Original Article

Detection of microorganisms on formalin-fixed and stored pathology tissues: A microbiological study

Gayathri Ramesh, Anuradha Katiyar, Ramamurthy Sujatha[1], Amrita Raj, Bhavana Gupta, Amit Kumar

Department of Oral and Maxillofacial Pathology, Rama Dental College Hospital and Research Centre, [1]Department of Microbiology, Rama Medical College, Kanpur, Uttar Pradesh, India

Abstract

Background: Formalin is widely used to fix histological preparations and as preservatives in embalming solutions and is an age-long practice in medical laboratories. It is generally accepted that the risk of contracting infections is relatively high among medical laboratory workers and pathologists. Recent studies have, however, suggested that formalin does not effectively inactivate all kinds of microbes in formalin-fixed tissue (FFT). Long time preserved tissues in formalin may develop growth of microbes on the surface of the formalin.

Aims and Objectives: The purpose of the study is to determine the growth of microorganisms on the surface of FFTs.

Materials and Methods: Fifty-one containers of 10% formalin with fixed tissues and undiscarded formalin solution not containing tissues of years 2013–2015 (17 in each year) were selected, and samples for inoculation onto the cysteine lactose electrolyte deficient agar plates were taken from the surface of the FFT using sterile cotton tips. The growth of the colonies was checked for after 48 h.

Results: Out of 51 samples from 2013 to 2015, 17 had shown growth of microbial colonies. Six out of 17 samples of 2013, 7 out of 17 of 2014 and 4 out of 17 samples of 2015 had colonies of microbes on agar plates. Gram-negative bacilli, *Bacillus subtilis* and micrococci were mostly found.

Conclusion: There were viable microbes on the surfaces of formalin solution containing pathology tissue. Since cross-contamination by microbes may occur during regrossing or processing, protocols to decrease cross-contamination should be instituted.

Keywords: Embalming, fixation, formalin, microbes, pathology, preservative

Address for correspondence:
Dr. Gayathri Ramesh, Department of Oral and Maxillofacial Pathology, Rama Dental College Hospital and Research Centre, Kanpur - 208 024, Uttar Pradesh, India. E-mail: amug3r@yahoo.co.in

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INTRODUCTION

The process of embalming and preserving tissue samples is necessary so that pathologists are able to work on tissues for an extended period of time without the risk of decay, tissue loss and pathogen transmission.[1] Modern pathology is built around the principle of preserving tissues such that the *in vivo* molecular status is maintained at levels representative of the disease state. Tissues are immersed in a solution of fixative which slowly inactivates biological activities, thus preserving the sample.[2] Modern embalming practices involve the use of fixative agents, most commonly 10% buffered formalin. Formalin is a potent disinfectant

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that targets the amine functional groups in proteins, thereby denaturing them. Research into the efficacy of modern embalming processes in destroying pathogenic agents is ongoing and largely incomplete.[1]

Formaldehyde is bactericidal, sporicidal and virucidal, but it works more slowly than glutaraldehyde. It has long been considered to be sporicidal by virtue of its ability to penetrate into the interior of bacterial spores. Low concentrations of formaldehyde are sporostatic and inhibit germination.[3]

It is difficult to pinpoint accurately the mechanism(s) responsible for formaldehyde-induced microbial inactivation. Clearly, its interactive and cross-linking properties must play a considerable role in this activity.[4]

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria (mycobacteria, nonsporulating bacteria and bacterial spores) to antibacterial agents. As a result, resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes).[3]

The development of resistance to antimicrobial agents and biocides is particularly warning problem which is compounded by cross-resistance mechanisms (between antibiotic and biocide) that may exist in certain bacteria such as pathogenic strains of *Escherichia coli*.[5]

Hence, the purpose of this study was to determine if any potentially pathogenic bacterium might be present on the surface of formalin-fixed tissues (FFTs) stored in tissue bank.

MATERIALS AND METHODS

Fifteen tissue samples each preserved in 10% formalin of the years 2013, 2014 and 2015 were retrieved for detection of microorganism on surface of tissues. Six samples of 10% formalin once used for tissue fixation but undiscarded and presently without tissue (two samples each of 2013, 2014 and 2015) were taken as controls. The surface of the formalin solution of control was swiped with sterile cotton tips and was directly inoculated onto the agar plates. The 10% formalin solution with FFT in it was discarded first and then the tissue samples were lifted from the container using sterile tweezers. Surface of the tissues was swiped using sterile loop inoculators, and after collection, each sample was inoculated in cysteine lactose electrolyte deficient (CLED) agar plates as it is a suitable media to grow both Gram-positive and Gram-negative bacteria (Gram-positive cocci, Gram-negative cocci, Gram-positive bacilli and Gram-negative bacilli) and yeast-like candida. The inoculated agar plates were incubated at 37°C for 48 h. Once the samples were cultured, morphologic characteristics of isolated individual colonies were recorded. Slides of the individual colonies were Gram stained to confirm purity and to determine bacterial morphology. Once a pure culture was confirmed, the postisolation sample was tested for biochemical reactions. The microbes isolated and identified in both controls and FFT samples based on their morphology on CLED agar plates, Gram's staining and biochemical reactions which were applied are shown in Table 1.

Table 1: Morphological characteristics of isolated and identified bacteria from formalin-fixed tissues and controls

Type of bacteria	Morphology of cultured bacterial colonies on CLED agar	Morphology of bacteria on Gram-stain	Biochemical test results applied				
E. coli	Lactose-fermenting pink flat and dry colonies	GNB	Indole test - positive	Citrate test - negative	TSI test A/A	Urease test - negative	Mannitol/motility test - fermented/with motile
K. oxytoca	Lactose-fermenting pink mucoid dome-shaped colonies	GNB	Indole test - positive	Citrate test - positive	TSI test A/A with gas	Urease test - positive	Mannitol/motility test - fermented/nonmotile
C. freundii	Late lactose-fermenting and moist colonies	GNB	Indole test - negative	Citrate test - positive	TSI test A/A with H_2S	Urease test - negative	Mannitol/motility test - fermented/motile
Diphtheroids	Whitish dry colonies	GPB, short and thick, very little pleomorphism	Fermentation of glucose - positive			Fermentation of sucrose - positive	
B. subtilis	Whitish dry colonies	GPB, short chains	Hemolysis on blood agar - well marked	Fermentation of salicin - positive			McFadyean's reaction - negative
CoNS	Small and dry colonies	GPC in cluster	Catalase test - positive	Coagulase test - negative	Urease test - negative		DNAse test - negative
Micrococci	Big round smooth colonies	GPC in tetrads	Catalase test - positive	Coagulase test - negative	Urease test - negative		O/F test - oxidative

TSI-A/A: Triple-sugar iron acid/acid, O/F: Oxidative/fermentative, *K. oxytoca*: *Klebsiella oxytoca, B. subtilis*: *Bacillus subtilis, C. freundii*: *Citrobacter freundii*, CoNS: Coagulase-negative *Staphylococcus*, GPC: Gram-positive cocci, GPB: Gram-positive bacilli, GNB: Gram-negative bacilli, CLED: Cysteine lactose electrolyte deficient, *E. coli: Escherichia coli*

RESULTS

Out of 51 samples (17 samples each) between the years 2013 and 2015, 17 samples exhibited bacterial growth on FFTs and controls. The six undiscarded formalin control samples not containing tissue showed growth of microorganisms, one each of *E. coli* [Figure 1] in the year 2013 sample and coagulase-negative *Staphylococcus* (CoNS) in the sample of year 2014 [Tables 1 and 2]. In the 15 FFT samples of the year 2013, 5 samples (one each of micrococci, *Bacillus subtilis* [Figure 2], diphtheroids, *E. coli* and *Citrobacter freundii* [Figure 3]) demonstrated growth on CLED agar. While in the samples of year 2014, out of 15 samples, 6 exhibited the presence of microbes (two each of micrococci and *B. subtilis*, one each of diphtheroids and *Klebsiella oxytoca* [Figure 4]). Out of 15 samples of the year 2015, 4 had viable organisms one each of micrococci, *C. freundii* and two of *B. subtilis* [Table 2].

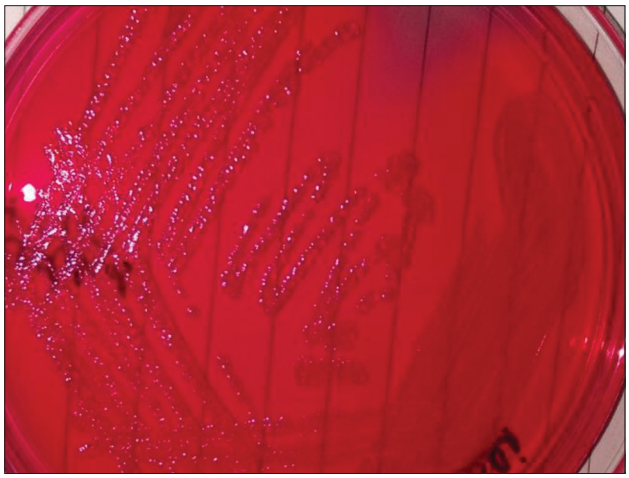

Figure 1: Cysteine lactose electrolyte deficient agar showing pink, lactose-fermenting colonies of *Escherichia coli*

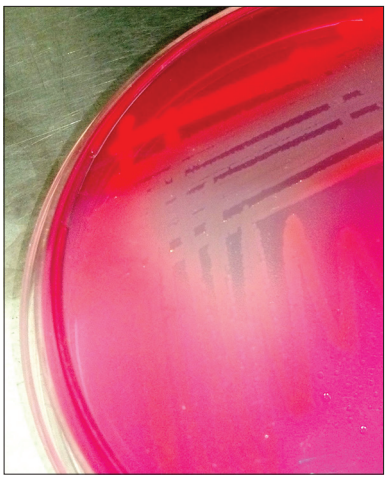

Figure 3: Cysteine lactose electrolyte deficient agar showing pale, lactose-fermenting colonies of *Citrobacter freundii*

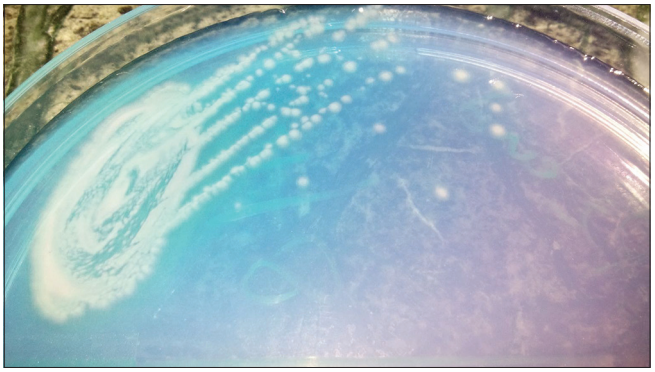

Figure 2: Cysteine lactose electrolyte deficient agar showing dry colonies morphologically resembling spores of *Bacillus subtilis*

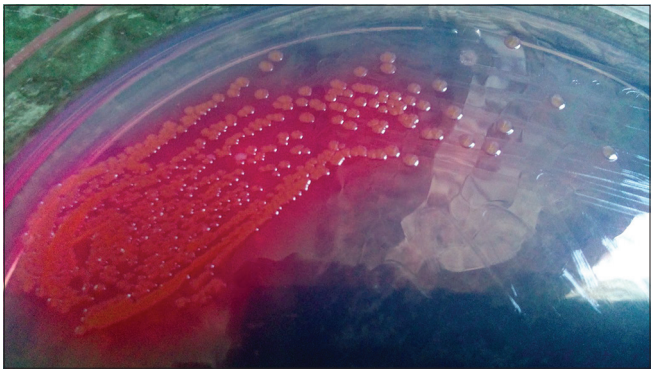

Figure 4: Cysteine lactose electrolyte deficient agar showing mucoid, lactose-fermenting colonies of *Klebsiella oxytoca*

Table 2: Distribution of cultured bacteria from formalin-fixed tissue samples and controls

Type of samples	Year of samples			
	Type of bacteria	2013 (17)	2014 (17)	2015 (17)
Undiscarded formalin samples as controls (6)	No growth (4)	1	1	2
	GPC (1)	-	CoNS (1)	-
	GPB (0)	-	-	-
	GNB (1)	*E. coli* (1)	-	-
Formalin-fixed tissue samples (45)	No growth (30)	10	09	11
	GPC (4)	Micrococci (1)	Micrococci (2)	Micrococci (1)
	GPB (7)	*B. subtilis* (1) Diphtheroids (1)	*B. subtilis* (2) Diphtheroids (1)	*B. subtilis* (2)
	GNB (4)	*E. coli* (1) *C. freundii* (1)	*K. oxytoca* (1)	*C. freundii* (1)

K. oxytoca: Klebsiella oxytoca, B. subtilis: Bacillus subtilis, C. freundii: Citrobacter freundii, CoNS: Coagulase-negative *Staphylococcus*, GPC: Gram-positive cocci, GPB: Gram-positive bacilli, GNB: Gram-negative bacilli, *E. coli: Escherichia coli*

The 45 samples of FFT contained cystic specimens (14), followed by reactive lesions (12), malignant tumors (11) and benign lesions (6) [Table 3].

The cystic samples showed growth in seven, three of the year 2013 (one each of *B. subtilis*, *E. coli* and *C. freundii*) and three of year 2014 (two of *B. subtilis* and one of micrococci). Out of 12 reactive lesions, 5 showed microbial growth (one each of micrococci, diphtheroids and *K. oxytoca*) in the samples of the years 2013 and 2014 while two of *B. subtilis* in the samples of year 2015 were also observed. Three out of 11 malignant tumor samples showed growth (two of micrococci [one each of year 2014 and 2015] and one of *C. freundii* of year 2015). Only one sample out of six benign lesions showed microbial growth (diphtheroids in the sample of year 2014) [Table 3].

DISCUSSION

Formaldehyde (methanal, CH_2O) is a monoaldehyde that exists as a freely water-soluble gas. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam.[3]

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported.[3]

Table 3: Type and number of samples and positive microbial cultures from formalin-fixed tissues of years 2013-2015

Sample	2013 (17)	2014 (17)	2015 (17)
Control (6)			
Number of samples	2	2	2
Number and type of growth	*E. coli* (1)	CoNS (1)	No growth
Reactive lesions (12)			
Number of samples	5	3	4
Number and type of growth	Micrococci (1) Diphtheroids (1)	*K. oxytoca* (1)	*B. subtilis* (2)
Benign tumors (6)			
Number of samples	1	2	3
Number and type of growth	No growth	Diphtheroids (1)	No growth
White lesions (2)			
Number of samples	1	1	0
Number and type of growth	No growth	No growth	-
Malignant tumors (11)			
Number of samples	3	4	4
Number and type of growth	No growth	Micrococci (1)	Micrococci (1) C. freundii (1)
Cysts (14)			
Number of samples	5	5	4
Number and type of growth	*B. subtilis* (1) *E. coli* (1) *C. freundii* (1)	*B. subtilis* (2) Micrococci (1)	No growth

K. oxytoca: *Klebsiella oxytoca*, *B. subtilis*: *Bacillus subtilis*, *C. freundii*: *Citrobacter freundii*, CoNS: Coagulase-negative *Staphylococcus*, *E. coli: Escherichia coli*

Earlier studies have shown that despite the use of fixative agents, several disease-causing agents may remain viable in preserved tissues.[1] Hence, the goal of this study was to determine if bacteria could be recovered from the FFTs.

In the present study, it was observed that few FFT samples retrieved from the tissue storage bank did show microbial growth on culture plates. Cystic samples, followed by reactive lesions and malignant tumors, predominantly showed microbial growth. The predominant samples cultured were of *B. subtilis*, micrococci, diphtheroids, *E. coli* and *C. freundii*. One sample each of CoNS and *K. oxytoca* were also isolated.

One important observation done in our study is that even the undiscarded 10% formalin sample without tissue also yielded microbial growth such as CoNS and *E. coli*.

All of the organisms identified on the surface of FFTs can be opportunistic pathogens such as diphtheroids and micrococci can be an opportunistic pathogen in immunocompromised hosts. Microorganisms such as CoNS may cause septicemia and subacute bacterial endocarditis and *B. subtilis* causes eye infections and septicemia. *E. coli* can cause urinary tract infection (UTI), diarrhea, pyogenic infection such as wound infections and septicemia. *K. oxytoca* causes UTI, wound infections, bronchopneumonia, nosocomial infections, meningitis, septicemia and rarely diarrhea and also *C. freundii* may cause UTI, gallbladder and middle ear infections.[6]

Pathogenicity can occur if the microbes are found in an immunocompromised host or with simple overgrowth. The number and variety of bacteria recovered suggests that if pathogenic organisms had been present previously, they could survive during fixation process.[1]

Gram-negative bacteria tend to be more resistant than Gram-positive organisms, such as staphylococci. Bacterial spores of the genera *Bacillus* and clostridium have been widely studied and are invariably the most resistant of all types of bacteria to antiseptics and disinfectants.[3]

Formalin 37% is the most active chemical disinfectant against most types of microorganisms such as bacteria and their spores, fungi and viruses.[7]

In a study done by Soliman *et al*.,[7] to compare the effect of five disinfectants in relation to the presence or absence of organic matter as an extra challenge to the action of disinfectants found that when formalin was used alone, it required the removal of the organic matter that can retard its action, especially against highly powerful organisms

such as *Pseudomonas aeruginosa* (achieved the action after 20 min). On the contrary, when it was added to other compounds such as a mixture of quaternary ammonium compound and glutaraldehyde, it gave the ultimate compound, and this was clear in the results of Incospect IC 22XA. However, these are nonenvironmentally safe compounds.[7]

In general, Gram-positive bacteria observed in this study to be more susceptible to antimicrobial agents, and Gram-negative bacteria, for example, *E. coli* are generally less susceptible to formalin because of their complex cell wall, in which the outer membrane of Gram-negative bacteria acts as permeability barrier in limiting or preventing the entry of many chemicals.[5]

Since concentration exponent of peroxygen and aldehyde disinfectants is small and their values are about 0.5 and 1, respectively, then it could be assumed that the effect of dilution in diminishing antimicrobial properties would be minimal if compared with alcohol and phenols.[8]

If the assumption of Gundersen *et al.* (1996)[9] is true that protease activity is responsible for the loss in bacterial abundance in formalin-fixed samples, then nonnucleoid-visible cells are most affected by protease activity. Protease, however, should act on both nucleoid and nonnucleoid cells. Thus, it is likely that nonnucleoid-visible bacteria are the result of autolysis of bacteria which continues after formalin fixation.[9]

Tabaac *et al.*[1] sampled tissues for microbiological contaminants from routinely preserved cadavers before examination and dissection by anatomy students. The results indicated that cadavers processed with 10% buffered formalin had viable organisms on their surfaces that can be a source of contamination of laboratory equipment and clothing. The authors concluded that given the diversity of bacterial species cultured, preserved cadavers used for anatomy education as well as research must be considered a possible source for dissemination of bacterial organisms.[1]

Bartos *et al.*[10] conducted a study to assess the presence of mycobacteria in tissue samples from four cadavers fixed with formalin, and tissue samples from a recently deceased unpreserved individual, who had a history of human tuberculosis infection, undergoing a postmortem (cause of death not related to tuberculosis).

Microscopy examination after the Ziehl–Neelsen staining and culture examination for the presence of mycobacteria was negative in all 22 tissue samples from the 4 embalmed cadavers. However, with polymerase chain reaction analysis, specific for Mycobacterium avium subsp. avium was positive in both tissue samples with and without tuberculous lesions. The authors concluded that importance to all regulations and rules related to sanitary procedures for the protection of staff and students working on cadavers with tuberculous lesions in the parenchymatous organs should be reiterated.[10]

Oke *et al.*[11] conducted a study to test the inactivation ability of 10% formalin with and without 75% ethanol pretreatment on multidrug-resistant strain of *Mycobacterium tuberculosis* (MDR-Tb) and Multidrug-sensitive *M. tuberculosis*. MDR-Tb strains resisted inactivation by 10% formalin but were inactivated by the treatment with 75% ethanol 2 h before 10% form.

Their results suggested that there is a risk of contracting tuberculosis from tissue that has been fixed in formalin if aerosolization or accidental inoculation should occur. The authors concluded that the result of their investigations was in agreement with the suggestions of other workers for the use of alcohol in addition to the conventional 10% formalin fixation of tissues to prevent occupational hazards to medical laboratory scientists, pathologists, anatomists and medical educators. This is pertinent more so now that more people die due to MDR-Tb infections.[11]

CONCLUSION

There were viable bacteria on surfaces of some biopsy tissues and controls. This is of concern because pathologists and anatomists across the world may be exposed to potentially pathogenic organisms every time they work with tissues. It has been suggested that the preservation technique is inadequate to eradicate all microorganisms. Universal precautions to prevent dissemination of organisms from tissues must be put in place in all pathological laboratories. Cross-contamination of tissues by microbes may also occur during processing; protocols to decrease cross-contamination should be instituted. Our current findings raise the need for continued investigation of the role of pathologists in dissemination of pathologic organism.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Tabaac B, Goldberg G, Alvarez L, Amin M, Shupe-Ricksecker K, Gomez F. Bacteria detected on surfaces of formalin fixed anatomy cadavers. Ital J Anat Embryol 2013;118:1-5.
2. Chafin D. Two-temperature formalin fixation preserves activation states efficiently. Recent Results Cancer Res 2015;199:107-17.
3. McDonnell G, Russell AD. Antiseptics and disinfectants: Activity, action, and resistance. Clin Microbiol Rev 1999;12:147-79.
4. Russell AD, Chopra I. Understanding Antibacterial Action and Resistance (Ellis Horwood Series in Pharmaceutical Technology). 2nd ed. Chichester, England: Ellis Horwood; 1996.
5. Saleh RH, Naher HS, Al-Jubory SA. A study of efficacy of disinfectants and bacterial contamination in AL-Hilla teaching hospital. Med J Babylon 2014;9:890-900.
6. Baveja CP. Text Book of Microbiology. 4th ed. Haryana, India: Arya Publications, Avichal Publishing Company; 2013.
7. Soliman ES, Sobeih MA, Ahmad ZH, Hussein MM, Moneim HA. Evaluation of commercial disinfectants against bacterial pathogens isolated from broiler farms. Int J Poult Sci 2009;8:728-32.
8. Eissa ME, Ashour MS, Mansy MS. Neutralizer evaluation study of some microbial isolates against two strong disinfectants with and without the presence of synthetic detergent. World Appl Sci J 2012;20:823-31.
9. Gundersen K, Bratbak G, Heldal M. Factors influencing the loss of bacteria in preserved seawater samples. Mar Ecol Prog Ser 1996;137:305-10.
10. Bartos M, Pavlikova H, Dvorska L, Horvath R, Dendis M, Flodr P, *et al*. Risk assessment of mycobacterial infections (human tuberculosis and avian mycobacteriosis) during anatomical dissection of cadavers. Vet Med 2006;51:311-9.
11. Oke AJ, Adeyemo AD, Oke AA. Multidrug-resistant human *Mycobacterium tuberculosis* strain resisted inactivation by 10% formalin. Open Access Sci Rep 2012;1:344.