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

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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. 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. Modern embalming practices involve the use of fixative agents, most commonly 10% buffered formalin. Formalin is a potent disinfectant...
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*.\[^3\]

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                      |
|                  |                                                      |                                     | 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             |
|                  |                                                      |                                     | Mannitol/motility test - fermented/without motile |
| *C. freundii*    | Late lactose-fermenting and moist colonies           | GNB                                 | Indole test - negative           |
|                  |                                                      |                                     | Citrate test - positive          |
|                  |                                                      |                                     | TSI test A/A with H₂S             |
|                  |                                                      |                                     | Mannitol/motility test - fermented/with motile |
| Diphtheroids     | Whitish dry colonies                                 |                                     | Fermentation of glucose - positive|
|                  |                                                      |                                     | Fermentation of sucrose - positive|
| *B. subtilis*    | Whitish dry colonies                                 | GPB, short chains                   | Hemolysis on blood agar - well marked|
|                  |                                                      |                                     | Fermentation of salcin - positive|
|                  |                                                      |                                     | McFadyean’s reaction - negative   |
|                  |                                                      |                                     | DNAse test - negative            |
| CoNS             | Small and dry colonies                               | GPC in cluster                      | Catalase test - positive         |
|                  |                                                      |                                     | Coagulase test - negative         |
|                  |                                                      |                                     | Mannitol/motility test - fermented/without motile |
| Microcacci       | Big round smooth colonies                            | GPC in tetrads                      | Catalase test - positive         |
|                  |                                                      |                                     | Coagulase test - negative         |
|                  |                                                      |                                     | Mannitol/motility test - fermented/without motile |
|                  |                                                      |                                     | O/F test - oxidative             |

\[^1\]TSI-A/A: Triple-sugar iron/acid, D/O: 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

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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*](image1)

![Figure 2: Cysteine lactose electrolyte deficient agar showing dry colonies morphologically resembling spores of *Bacillus subtilis*](image2)

![Figure 3: Cysteine lactose electrolyte deficient agar showing pale, lactose-fermenting colonies of *Citrobacter freundii*](image3)

![Figure 4: Cysteine lactose electrolyte deficient agar showing mucoid, lactose-fermenting colonies of *Klebsiella oxytoca*](image4)

| 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) | *B. subtilis* (2) | *B. subtilis* (2) |
| | GNB (4) | Diphtheroids (1) | Diphtheroids (1) | - |
| | | *E. coli* (1) | *K. oxytoca* (1) | *C. freundii* (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, CH₂O) 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]

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

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

Earlier studies have shown that despite the use of fixative agents, several disease-causing agents may remain viable in preserved tissues.[5] 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.[5]

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.[9]

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.[9]

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.
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