

## ORIGINAL ARTICLE

## Antimicrobial activity of UMFix tissue fixative

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**Aims:** The aim of this study was to determine the antimicrobial effects of UMFix, an alcohol based tissue fixative, on various microorganisms. The UMFix solution was compared with 10% neutral buffered formalin.

**Methods:** Standard methods to determine microorganism colony counts were performed after exposure of the microorganisms to UMFix and 10% neutral buffered formalin.

**Results:** After a short exposure, UMFix rapidly killed vegetative bacteria, yeasts, moulds, and viruses. Bacterial spores were resistant to killing by UMFix. All organisms were killed by the 10% neutral buffered formalin preparation.

**Conclusions:** UMFix was microbicidal for vegetative bacteria, yeasts, and aspergillus species after a short exposure, although it was not active against spore forming bacillus species. The methanol content of the fixative was responsible for the killing effect of this fixative. No killing was seen when polyethylene glycol was used alone.

UMFix is a recently described tissue fixative developed at the University of Miami, USA.<sup>1</sup> It is an alcohol based preservative that was shown to provide comparable histomorphology to that seen after formalin fixation. Other advantages include the lack of toxicity on laboratory personal and its ability to preserve RNA, DNA, and protein in tissue after fixation, processing, and paraffin wax embedding.

Formaldehyde, the most commonly used tissue fixative, was originally used as an antiseptic and was later discovered to have fixative properties.<sup>2</sup> The routinely used 10% formalin is a 3.7% solution of formaldehyde in water.

For over a century, 10% formalin has been considered the best fixative for histopathology; however, from the beginning its numerous toxic effects were recognised.<sup>3</sup> Formaldehyde is a known carcinogen. It is irritating to the eyes, respiratory mucosa, and skin. It was also reported to cause impaired memory and dexterity, and could be fatal if ingested.<sup>4</sup> Therefore, human exposure must be monitored.

"Probably the greatest advantage of UMFix is that it preserves RNA as well as DNA, providing a quality comparable to fresh tissue"

In addition to its toxic effects, other drawbacks of formalin have become clear in recent years. Modern pathology no longer relies solely on histomorphology to render diagnosis. Analysis of DNA, RNA, and protein from the tissue specimen is becoming increasingly important, not only for the classification of pathological processes, but also for prognosis and individualised treatment.<sup>5–6</sup> However, RNA is degraded in formalin fixed tissue, precluding molecular analysis.<sup>7</sup> In addition, formalin destroys or distorts many protein epitopes, hindering its use in proteomics studies. Even DNA from formalin fixed tissue is of suboptimal quality because formalin has been shown to cause chemical modification of DNA.<sup>7</sup>

All these factors have led to the development of alternative fixatives. At first, ethyl alcohol based fixatives were introduced.<sup>7</sup> However, although less toxic, these fixatives do not preserve RNA well in tissue. Recently, a new alcohol based tissue fixative, comprising a mixture of methanol and polyethylene glycol and named UMFix, was developed at the

University of Miami, USA,<sup>1</sup> and has been used as an alternative to formalin.

UMFix was shown to provide histomorphology comparable to formalin. Because protein is better preserved when this fixative is used, the results of immunoperoxidase studies are better than with similar material preserved in formalin. Probably the greatest advantage of UMFix is that it preserves RNA as well as DNA, providing a quality comparable to fresh tissue.<sup>1</sup> Using UMFix preserved, paraffin wax embedded tissue, intact RNA can be extracted from the same paraffin wax block that is used to render diagnosis. In addition, similar to ethyl alcohol based fixatives, UMFix is much less toxic than formalin; it does not irritate the skin or mucosa, and is not carcinogenic. However, it can be toxic if ingested in large quantities.

The antimicrobial effects of this fixative have not been investigated. It is well known that 70% wt/vol alcohol serves as a disinfectant. In addition, it was shown that mixing methanol with a small amount of hypochlorite gives superior sporocidal activity to mixing methanol with ethanol.<sup>8–9</sup> In this report, we investigated the effect of UMFix on several different types of microorganism.

## MATERIALS AND METHODS

### Organisms

Laboratory clinical isolates of *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Candida albicans*, *Cryptococcus neoformans*, aspergillus species, and herpes simplex virus (HSV) were tested. A spore suspension of *Bacillus subtilis* ATCC 6633 (subtilis spore suspension; Difco Laboratories, Detroit, Michigan, USA) and the stern strain of *Bacillus anthracis* were also examined.

### Reagents

UMFix (Sakura Finetek USA Inc, Torrance, California, USA) is a mixture of methanol and polyethylene glycol at a predetermined ratio that has recently been developed at University of Miami, USA (US patent application 10/141,780).<sup>1</sup> In use solutions of 10% neutral buffered formalin and

**Abbreviations:** Ct, threshold cycle; HSV, herpes simplex virus; PCR, polymerase chain reaction

**Table 1** Viable counts of vegetative organisms after a 20 minute exposure to 10% neutral buffered formalin and to UMFix

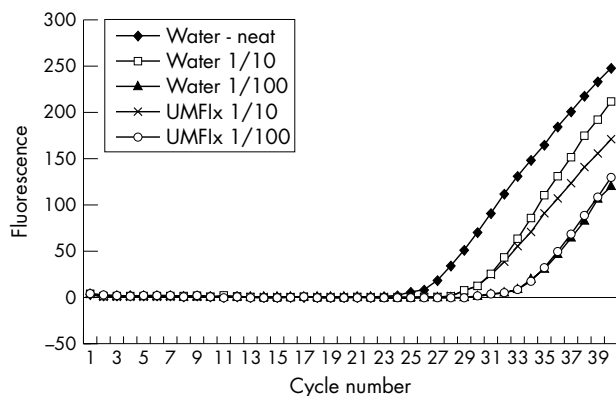
Organism	Initial CFU/ml	10% formalin CFU/ml	UMFix CFU/ml
<i>Staphylococcus aureus</i>	$3.7 \times 10^7$	0	0
<i>Enterococcus faecium</i>	$1.8 \times 10^8$	0	0
<i>Escherichia coli</i>	$3.3 \times 10^7$	0	0
<i>Mycobacterium fortuitum</i>	$2.3 \times 10^8$	0	0
<i>Mycobacterium chelonae</i>	$2.5 \times 10^7$	1 colony	0
<i>Candida albicans</i>	$3.3 \times 10^7$	0	0
<i>Cryptococcus neoformans</i>	$1.7 \times 10^7$	0	0
<i>Aspergillus fumigatus</i>	$2.7 \times 10^5$	0	0
<i>Aspergillus niger</i>	$2.4 \times 10^6$	0	0

CFU, colony forming units.

UMFix were prepared. All dilutions were performed using sterile deionised water. Organism suspensions were prepared from 5% sheep blood agar plates that were incubated overnight at 35°C. The suspension was washed once with sterile deionised water.

**Preparation of bacterial and fungal organisms**

A heavy suspension of each organism, equal to approximately a McFarland 2–10 standard, was prepared in buffered saline. A 100 µl aliquot of each organism was added to 5 ml of each test solution. The zero time control tube was diluted in sterile water and cultured to determine the initial number of colony forming units/ml of each organism. After 20 minutes, each tube was cultured in 5% sheep blood agar or Sabouroud dextrose agar to determine the viable counts remaining after this short treatment. The dilution schedule for the tubes containing the fixatives was as follows: 10 µl and 100 µl were plated directly on to the agar plate. A 10 fold dilution of the test tube was prepared and 100 µl of this solution was plated on to the agar plates. This last dilution was performed to negate inhibitor carryover effects of the different fixatives. The plates were incubated for a period of seven days before a final negative recording was made. Spore suspensions and vegetative cells of *B subtilis* and *B anthracis* were prepared to an approximate McFarland 0.5 standard. The sampling times and methods were similar to those outlined above. We also tested spore suspensions in UMFix that had been subjected to the microwave process used for tissue processing to determine whether it enhanced the killing of bacterial spores.<sup>10</sup>



**Figure 1** Real time polymerase chain reaction of pGEM herpes simplex virus plasmid suspended in water and UMFix (all dilutions were made with water).

**Table 2** Viable count of *Bacillus subtilis* spore suspension and vegetative organisms after exposure to 10% neutral buffered formalin and UMFix

Organism	Time of exposure	Initial CFU/ml	10% formalin CFU/ml	UMFix CFU/ml
Spores	0	$4.6 \times 10^7$		
	20 min	ND	$2.3 \times 10^7$	$2.5 \times 10^7$
	24 hour	$3.3 \times 10^7$	0	$2.4 \times 10^7$
Vegetative	0	$5.4 \times 10^7$		
	20 min	ND	0	0
	24 hour	$4.8 \times 10^7$	0	0

CFU, colony forming units; ND, not determined.

**Preparation of HSV**

The virus was grown on A549 cells until the cytopathic effect was confluent on the cell monolayer. The suspension was frozen at -80°C to lyse cells and then centrifuged at 375 xg to remove cellular debris. A 100 µl aliquot was added to 2 ml of UMFix and 2 ml of sterile saline. The suspension was held for 20 minutes, after which 50 µl of each suspension was added to the A549 cell monolayer to determine viability. In addition, the HSV specific DNA concentration was determined from each suspension using a real time polymerase chain reaction (PCR) assay. Because real time PCR is a quantitative procedure, the original inoculum of virus was tested to determine whether the viral nucleic acid was affected by any of the treatment procedures. PCR was performed using the HSV forward and reverse primers, which are directed at a 92 bp region of the HSV polymerase gene sequence (J Jordan, personal communication, 2002). The internal oligonucleotide probe was labelled with the fluorescent dye 5-carboxyfluorescein (FAM) on the 5' end and a 3' minor groove binder/non-fluorescent quencher. The primers and hybridisation probe were synthesised by Applied Biosystems (Forest City, California, USA). Each 25 µl PCR reaction chamber contained one OmniMix HS PCR bead (Cepheid, Sunnyvale, California, USA), 0.5µM HSV forward and reverse HSV primers, 0.5µM probe, and deionised water to a final volume of 20 µl. In a separate work area and using a CleanSpot PCR/UV workstation (Coy Laboratory Products, Grass Lake, Michigan, USA), 5 µl of each sample was added to the reaction chamber. The PCR reaction was performed on the Smart Cycler instrument (Cepheid), and the protocol included an initial denaturation step at 95°C for 240 seconds, which was followed by 95°C for 15 seconds and 60°C for 30 seconds for 45 cycles. Fluorescence measurements are made during every cycle. The threshold cycle (Ct) is the cycle at which there is a significant increase in fluorescence, and this value is associated with an exponential growth of the PCR

**Table 3** The killing action of UMFix on herpes simplex virus and its effect on nucleic acid detection by real time PCR

HSV diluent (duplicate test)	Viability after exposure for 20 minutes	Real time PCR Ct*
UMFix-1	No growth	30.7
UMFix-2	No growth	30.7
Saline-1	Growth	30.8
Saline-2	Growth	31.7
HSV (original inoculum)	Growth	20.5

\*Ct, cycle at which there is a significant increase in fluorescence, which is associated with an exponential growth of PCR product during the log linear phase. HSV, herpes simplex virus; PCR, polymerase chain reaction.

product during the log linear phase. A positive and negative control was included in each run.

## RESULTS

All non-spore forming bacteria were effectively eradicated within 20 minutes of exposure to 10% neutral buffered formalin and UMFix (table 1). There was one colony of *M fortuitum* remaining after this short exposure; however, there was a significant seven log killing of the organisms. When this experiment was repeated, there was complete killing for both fixatives. The yeast suspensions were as susceptible to the killing action of formalin and UMFix as were the vegetative bacteria (table 1). Two conidia suspensions prepared from *Aspergillus fumigatus* and *Aspergillus niger* were as susceptible to the fixative reagents as the vegetative yeast cells.

When the *B subtilis* spore suspension was used as the test organism, there was no apparent killing within the first 20 minutes of exposure to both of the fixatives (table 2). However, the 10% neutral buffered formalin did kill the spore suspension when the exposure time was prolonged, whereas UMFix had no effect on the spore suspension—there was no significant difference between the colony forming units/ml in the water control and UMFix. Similar results were obtained when the spore suspension of *B anthracis* was tested in these two fixatives. No killing was evident for the spore suspension, but the vegetative cell suspension was effectively killed within 20 minutes.

HSV was killed within 20 minutes of exposure to UMFix (table 3). To determine the HSV specific DNA content of the suspensions, a 1/50 dilution of each suspension was examined by real time PCR. Previous results using a pGEM HSV plasmid showed that UMFix had no detrimental effect on the nucleic acid. The plasmid was treated with UMFix then diluted in water before testing (fig 1). Because of its high alcohol content, the UMFix solution is inhibitory to the PCR reaction, but once it is diluted 1/10 or 1/100 in water, the real time PCR reaction goes to completion. The Ct for the sample that was originally suspended in UMFix was the same as the sample diluted in water, indicating that the fixative did not alter the HSV nucleic acid. The Ct values for the 1/10 and 1/100 dilutions of the control were 31.32 and 34.95, respectively, whereas the Ct values for the 1/10 and 1/100 dilutions of the UMFix treated sample were 31.48 and 35.07, respectively. The results shown in table 3 demonstrate that the HSV viral particle DNA was recoverable after treatment. The mean Ct values for the control sample and

## Take home messages

- UMFix is a newly developed fixative based on methanol and polyethylene glycol that has improved nucleic acid preserving properties and safety aspects compared with formalin based fixatives
- UMFix was found to have a microbicidal effect on vegetative bacteria, mycobacteria, yeast, and viruses after a short exposure, but has no sporocidal effect
- Because no killing was seen when polyethylene glycol was used alone, methanol must be responsible for the bactericidal properties of this fixative

UMFix sample were 30.7 and 31.2, respectively. In addition, the original HSV inoculum gave a Ct of 20.5. It is known that each log dilution of target in the real time PCR assay represents approximately a 3.5 Ct difference.<sup>11</sup> The final dilution of the virus that was tested in the saline and UMFix was 1/1000. The value obtained in the solutions is approximately 10.5 Ct units higher and consistent with the dilutions used in the assay.

To determine the agent responsible for the bactericidal activity of UMFix, each active ingredient was tested against *Staphylococcus aureus*, *Enterococcus faecium*, and *Escherichia coli* (table 4). We found that a concentration of 50–60% was bactericidal for these organisms. The polyethylene glycol had no bactericidal activity when it was tested alone.

## DISCUSSION

The antimicrobial properties of alcohol are well established. Therefore, it is not surprising to find that UMFix, which has a high methanol content, kills vegetative bacteria, mycobacteria, and fungi. Ethyl and isopropyl alcohols are commonly used for disinfection; they exhibit a broad spectrum of activity against vegetative bacteria, fungi, and viruses. The relative activity of the aliphatic alcohols (methanol, ethanol, or propanol) has been shown to increase with an increase in the molecular weight. A 70–80% ethyl alcohol solution was bactericidal to vegetative bacteria within 90 seconds in suspension tests.<sup>12</sup> Isopropyl alcohol was found to be slightly more bactericidal than ethyl alcohol.<sup>13</sup> It is also known that they have little or no activity against bacterial spores. It was shown that methanol in concentrations up to 95% is not effective at killing anthrax spores.<sup>14</sup> These data were substantiated by Morton, who found that *B anthracis* spores were completely resistant to methanol at concentrations of 0.004% to 95%, but were killed by ethanol and isopropanol.<sup>13</sup> The sporocidal activity of alcohols could be increased by the addition of sodium hypochlorite to the alcohol.<sup>8,9</sup> Our results showing that UMFix is not an effective killer of bacterial spores is in line with expectations based on this previous research. Because UMFix is used in combination with rapid tissue processing, which incorporates microwave energy,<sup>10</sup> we tested whether this facilitated the killing of spores. However, we found no significant reduction of spore survival, so that microwave energy does not appear to contribute to the bactericidal activity.

It is well documented that enveloped viruses are susceptible to the killing effects of alcohol, and we found that HSV was killed by UMFix. It has previously been shown that a concentration of 70% ethanol for 10 minutes is sufficient to inactivate poliovirus type 1, coxsackie B-1, ECHO 6, adenovirus type 2, HSV, vaccinia virus, and influenza virus (Asian strain).<sup>15</sup> Previous studies also showed that human immunodeficiency virus infectivity was undetectable after

**Table 4** The killing action of various concentrations of methanol against *Staphylococcus aureus*, *Enterococcus faecium*, and *Escherichia coli* for two time periods of exposure

Methanol reagent (%)	<i>S aureus</i>		<i>E faecium</i>		<i>E coli</i>	
	2 min	20 min	2 min	20 min	2 min	20 min
0	+	+	+	+	+	+
10	+	+	+	+	+	+
20	+	+	+	+	+	+
30	+	+	+	+	+	+
40	+	+	+	+	+	+
50	±	–	+	+	–	–
60	–	–	–	–	–	–
70	–	–	–	–	–	–
80	–	–	–	–	–	–
90	–	–	–	–	–	–
100	–	–	–	–	–	–

+, growth; ±, decreased growth; –, no growth.

one minute of exposure to 70% ethanol.<sup>16</sup> Our results suggest that methanol (as part of UMFix) has a similar efficacy to ethanol when their ability to kill viruses is compared.

“It is well documented that enveloped viruses are susceptible to the killing effects of alcohol, and we found that herpes simplex was killed by UMFix”

In conclusion, our results confirm that because of its methanol content, UMFix has a microbicidal effect on vegetative bacteria, mycobacteria, yeast, and viruses after a short exposure, but does not have a sporocidal effect. All of these studies were performed using cell free organisms. A recent study demonstrated that mycobacteria may remain viable in formalin fixed tissue for prolonged amounts of time.<sup>17</sup>

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