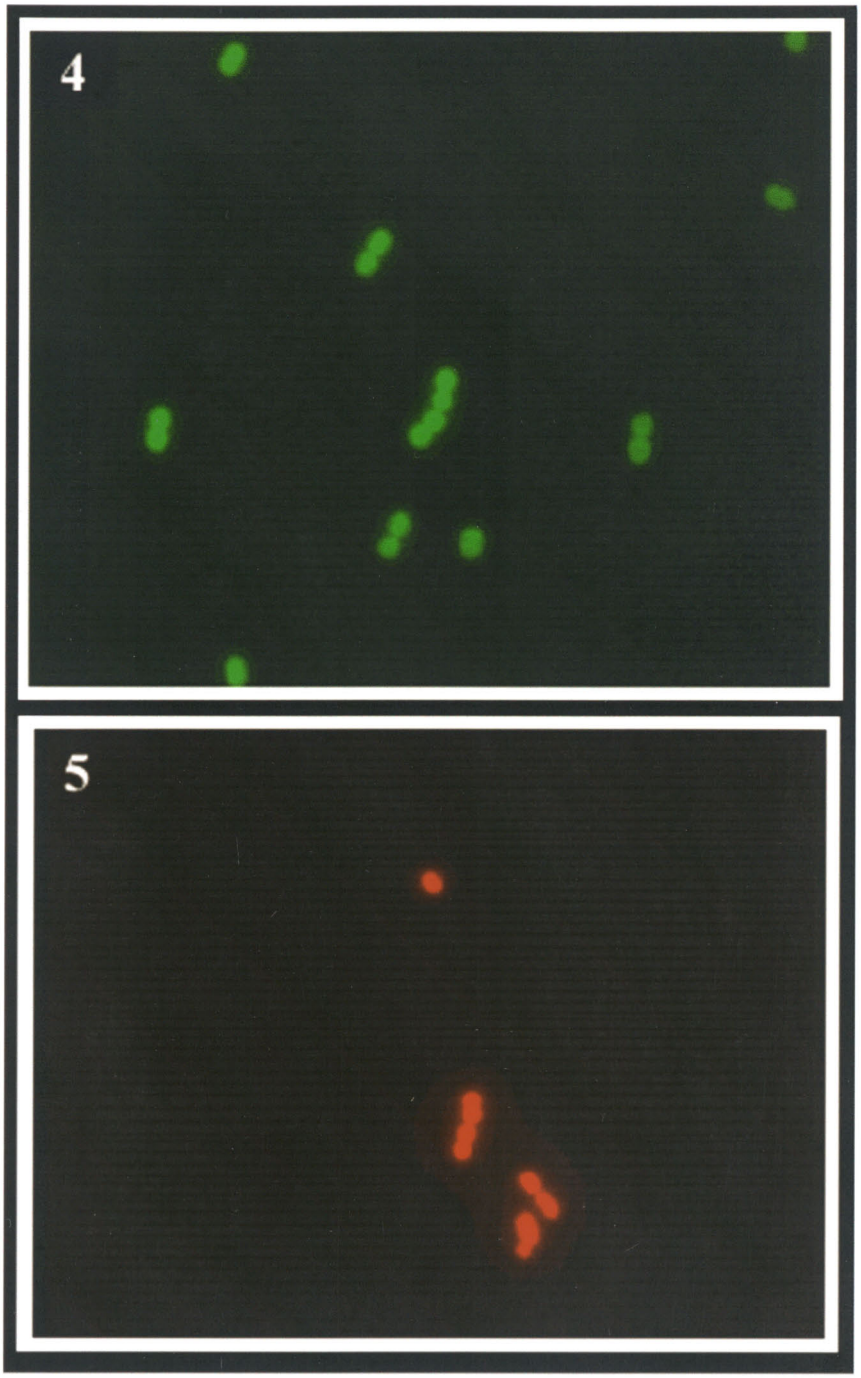


Fig. 4; Log phase E. faecalis cells stained with SYTO 9 and PI: Fig. 5; 70% isopropyl alcohol killed E. faecalis cells stained with SYTO 9 and PI.

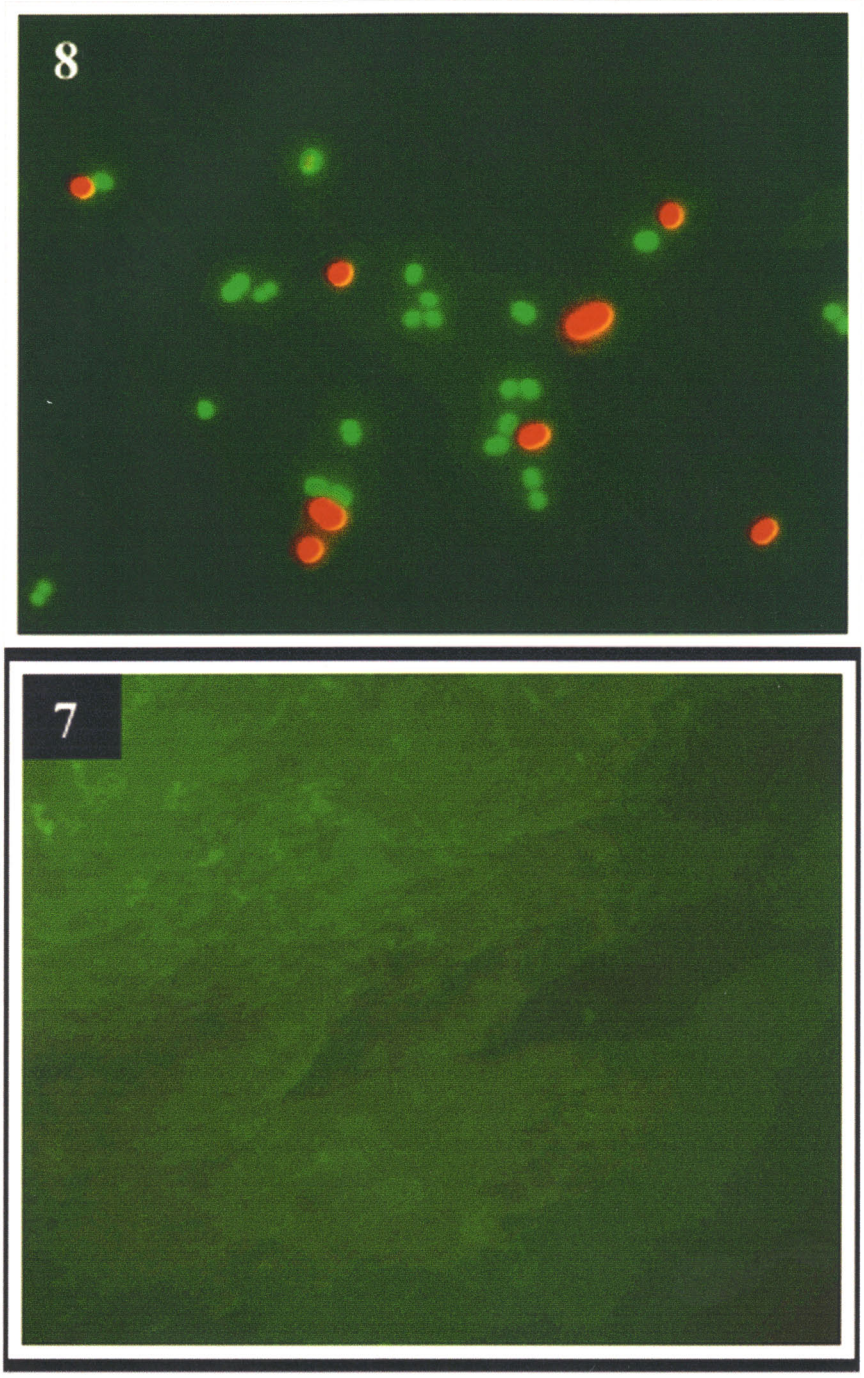


Fig. 6; 50/50 mixed population of live and killed E. faecalis cells stained with both SYTO 9 and PI:
Fig. 7; Sterile bovine dentin stained with SYTO 9 and PI.

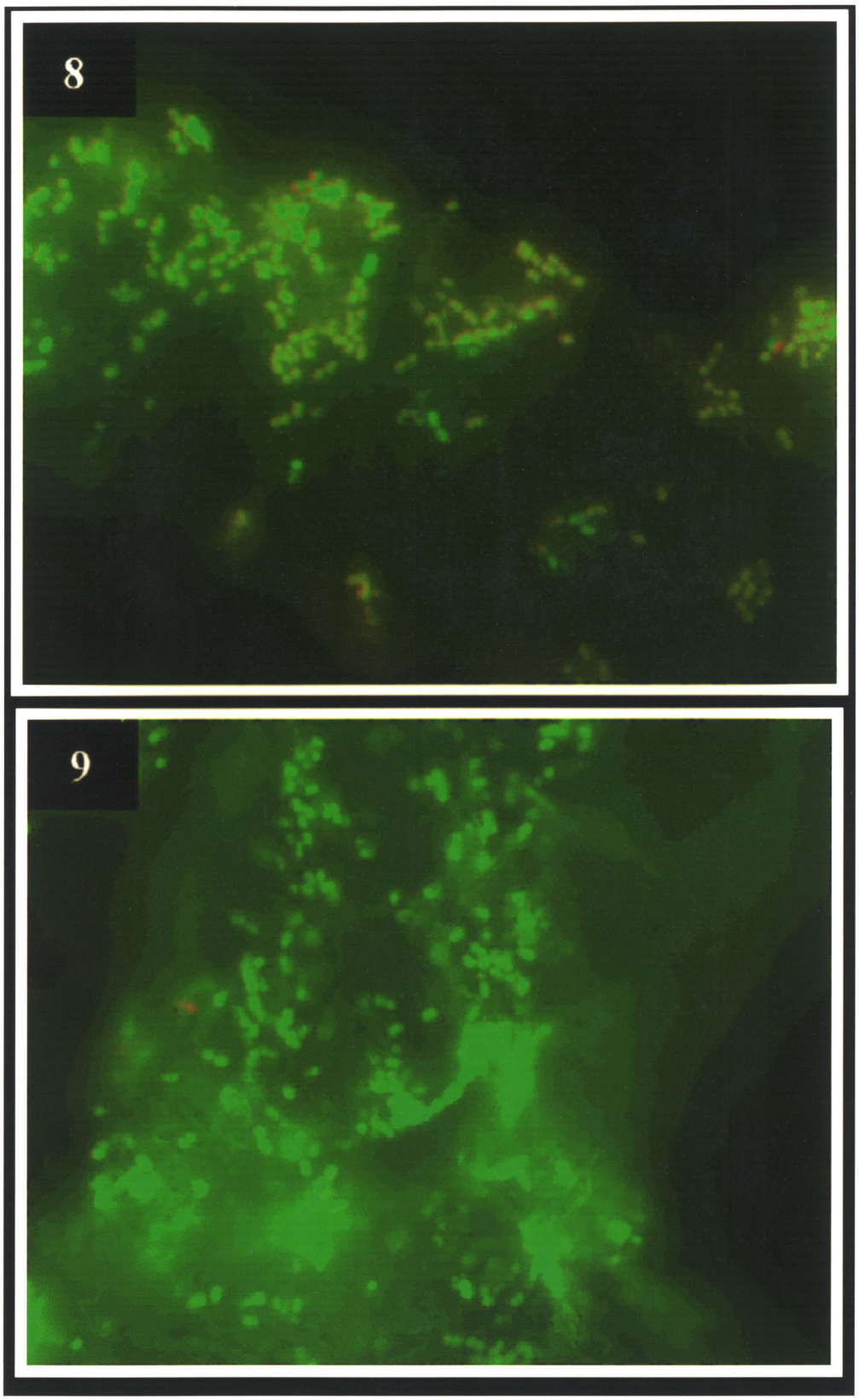


Fig. 8; Prestained E. faecalis cells placed with sterile bovine dentin: Fig. 9; E. faecalis cells placed in bovine dentin and then stained with SYTO 9 and PI

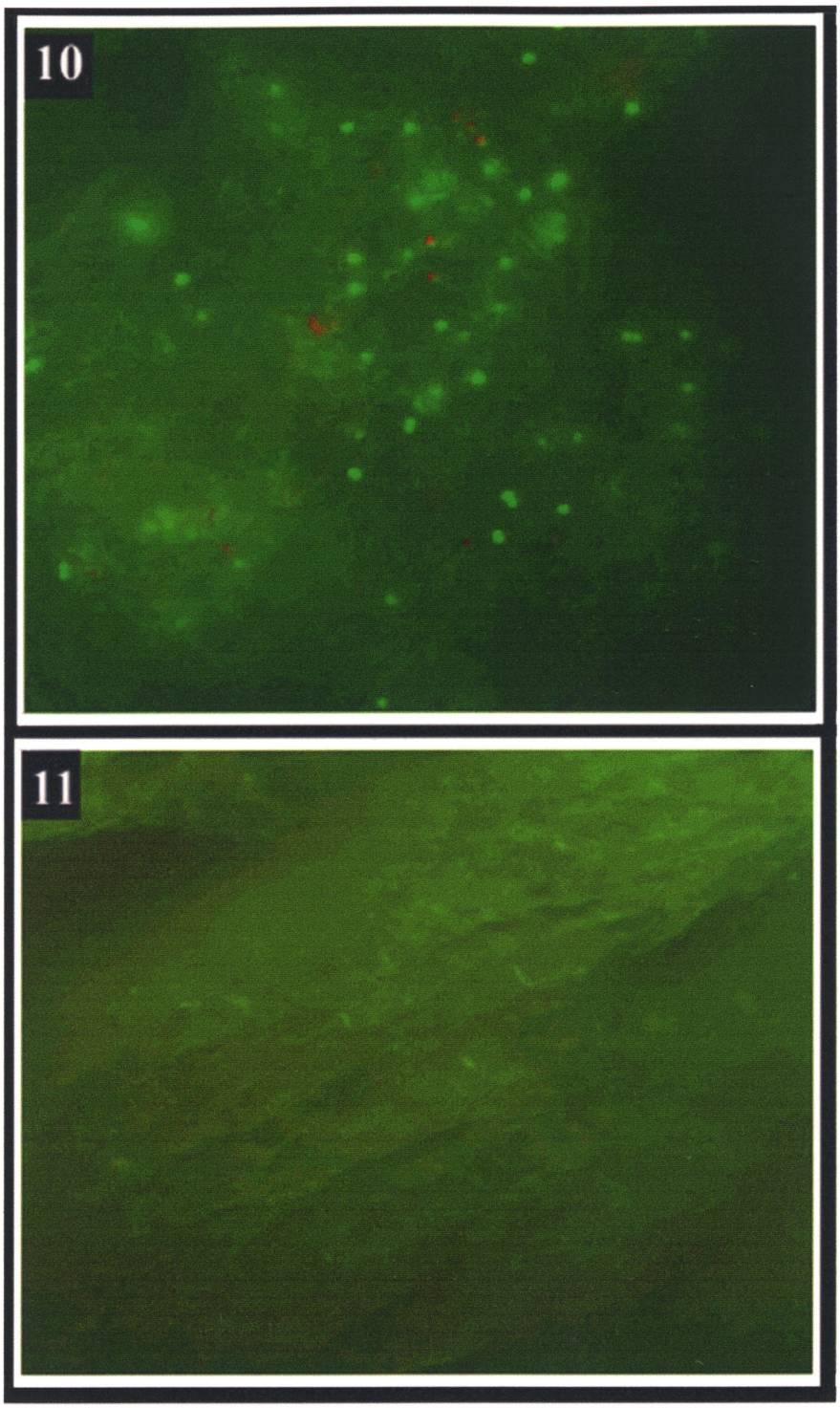
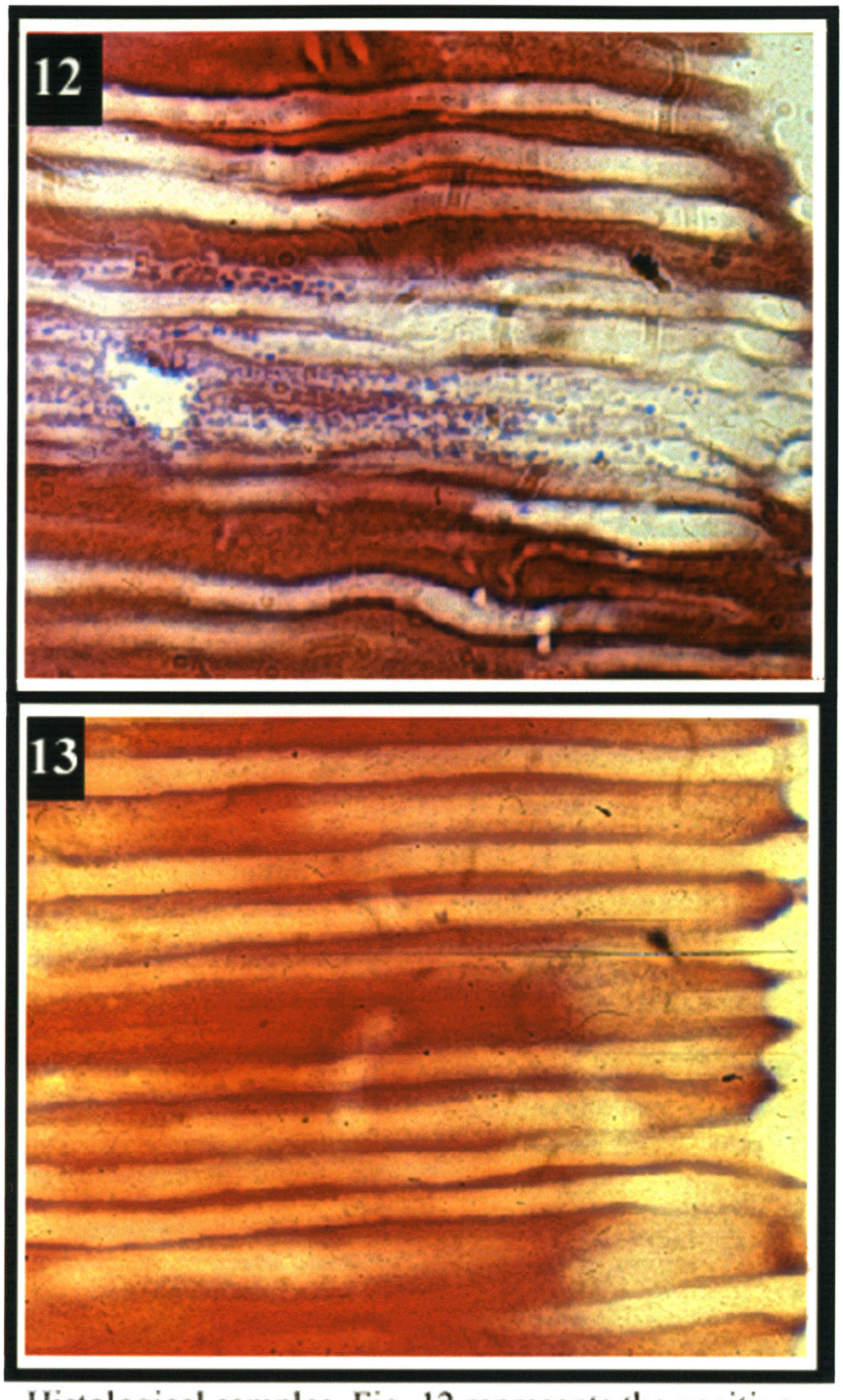


Fig. 10; Fluorescence of infected group showing live and dead E. faecalis cells.
Fig. 11; noninfected group.



Histological samples. Fig. 12 represents the positive control group with gram stained E. faecalis cells. Fig. 13 represents the negative control group showing no bacterial penetration of bacteria.

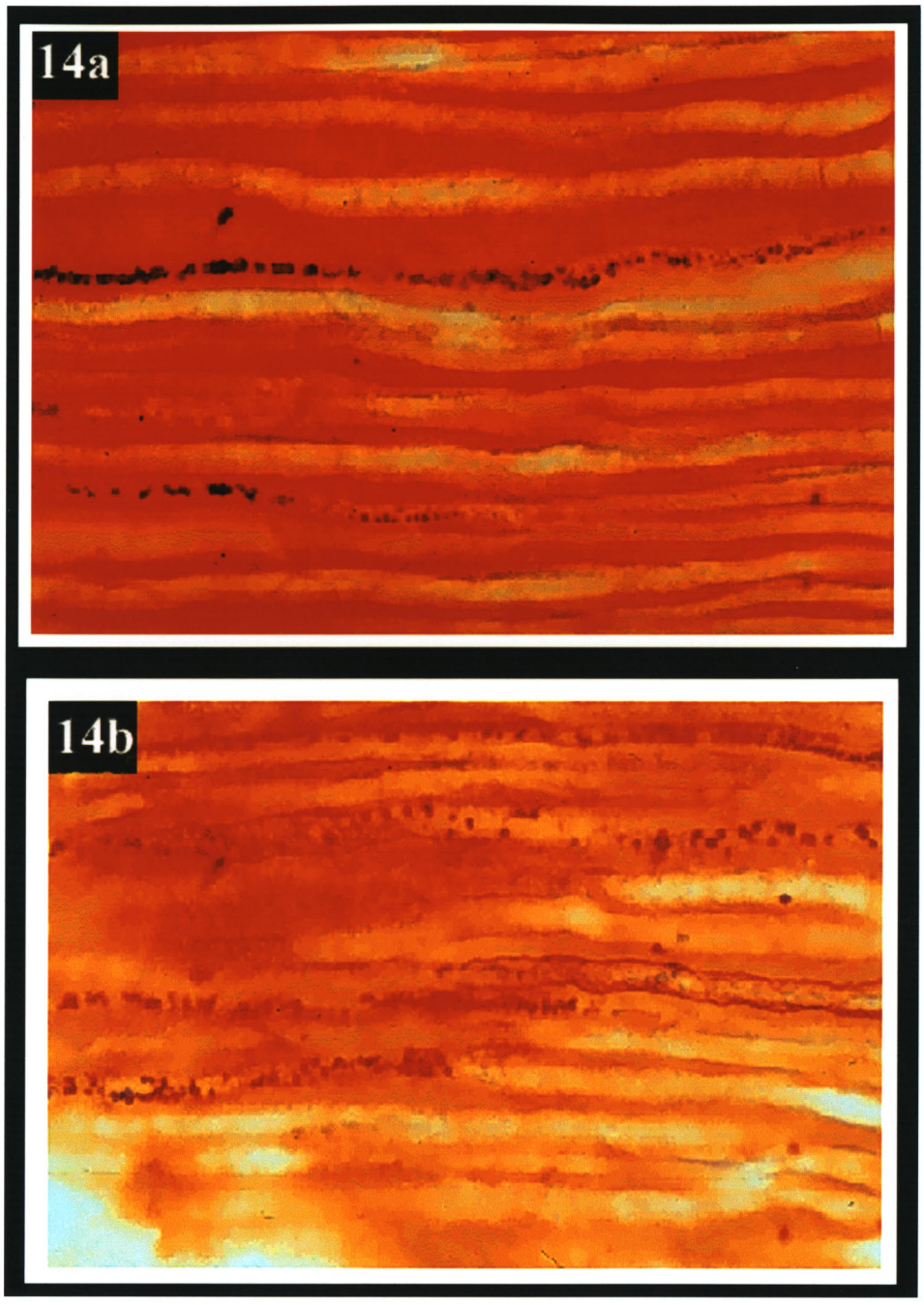


Fig. 14; 14a=Histological section of infected group 3:
14b=Histological section of infected group 4.

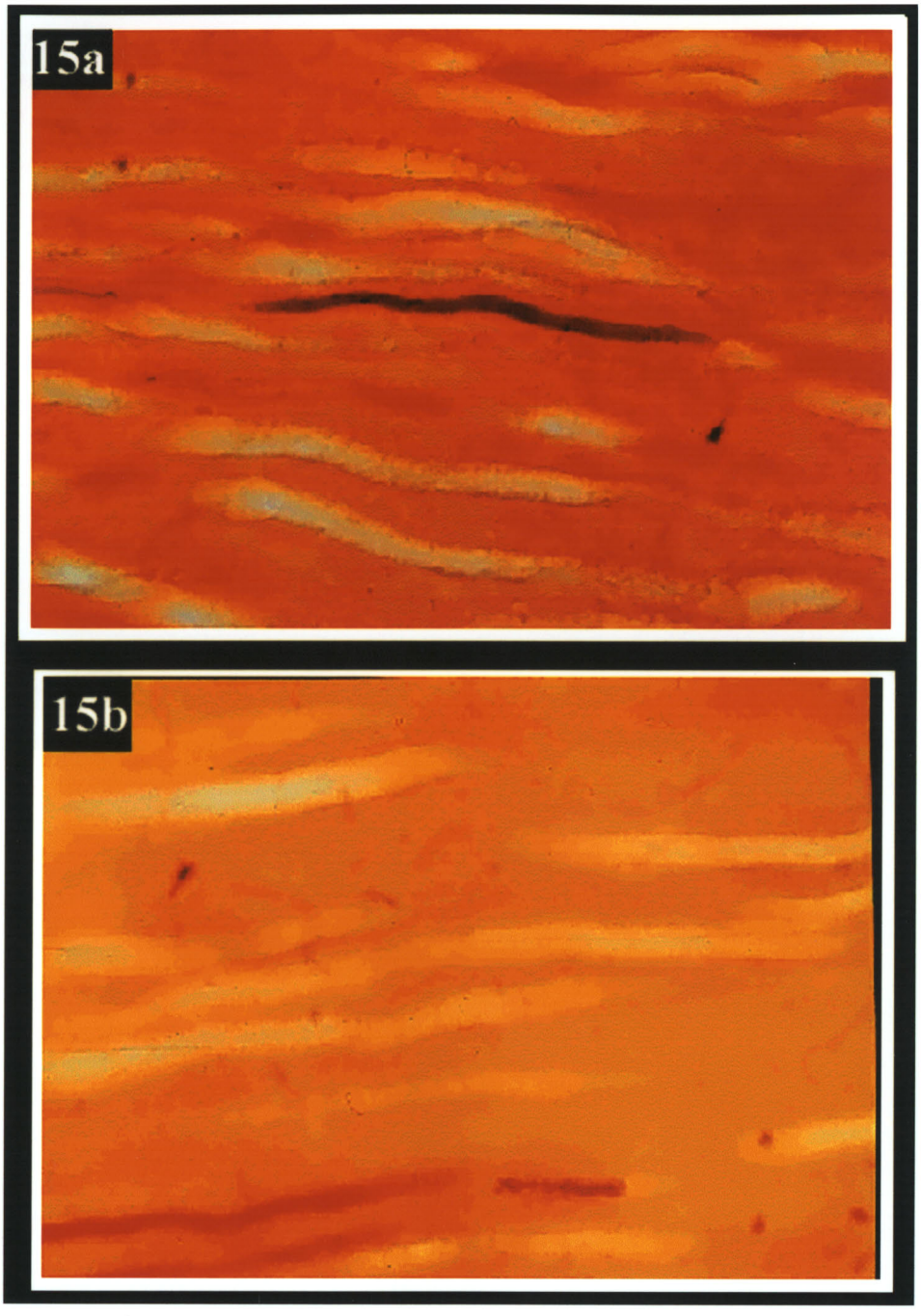


Fig. 15; 15a & 15b=Histological sections showing artifacts in noninfected group 2.

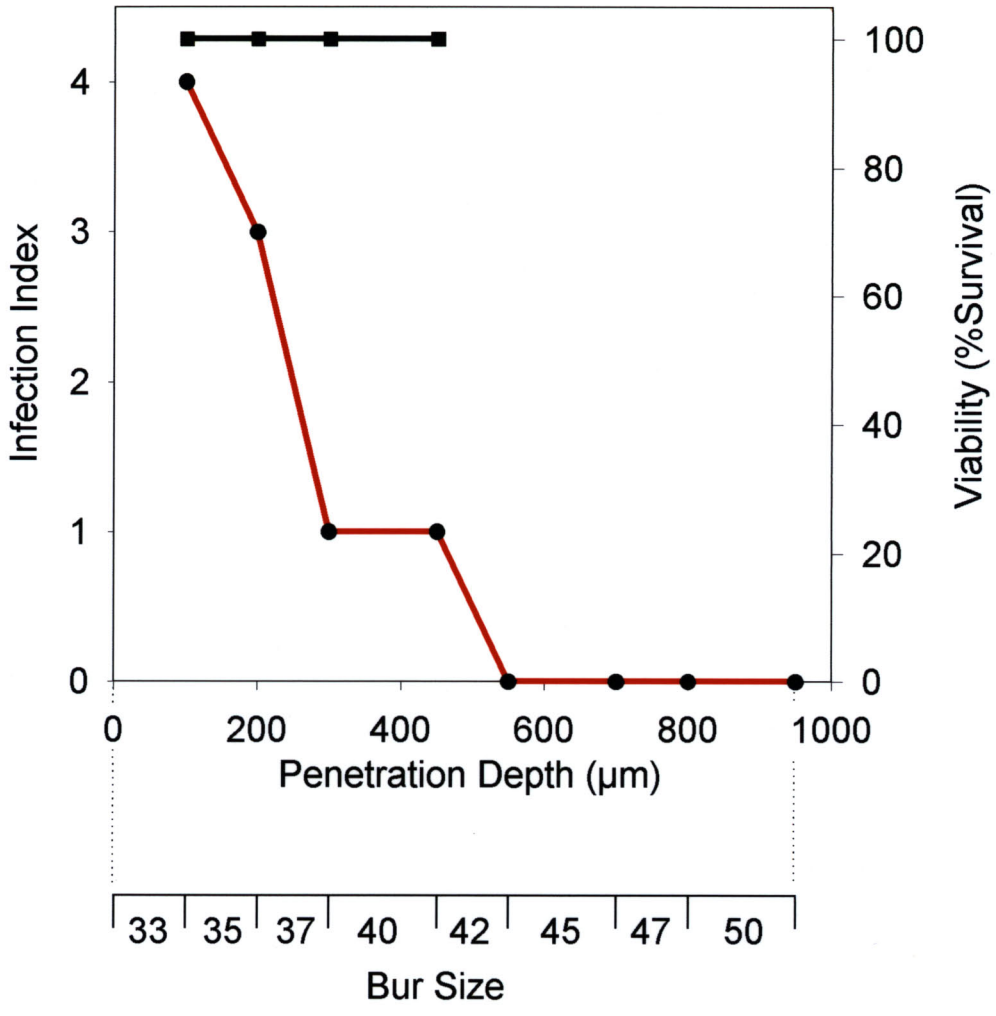


Fig. 16; Graph using the standard error of the mean for infectivity, bacterial penetration, and cell viability of group 1 (Positive control).

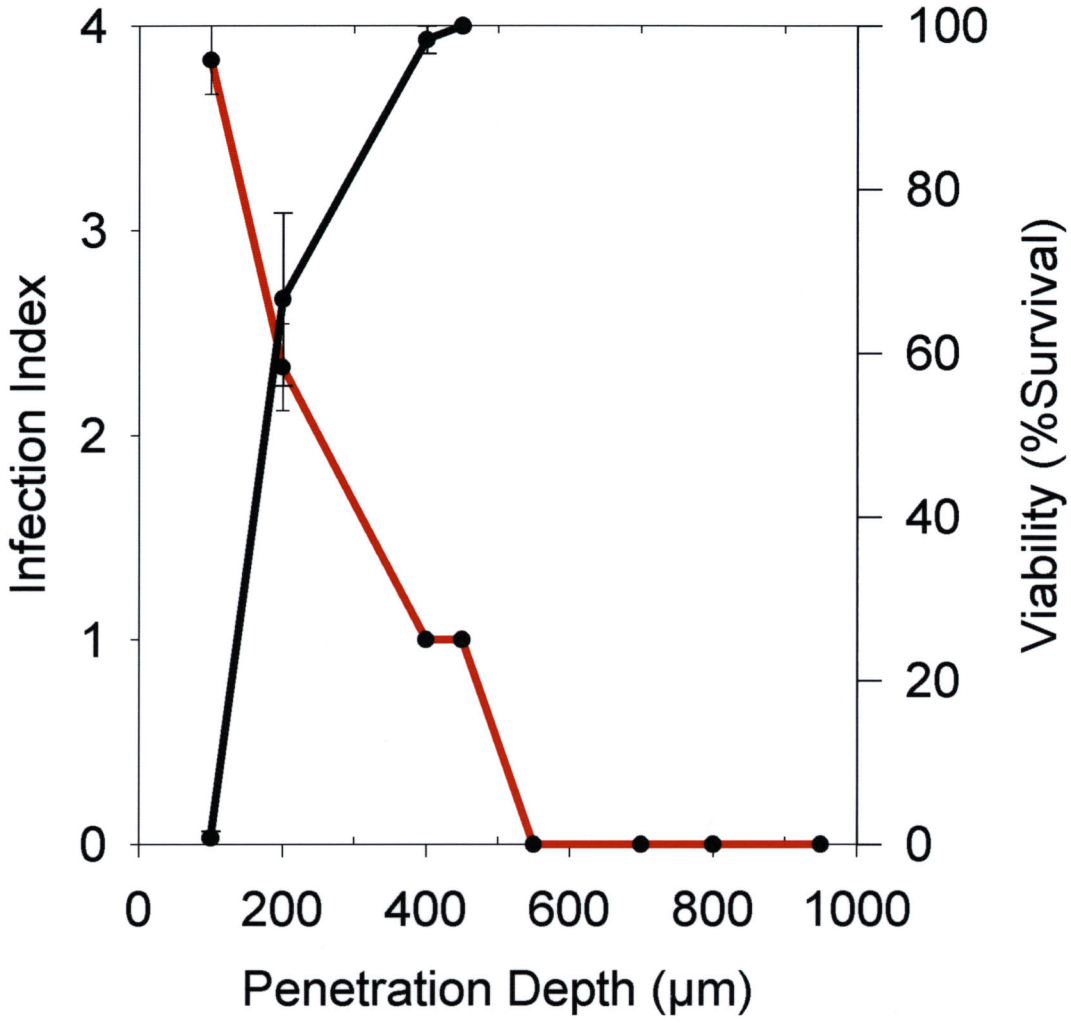


Fig. 17; Graph using the standard error of the mean for infectivity, bacterial penetration, and cell viability of group 3 (NaOCl).

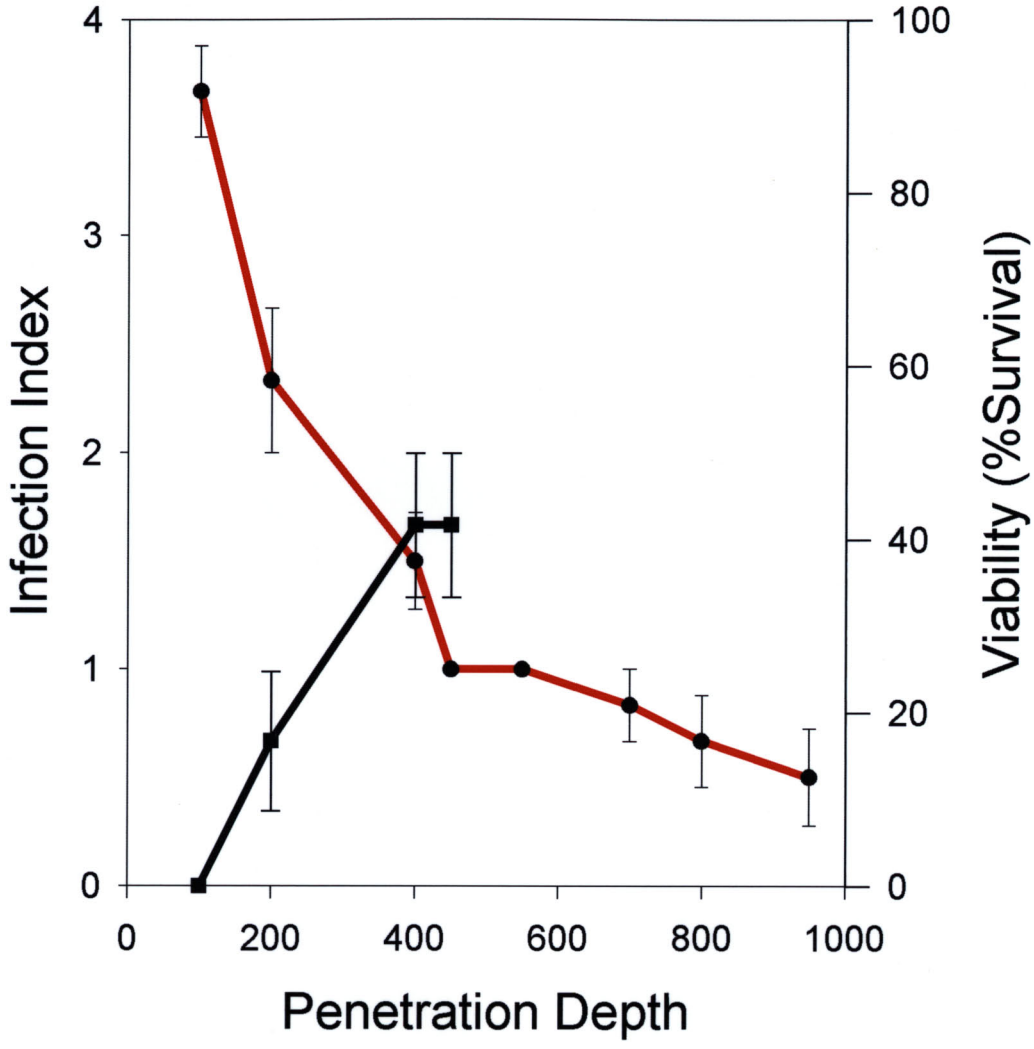
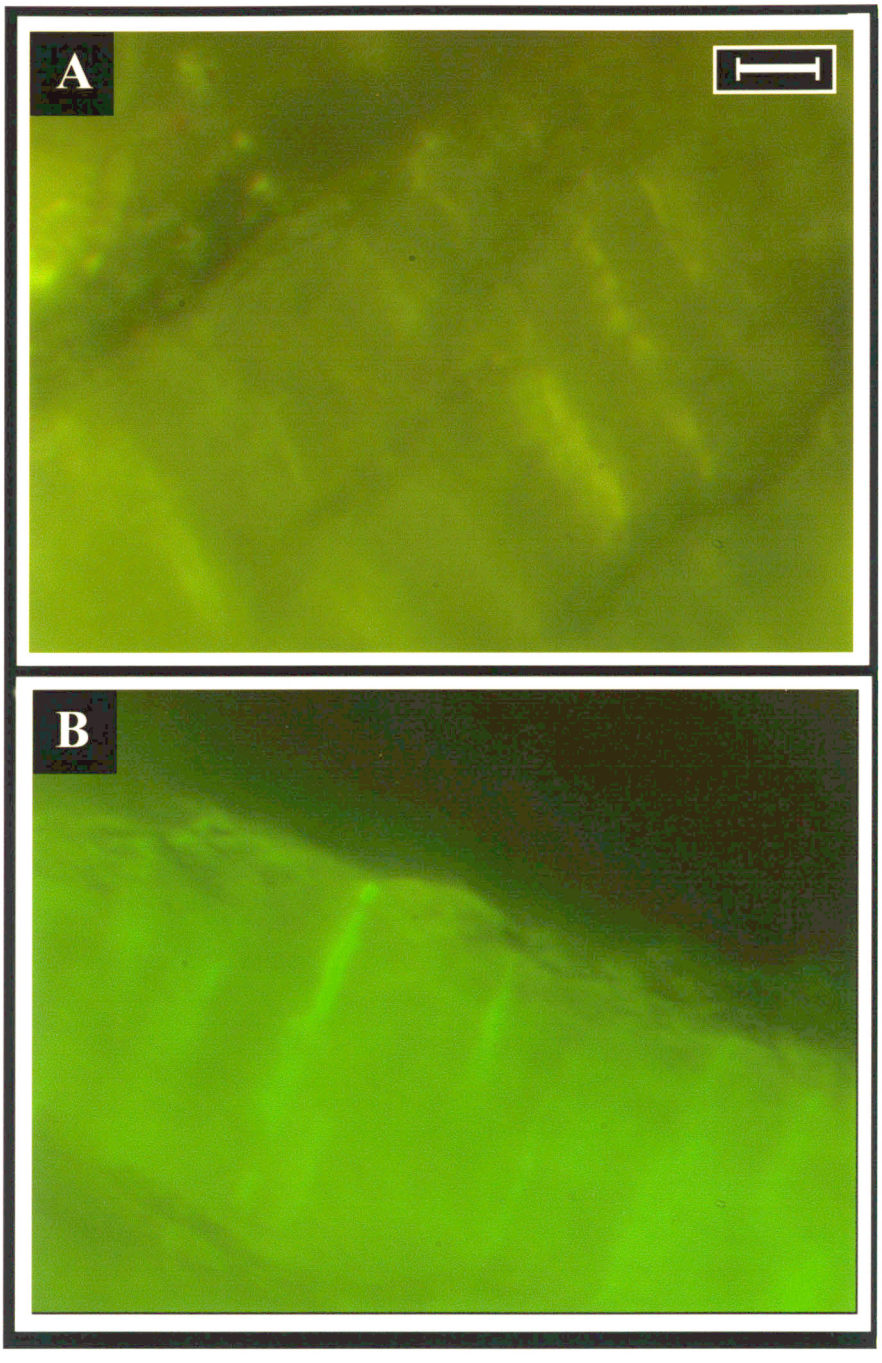
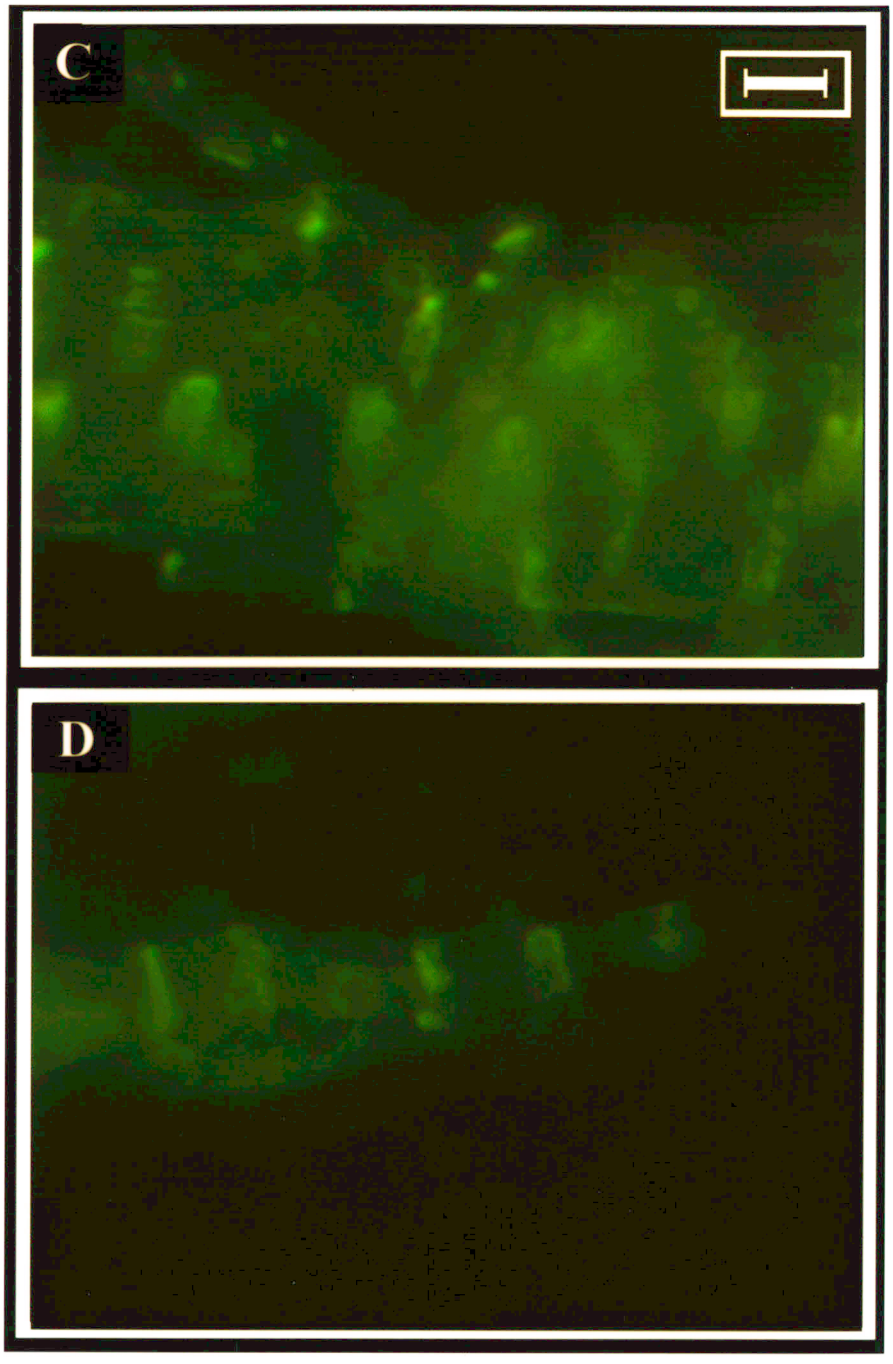


Fig. 18; Graph using the standard error of the mean for infectivity, bacterial penetration, and cell viability of group 4 (IKI).



Fig, 19; A=Negative control at a depth of 100µm:
B=Negative control at a depth of 100-200µm.



Fig, 20; C=Negative control at a depth of 200-300µm:
D=Negative control at a depth of 300-450µm.

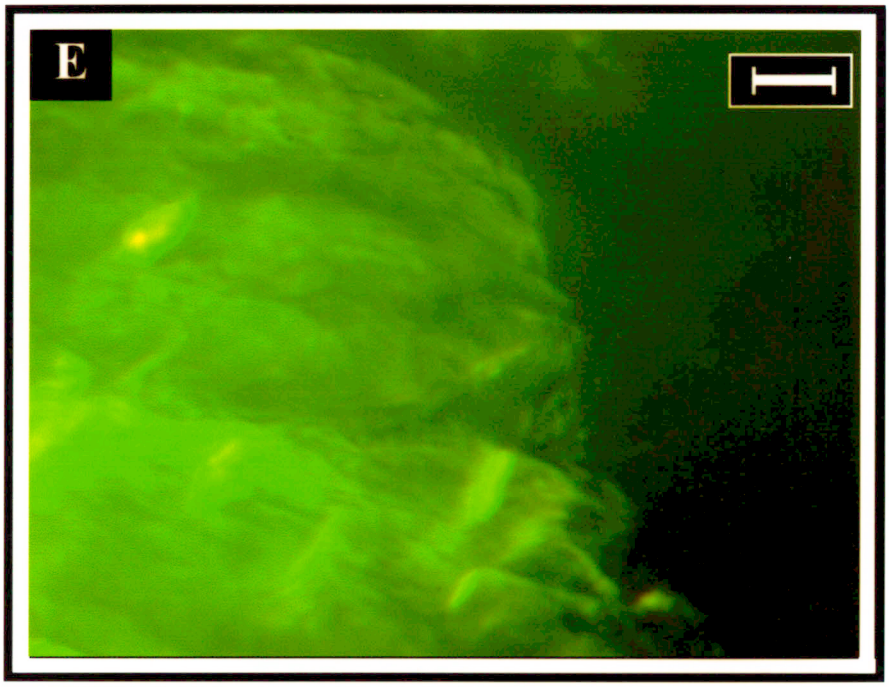


Fig. 21; E=Negative control at a depth of 450-550µm.

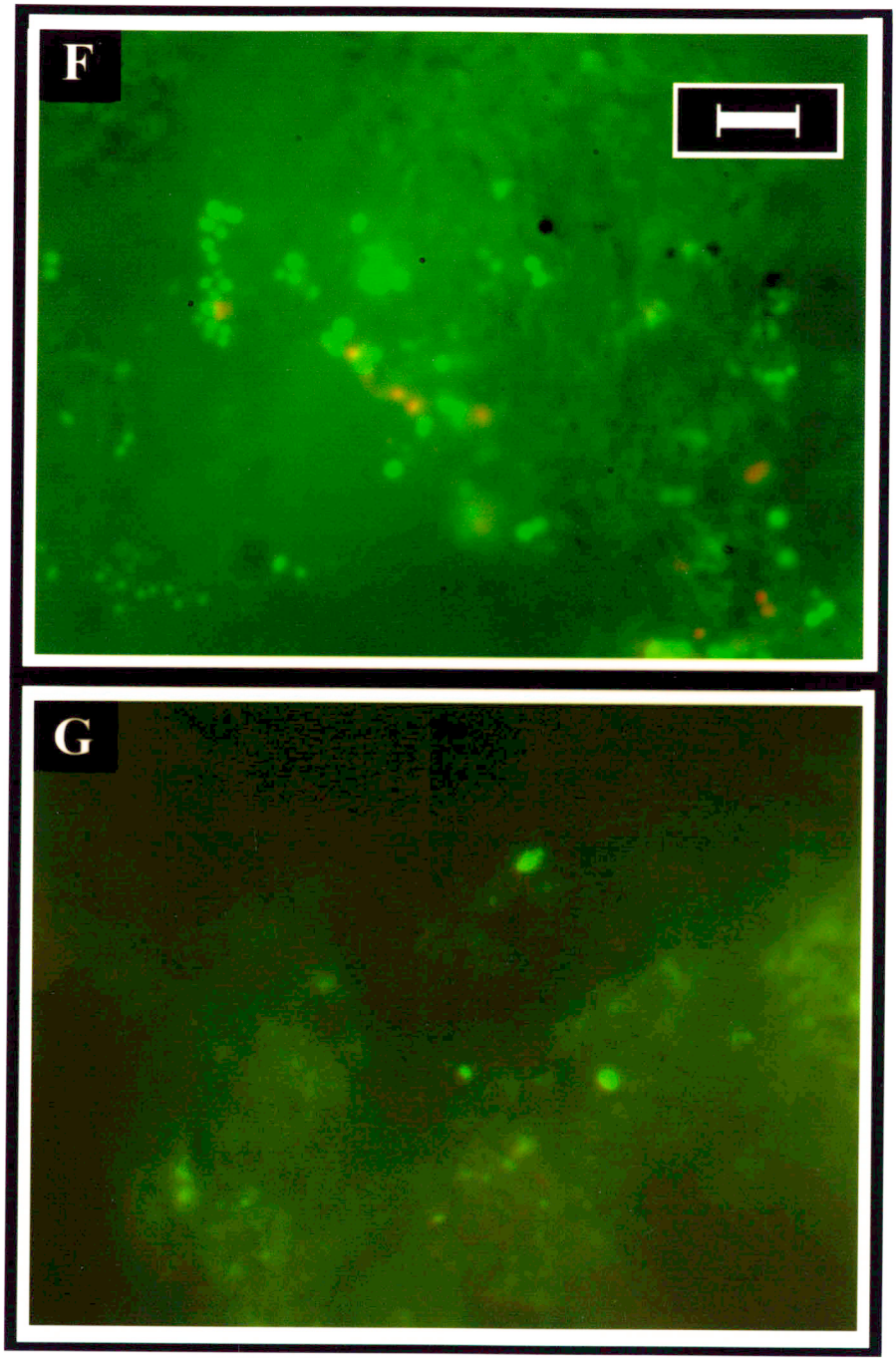


Fig. 22; F=Positive control at a depth of 100μm:
G=Positive control at a depth of 100-200μm

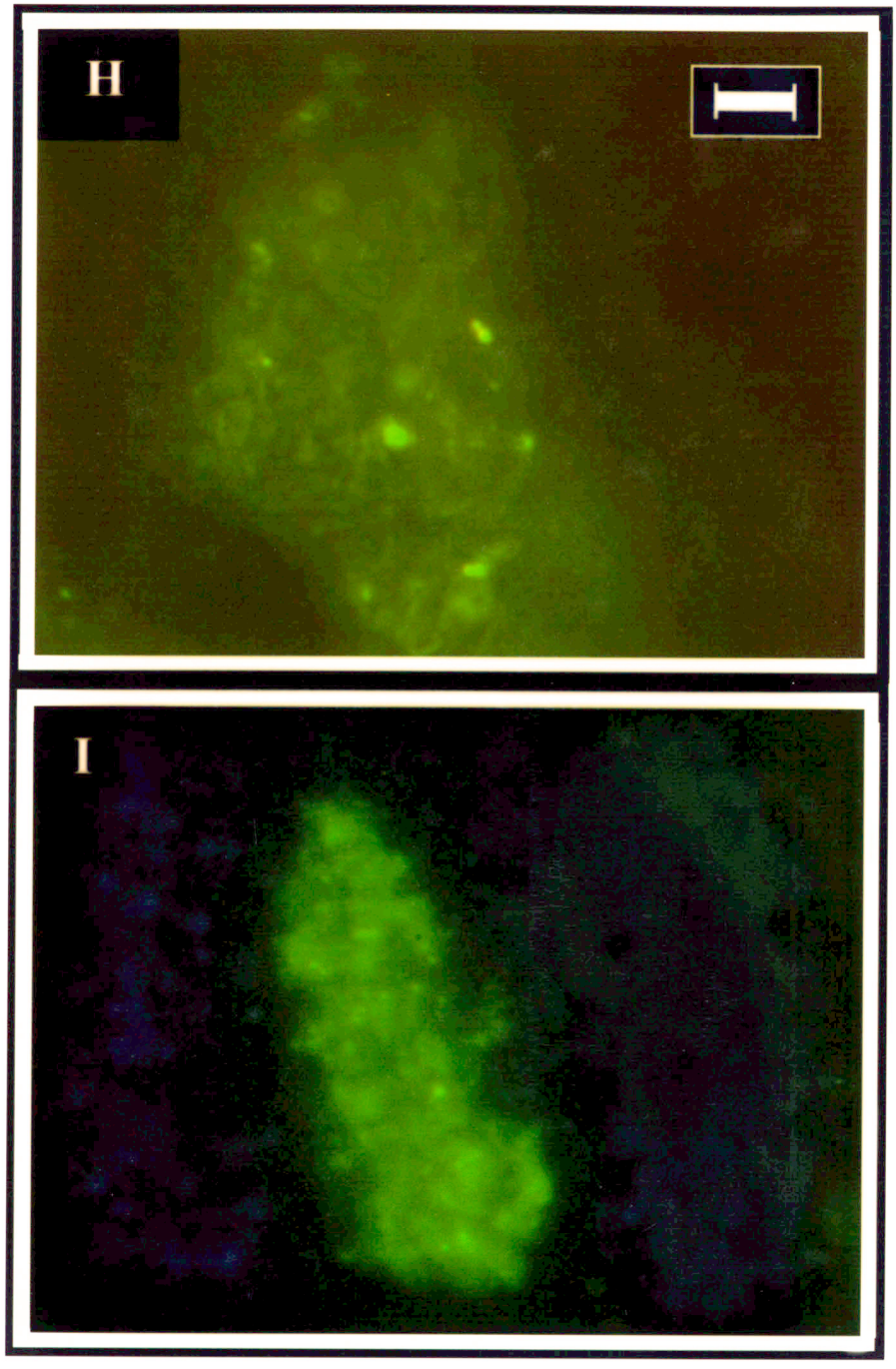


Fig. 23; H=Positive control at a depth of 200-300μm:
I=Positive control at a depth of 300-450μm

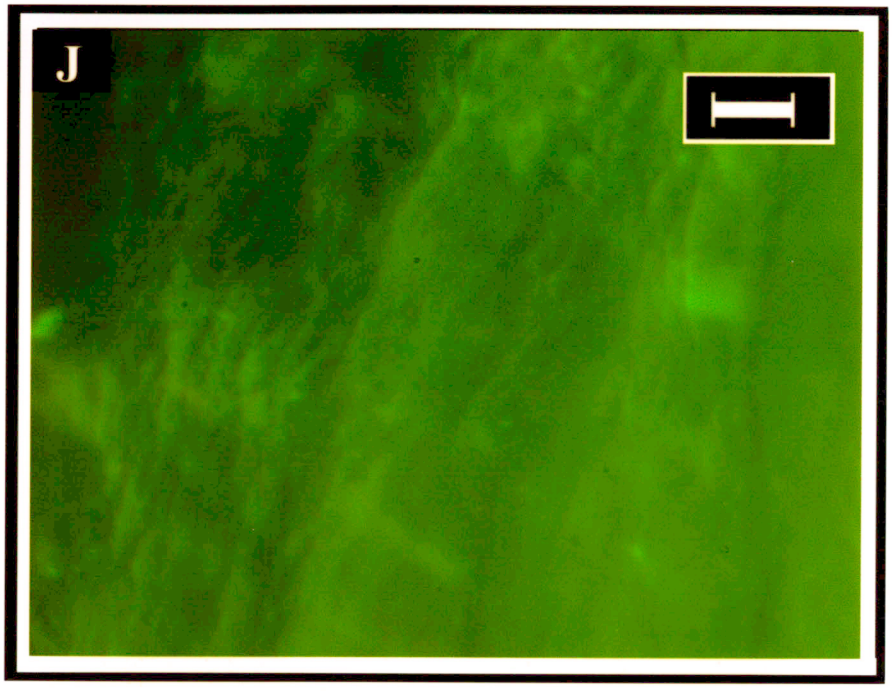


Fig. 24; J=Positive control at adepth of 450-550µm.

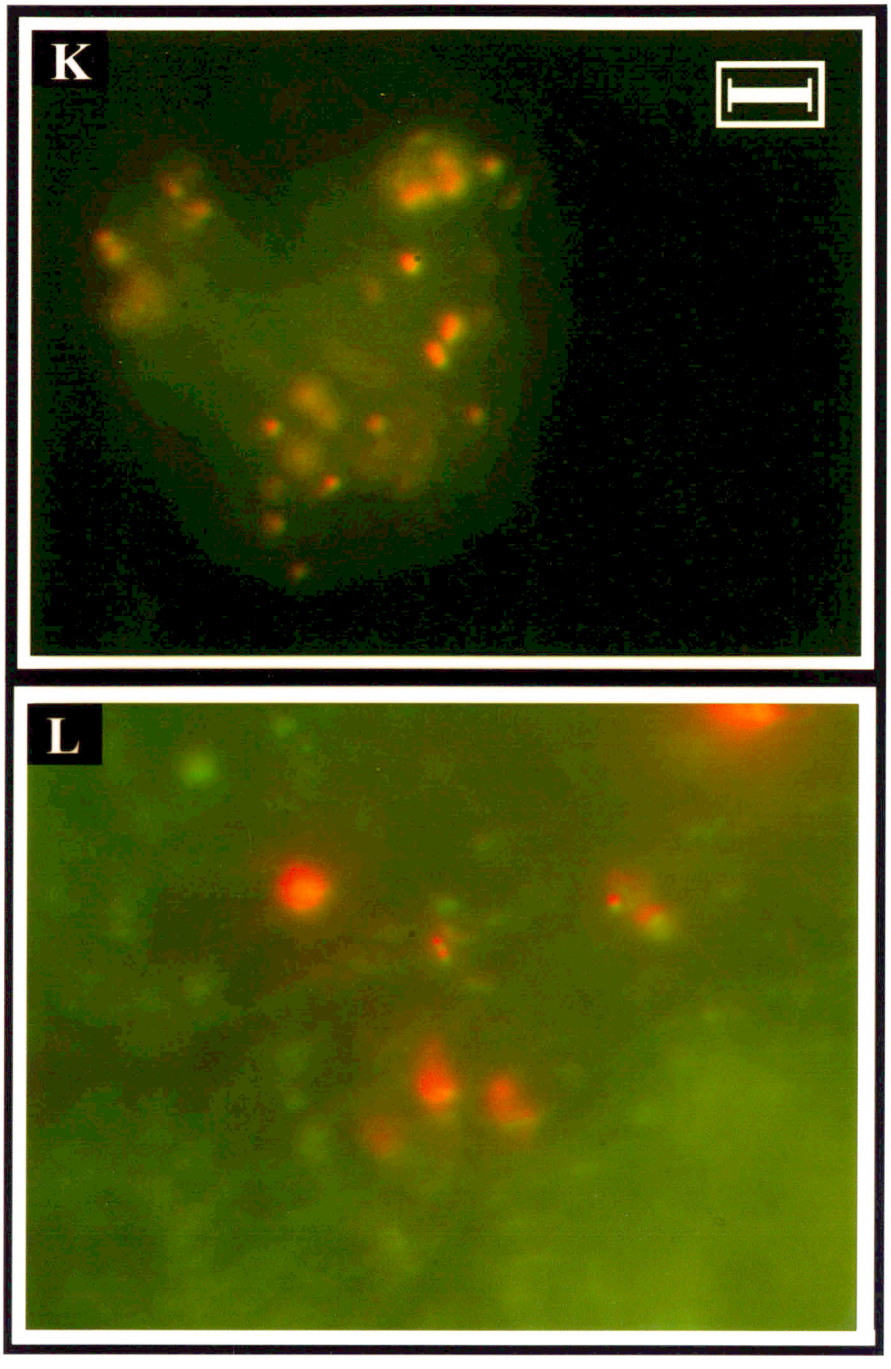


Fig. 25; K=1% NaOCl at a depth of 100μm:
L=1%NaOCl at a depth of 100-200μm.

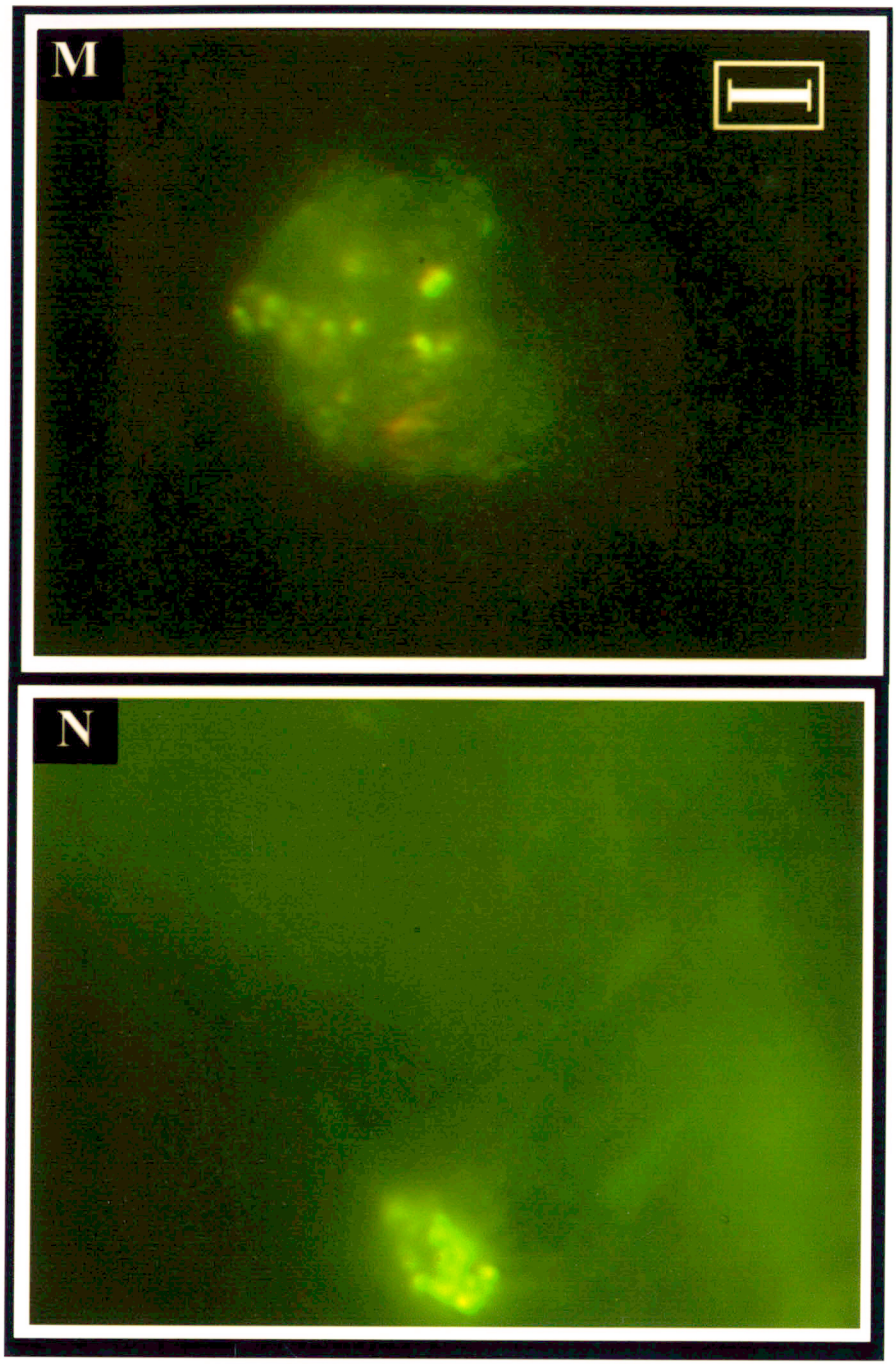


Fig. 26; M=1% NaOCl at a depth of 200-300μm:
N=1% NaOCl at a depth of 300-450μm.

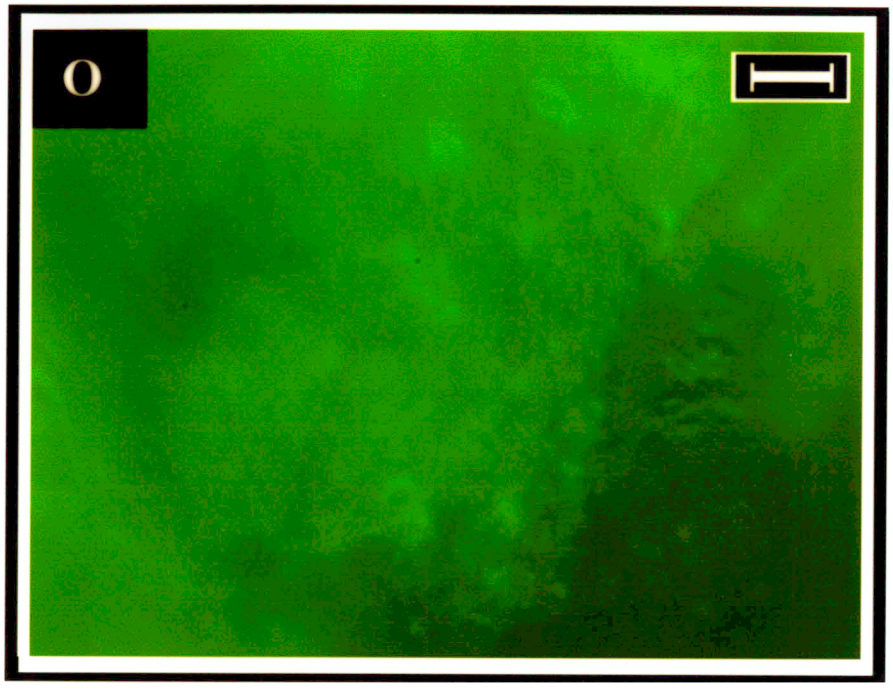


Fig. 26; O=1% NaOCl at a depth of 450-550μm.

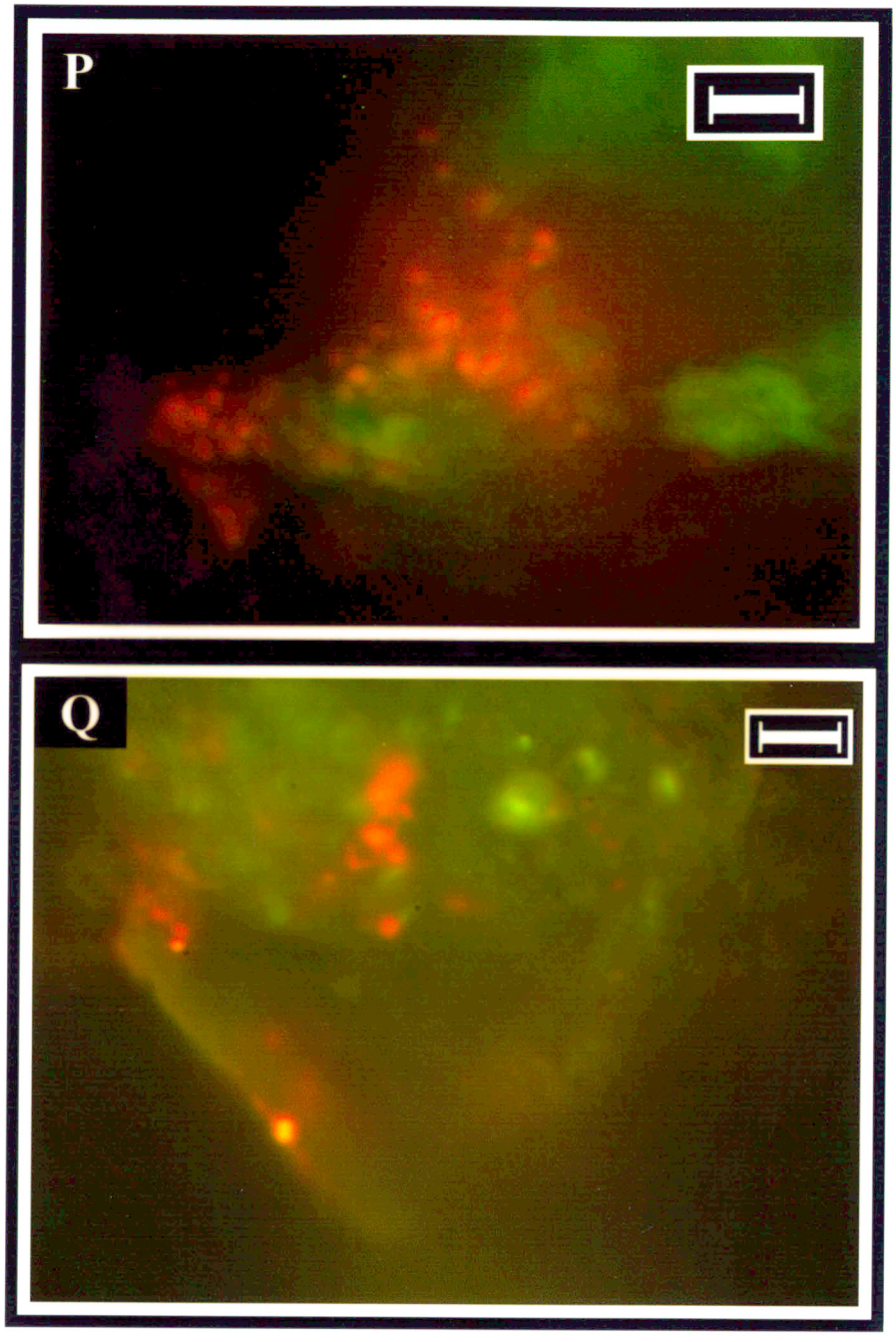


Fig. 28; P= 2% IKI at a depth of 100µm:
Q= 2% IKI at a depth of 100-200µm.

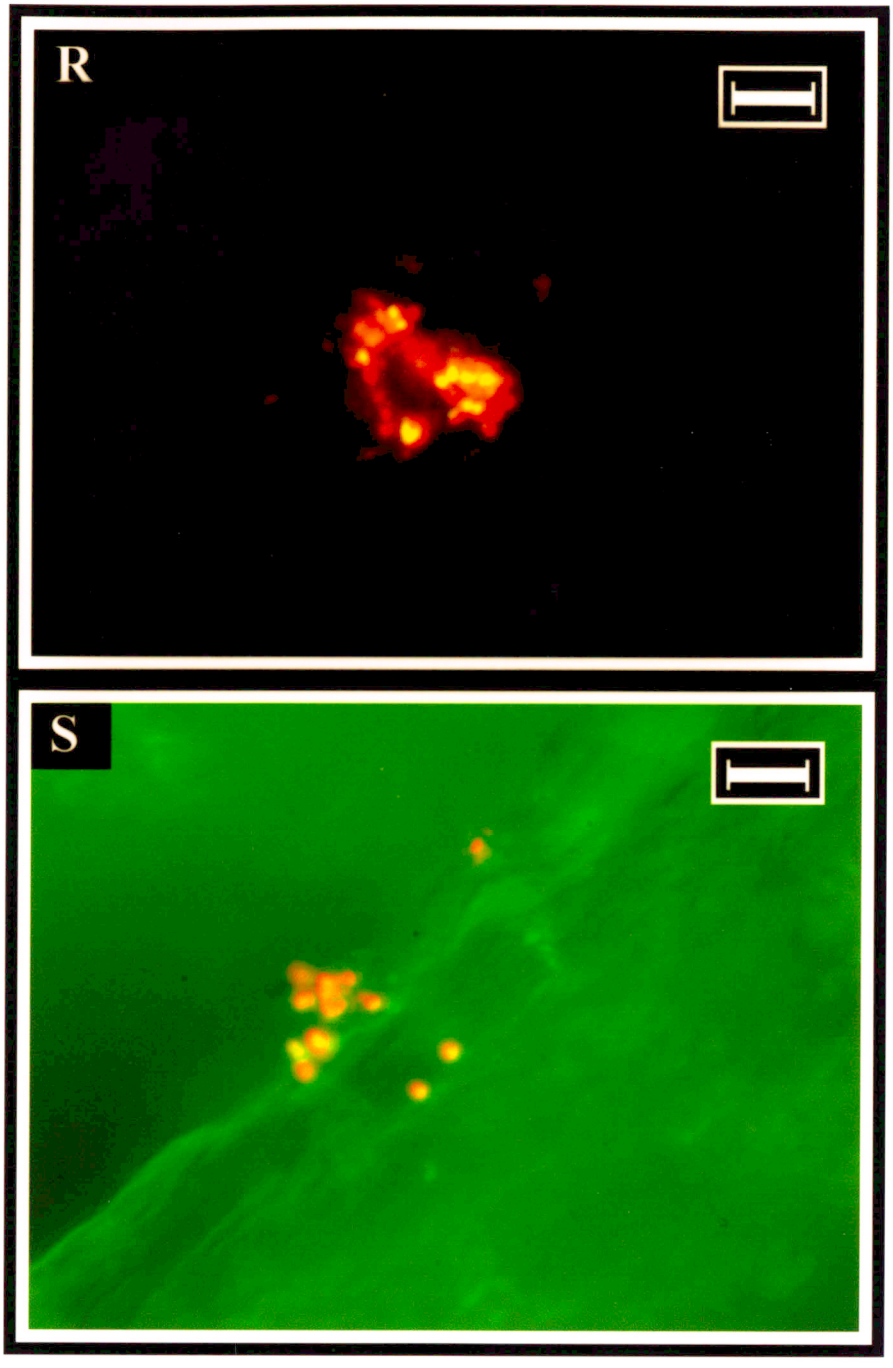


Fig. 29; R=2% IKI at a depth of 200-300μm:
S=2% IKI at adepth of 300-450μm.

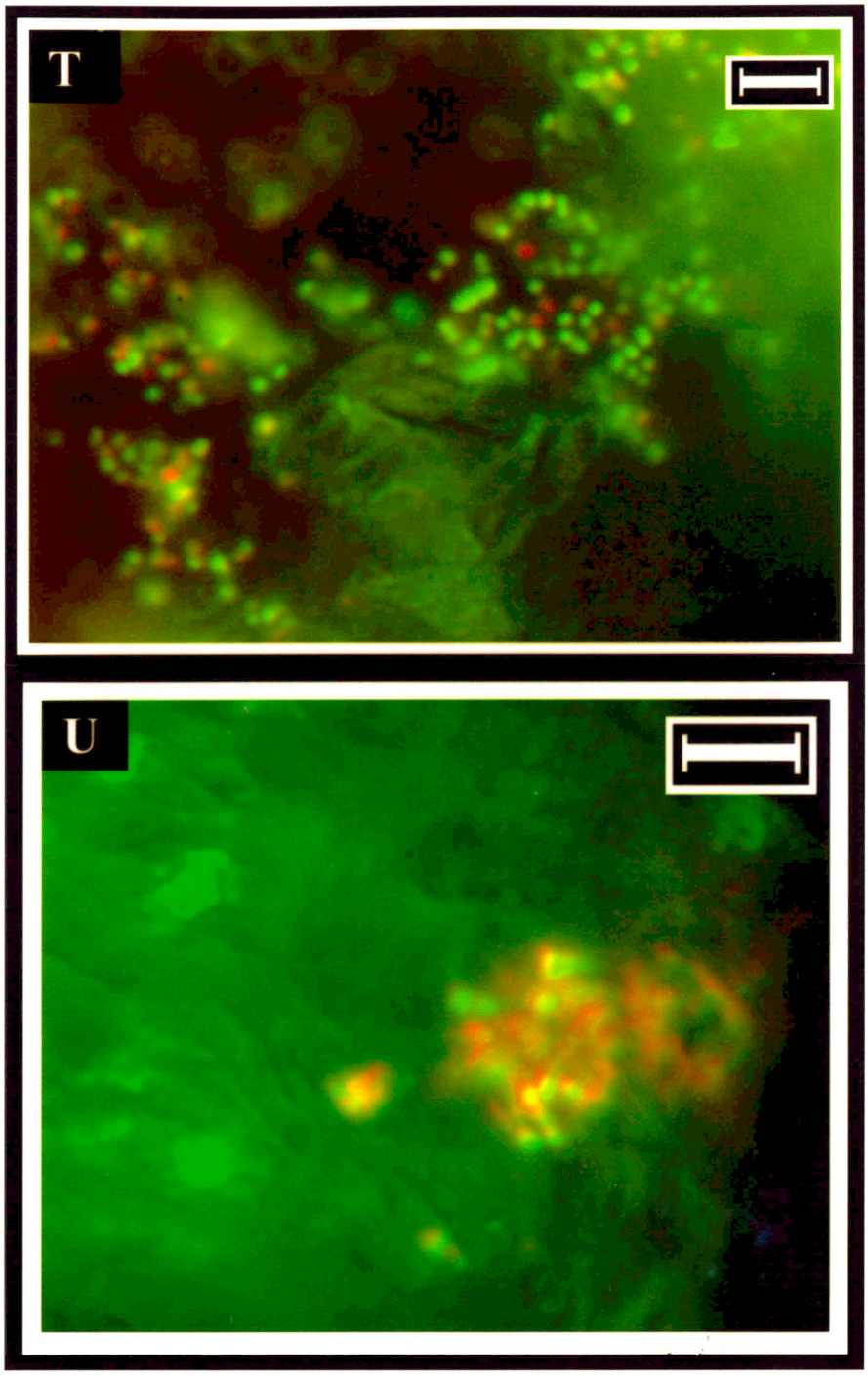


Fig. 30; T=2% IKI at a depth of 450-550µm:
U=2% IKI at adepth of 550-700µm.

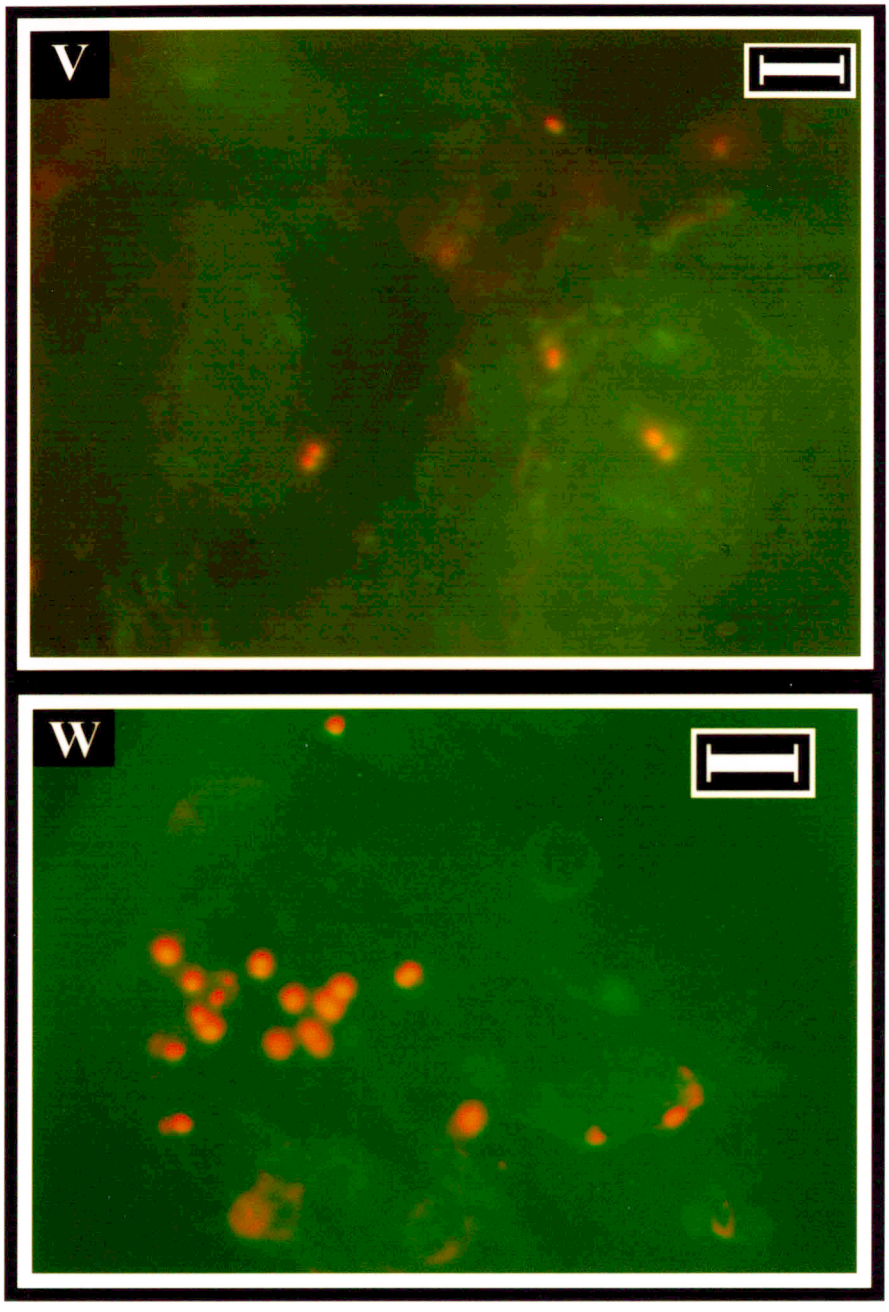


Fig. 31; V=2% IKI at a depth of 700-800μm:
W=2% IKI at a depth of 800-950μm.

DISCUSSION

The first part of this study was performed as a pilot investigation. There are no published studies using nucleic acid dyes to investigate the viability of microorganisms in dentin. These experiments were designed to control the growth characteristics of the test microorganism, and to gather information on the behavior of nucleic acid dyes in contact with a biological vehicle made of dentin. This information was critical for continuing the investigation to an *in-vitro* model.

The second part of this study evaluated the effectiveness of the dual stain method in differentiating live and killed *Enterococcus faecalis* cells in infected dentinal tubules after disinfecting procedures. The long range goal of this investigation is to look at the potential use of the fluorescent dual stain method in an *in-vivo* model that contains a synergistic population of endodontic pathogens trapped in dentinal tubules after obturation, and evaluate the ability of microorganisms to survive. The role of microorganisms in periapical inflammation has been well documented. Understanding how or if these microorganisms can survive inside dentinal tubules after incomplete disinfection may lead to treatment methods that will increase endodontic prognosis.

The use of *Enterococcus faecalis* as a test microorganism was based on previous studies (Spångberg et al 1967, Akpata et al 1982,

Meryon et al 1986, Haapasalo et al 1987, Ørstavik et al 1990, Safavi et al 1990, Vahdaty et al 1993, Tanriverdi et al 1997). The advantages of using a gram-positive facultative anaerobe are; tolerates exposure to oxygen, nutritional requirements are minimal, grows rapidly, thick cell wall, and penetrates dentinal tubules. In a recent paper by Sundqvuist in 1998, *E. faecalis* was recovered from several teeth that were considered endodontic failures. *E. faecalis* appears to have the mechanisms that allow it to survive as a monoinfection. Teeth undergoing endodontic treatment that are poorly isolated or left open for drainage are at risk of contamination with this bacterium and others.

The growth characteristics of *Enterococcus faecalis* were similar to that of *E-coli.* Luria Bertani agar supplemented with caso-amino acids and glucose was shown to provide adequate nutrients for the test microorganism to grow.

Ultraviolet light has been proven to be an efficient disinfection method for killing bacterial cells(E. J. Morris 1972, Robert Qualls et al 1982, and J. C. H. Chang et al 1985). The results obtained here are in agreement with the previous findings. However, 70 percent isopropyl alcohol proved to be easier in our hands, to work with, and less susceptible to contamination by error. The results were 99.9 percent and reliable.

The manufacturer, Molecular Probes, recommended calibration of the dyes to the test microorganism. We found that a concentration of 0.25μmol SYTO 9 was efficient in staining an *Enterococcus faecalis* cell population of 3.2 x 10^9 cells/ml. PI on the other hand, required a lesser concentration. PI required 0.002μmol for a cell population of 3.2 x 10^9 cells/ml. When the dyes were used in these ratios, efficient differentiation of live and killed cells was made easy.

Other investigators found different ratios of PI and FDA to produce better results (Jones et al 1985, Schupp et al 1987). SYTO 9 stains both dead and live cells. SYTO 9 can readily penetrate intact cell membranes allowing SYTO 9 to bind to the DNA of both populations. PI on the other hand, will be excluded from a cell with an intact membrane. PI will only stain cells with broken membranes. A probable cause for different ratios of dyes is that both cells stain with SYTO 9.

In this investigation, the dentin was observed to take up the SYTO 9 dye. The dentin appeared light green and the intensity level was well below the fluorescent emission of the bacteria. This indicated that differentiation of live and killed bacterial cells within dentin would be possible. Another observation was that the dentin contained bridges that appeared to transverse the dentinal tubules. These bridges emitted a signal of similar intensity as bacteria when viewed from a longitudinal angle

presenting a possible confusion when scoring the source of fluorescent emissions, but the morphology of the bridges were different and easily distinguishable from the microorganisms. Also, periradicular dentin around the dentinal tubules emitted an intense green signal similar to the dentinal bridges.

SYTO 9 and propidium iodide penetrated the dentinal shavings easily. In a study by Byers et al 1995, a fluorescent carbocyanine dye Di-I (1,1'-dioctadecyl-3,3,3',3'tetramethyindo-carbocyanine percholorate) and a gelatin embedment method were used to stain the processes of odontoblasts in dentinal tubules of rats and monkey teeth. The results were striking. They found that the dye stained odontoblasts and their processes deep into the dentinal tubules easily as well as osteocytes and cementocytes. The success of this process was dependent on perfusion-fixed jaws. This allows identification of the cell, but viability was lost during the fixation process.

The standardized bovine model first introduced by Haapasalo and Ørstivik in 1987 provided samples that were easily infected, and produced good penetration of microorganisms into the dentinal tubules. In our study, a different infection method was used. We removed the cementum and smear layer from both sides of the bovine sample. To avoid bacterial penetration on the outer circumference, dentin samples were placed on

sterile bone wax and surrounded by modified LB agar. Fresh log phase inoculum was introduced to the lumen every 12 hours. This promoted the flow of microorganisms through the tubules. As the agar lost moisture, fluid in the lumen was drawn through the dentinal tubules. This method provided a rapid infection time and avoided peripheral penetration of bacteria.

The colorization method used for the image micrographs was lengthy and required large amounts of memory. It is highly recommended to use a color camera if available. This will reduce time and computer requirements greatly. Also, since the images were taken from two different filters, the images may drift from their original position just enough to cause the micrographs to miss-a-line during colorization.

Since there has never been a study that evaluated vital staining of microorganisms in dentin, it is difficult to interpret our results. Our findings were that SYTO 9 and propidium iodide can readily and quickly differentiate live and killed microorganisms in the dentinal tubules. These results suggested that this approach could be used to further investigate the effectiveness of chemotheraputic agents *in-vitro*.

E. faecalis has been shown to resist treatment with $Ca(OH)_2$ (Ørstivik et al 1990, Safavi et al 1990). This study is in agreement with others. $Ca(OH)_2$ showed no effect on *E. faecalis* after 5 minutes or 1

week exposure. The infected dentinal samples that were challenged with $Ca(OH)_2$ were rendered un interpretable. The dentinal tubules, viewed by fluorescent microscopy, contained fluorescent activated matter that appeared in shape similar to fungal hyphe. A previous study has shown that $Ca(OH)_2$ will coagulate bacterial proteins. Whether the matter inside these tubules was coagulated proteins or fungal hyphe was not determined. The substance within the tubules was stained with SYTO 9, and appeared green.

The evaluation of 1% buffered NaOCl and 2% IKI provided useful information of the effect that these agents have on *E. faecalis* in dentinal tubules. The amount of dentinal shavings produced by the series of burs was very large. Random fields were examined in order to achieve a representative example of the effect of the agents used. The information gathered can only be interpreted as a small sample of a large population. The experimental time used is not representative of a clinical situation. Irrigation with NaOCl is present in most cases more than 5 minutes, and 2% IKI treatment is recommended for 10 minutes, Safavi et al (1990). Ørstavik found 5.25% NaOCl to be effective 300 microns into dentinal tubules. The length of time for treatment was not clear. In this study, *E. faecalis* was efficiently killed at depths of 100 micrometers by 1% NaOCl and 2% IKI as judged by

fluorescence microscopy. Between 100 and 300 micrometers, 1% NaOCl had little to no effect, whereas 2% IKI continued to kill bacteria to depths of 950 micrometers. At depths of more than 100 micrometers, viable bacteria were found using the dual nucleic acid method. 2% IKI was more effective in killing *E. faecalis* than 1% buffered NaOCl after a 5 minute treatment. The penetration of IKI was found to the level on 950 micometers. This is in agreement with Ørstavik who found IKI to penetrate 1000 micrometers into dentinal tubules. Killed bacteria were seen by fluorescence microscopy at 950 micrometers. Viable bacteria were also been seen at this penetration depth. The level of penetration of bacteria in the IKI group was considerably deeper than the other groups. In 3 of the 6 samples, bacterial penetration was found to the last bur depth. All dentin root samples were divided randomly and infected identically. The reason for the difference in penetration is unknown. A possible cause could be the surge of fluid IKI into the tubules pushing the bacteria deeper. One other cause might be the bur pushing the bacteria deeper as it rotates during the cutting process. The later might not be a factor due to the phenomenon only occurring in the IKI group. The sample size was also small.

The focus of this study was to evaluate the method of a dual nucleic acid stain. The results are encouraging for further investigation into this method for determining viability of endodontic pathogens in an *in-vivo* model.

SUMMARY

This study investigated efficiency of SYTO 9 and propidium iodide in differentiating between live and killed *E. faecalis* cells in bovine root samples. SYTO 9 and propidium iodide stained *E. faecalis* easily. Live and killed cells in a population were easily differentiated using the nucleic acid dyes. There was limited background staining. Dentin did absorb SYTO 9, but lacked the intense fluorescence emission that the microorganisms displayed. The bovine dentin samples contained peritubular and bridge formations that stained green with SYTO 9. These tubes and bridges emitted a signal similar in intensity as bacteria, but could be differentiated on a morphological basis. The fluorescent microscopy observation technique was rapid, and reliable.

Standardized bovine dentinal root samples were infected with *E. faecalis* for 9 days, and challenged by 1% NaOCl and 2% IKI for 5 minutes. Positive controls showed bacterial penetration to 450μm and contained 100% live cells. The negative control showed no signs of bacterial penetration in any of the samples.

The results showed that the dual nucleic acid method provided rapid and qualitative analysis of bacterial viability in an *in-vitro* bovine dentinal root model. 2% IKI killed *E. faecalis* at deeper depths than 1%

NaOCl after treatment for 5 minutes. 1% NaOCl and 2% IKI both effectively killed *E. faecalis* to a depth of 100 micrometers. At a depth of 300 micrometers, 1% NaOCl showed little or no effect. 2% IKI continued to kill bacteria to depth of 950μm. Results of this study are encouraging for further investigation into an *in-vivo* model that looks at the viability of microorganisms in endodontically treated teeth after obturation.

CONCLUSION

The dual stain method provided efficient analysis of bacterial cell viability in bovine dentinal root samples. SYTO 9 and propidium iodide stained the test microorganism readily in suspension as well as in bovine dentinal tubules. The modified infection technique provided fast and reliable penetration of microorganisms into dentin samples. The results of these tests showed that SYTO 9 and propidium iodide can differentiate between live and killed *Enterococcus faecalis* cells in an *in-vitro* model. The results are fast and reliable.

Two way analysis at $p<0.001$ suggest that 2% IKI is more effective at killing *E. faecalis* at dentinal tubule depths between 100-450μm than 1% buffered NaOCl after 5 minutes of treatment. Both treatments were highly effective in killing *E. faecalis* at dentinal tubule depths of 100μm.

This study provides information that encourages the use of this method over conventional culturing techniques for evaluation of bacterial viability in endodontically treated teeth. 2% IKI penetrates farther into dentinal tubules than 1% NaOCl after 5 minutes. Further investigations are needed to understand the survival of microorganisms that are trapped in root canal system after endodontic treatment

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