

# Finding an Alternative to Formalin for Sterilization of Extracted Teeth for Teaching Purposes

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**Abstract:** Formalin is a known carcinogen, so there is a need to establish whether a safer alternative is available for the sterilization of human teeth destined for use in clinical training. Any disinfectant that is not capable of sterilizing 100 percent of the samples tested should be considered a failure. In this study, biofilms of oral bacteria were grown on previously autoclaved extracted human teeth. These biofilm-laden teeth were then screened against a range of disinfectants for an exposure time of seven days in a laboratory refrigerator. Culture methods were employed to validate the sterility of the tooth samples. Five percent Virkon and Gigasept PA proved effective against the laboratory model of disinfection and were carried forward to challenge freshly extracted human teeth. Gigasept PA was the only disinfectant that sterilized 100 percent of the tooth samples. Gigasept PA should be considered a safer and effective alternative to formalin for the sterilization of extracted teeth destined for teaching purposes.

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Extracted teeth are frequently collected under appropriate ethical guidelines for research purposes, clinical trials, product testing, and educational use. In the educational setting, extracted teeth enable a wide variety of practical and technical skills to be taught in the preclinic and are highly valued to those students who use them.<sup>1</sup> This prior tactile knowledge of the dentition is important for dental students to experience before they are asked to remove enamel from patients as part of their training.

The potential for the transmission of microorganisms within the dental clinical practice is well documented.<sup>2</sup> However, this risk reaches beyond the dental clinic since viable microorganisms have been detected from aspirates originating from extracted teeth used by students during their preclinical training.<sup>3</sup> These concerns led to the U.S. Centers for Disease Control and Prevention guidelines for infection control of extracted teeth used for research and teaching to support safe dental practice, which stipulate that all extracted teeth must be sterilized prior to use for teaching.<sup>4</sup> The definitive method of sterilization for the complete inactivation of bacteria is autoclaving with high-pressure steam, typically at 121° C for a minimum of fifteen minutes. However, autoclaving affects the mechanical properties of the teeth, causing them to become brittle and routinely

fracture after repeated sterilization cycles when used for in vitro experiments.<sup>5</sup>

At Liverpool University Dental Hospital (LUDH), the current policy is to place extracted teeth in 10 percent formalin (Sigma-Aldrich, Poole, UK) for seven days before incorporating them into the phantom head teaching suite. This procedure has been previously shown to sterilize the teeth.<sup>6-8</sup> Formaldehyde (CH<sub>2</sub>O) is an organic compound, a noxious, colorless gas at room temperature, that when mixed with aqueous solution forms formalin, which is classified as both cytotoxic and genotoxic, results in injury upon contact with skin and the eyes and can cause upper respiratory track irritation upon inhalation.<sup>9</sup> More importantly, formaldehyde has been classed by the International Agency for Research on Cancer as “carcinogenic to humans.”<sup>10</sup> Longitudinal studies of formaldehyde exposure have displayed evidence of an increase in the incidence of nasopharyngeal cancer<sup>11</sup> and a causal association with myeloid leukemia;<sup>12</sup> there is also limited evidence of an association with sinonasal adenocarcinoma.<sup>13</sup> In order to conform to current health and safety requirements, formalin solutions should only be handled within a fume-hood, which may not be available or convenient.

One of the fundamental questions surrounding the Control of Substances Hazardous to Health

(CSHH regulations, Health and Safety Executive, United Kingdom) is “Is there a safer alternative available?” The aims of this study were to develop a laboratory model capable of screening a range of commercially available disinfectants under reproducible conditions to determine if there is a suitable alternative to formalin that is capable of reducing the microbial load of modelled extracted teeth and ultimately freshly extracted teeth, to zero. Any disinfectant that is not capable of sterilizing 100 percent of the samples tested should be considered a failure.

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## Materials and Methods

LUDH has a long-standing ethical approval process for the retention of extracted teeth for “teaching and research purposes” with informed, signed consent from the patient. Extracted teeth without amalgam restorations were retained from various LUDH clinics before their transfer to the oral microbiology laboratories. Prior to any further handling, the teeth were first autoclaved en masse at 121° C for fifteen minutes in order to kill any potentially harmful bacteria. The teeth were then thoroughly rinsed with deionized water to remove excess soft tissue debris before being re-autoclaved in order to sterilize the teeth prior to use in laboratory assays.

Two milliliters of fresh human saliva were inoculated into 10 ml of brain heart infusion (BHI) broth (Sigma-Aldrich) and vortex mixed. The resulting mixture served as the inoculum. Sterile teeth were individually placed into 20 ml of BHI held within 25 ml universal containers before 100 µl of the inoculum was added. Employing a small headspace above the liquid broths encouraged the growth of facultative anaerobes without the need for anaerobic incubation.<sup>14</sup> The samples were then incubated in an orbital shaker at 37° C for seven days to form a liquid culture together with a biofilm of oral bacteria on the tooth’s surface.

Following incubation, the teeth were removed from the broth and gently rinsed with 10 ml of sterile phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK) applied by syringe to remove excess liquid culture and unadhered bacterial cells. This procedure was carried out in a class II safety cabinet (BioMAT-2, Medical Air Technology, Oldham, UK) to protect workers from potentially harmful aerosols. The teeth were then placed into 10 ml of a variety of commercially available disinfectants (prepared as per manufacturer’s instructions, unless otherwise

stated) for a period of one week whilst refrigerated at 4° C. The disinfectants used in this study were the following: sodium hypochlorite (NaOCl) at 1 percent and 5.25 percent (Sigma), saturated thymol solution (Sigma, 0.1 percent weight/volume), Microsol3 (Anachem, Luton, UK) (active ingredients: potassium peroxymonosulfate, sodium dodecylbenzenesulfonate and sulphamic acid), Virkon at 1 percent and 5 percent (Dupont RelyOn, Suffolk, UK) (active ingredients [proprietary information]: triquaternary cationics, amine salts, and halogenated compounds), and Gigasept PA (Schülke UK, Sheffield, UK) (active ingredient: peracetic acid). All of the commercially available disinfectants were made in accordance with the manufacturer’s recommendation, with the exception of the x5 strength Virkon solution. PBS was included as a negative control, along with the direct transfer of samples to fresh BHI.

Following exposure, the teeth (n=10-24) were extricated from the disinfectant, then rinsed in 10 ml of PBS to minimize the carryover of antimicrobial solution before being placed into 20 ml of fresh BHI. The samples were then returned to the orbital shaker at 37° C for seventy-two hours. Turbidity within the broth was assumed to originate from viable bacteria remaining on the tooth’s surface. Any samples that were deemed dubious at this juncture (i.e., slight turbidity without obvious suspended bacterial matter) were swabbed onto blood agar (Oxoid) to determine whether viable bacteria were present within the liquid growth medium.

Disinfectants that proved effective in the biofilm model were carried forward to evaluate their efficacy with teeth that had been freshly extracted from patients attending LUDH. Vials containing 10 ml of disinfectant were taken to the clinic and extracted teeth were placed directly into them. These samples were then processed as described previously (seven days contact time at 4° C followed by transfer to BHI).

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## Results

Tooth samples were prepared at various stages of the experiments for gram-staining and plating onto solid growth media to confirm the presence of known oral bacteria, such as *Streptococcus spp.* and *Actinomyces spp.* (data not shown). The protocol was validated by appropriate controls to confirm that a biofilm containing oral bacteria was present on the teeth and that the development of turbidity in the

BHI broths indicated the failure of the disinfectant to kill all of the incumbent bacteria. An indicative measurement of the microbial load present upon a typical tooth, following seven days' biofilm growth, was  $6.7 \times 10^7$  colony forming units.

Thymol was the least effective of the disinfectants tested in the in vitro biofilm model as it failed to sterilize any of the tooth samples. NaOCl was also shown to be ineffective at concentrations of both 1 percent and 5.25 percent. Microsol3 and Virkon at 1 percent proved effective disinfectants but failed to sterilize a number of samples. Using 1 percent Virkon that had been prepared seven days previously did not measurably affect its ability to disinfect teeth. Virkon at a concentration of 5 percent and Gigasept PA were the two most effective tooth disinfectants as they were able to sterilize 95 percent (i.e., one positive culture from twenty) and 100 percent of the samples, respectively (Table 1). Only the two most effective disinfectants, Virkon (5 percent) and Gigasept PA, were progressed from the in vitro experiments to testing their ability to sterilize freshly extracted teeth. Virkon (5 percent) failed to kill all bacteria in two out of the fourteen samples, whereas Gigasept PA was demonstrated to be 100 percent effective at sterilizing extracted teeth (Table 2).

**Table 1. Efficacy of disinfectants on extracted teeth laden with a biofilm of oral bacteria**

Treatment	Number of Model Teeth Sterilized/Tested	Percentage Sterilized
Gigasept PA	24/24	100%*
MicroSol3	18/20	90%
Sodium hypochlorite (1%)	3/21	14.2%
Sodium hypochlorite (5.25%)	5/14	35.7%
Thymol	0/10	0
Virkon (1% 7-day-old)	8/10	80%
Virkon (1% fresh)	16/21	76.2%
Virkon (5%)	19/20	95%*

\*Disinfectants carried forward to test against freshly extracted teeth.

**Table 2. Efficacy of disinfectants against freshly extracted teeth**

Treatment	Number of Extracted Teeth Sterilized/Tested	Percentage Sterilized
Gigasept PA	14/14	100%
Virkon (5%)	12/14	85.7%

## Discussion

For the purposes of this discussion, disinfection refers to an action that reduces the microbial load present on the surface of an object, whereas sterile refers to an object without a detectable microbial load. By this definition, it is possible to disinfect an object to the point at which it becomes sterile. If this action is executed within the confines of a validated procedure, it can be termed sterilization as opposed to (mere) disinfection.

There have been a number of studies on the sterilization of extracted human teeth. Two of these used freshly extracted teeth immersed into a range of agents.<sup>6,8</sup> A third study artificially seeded the pulp chamber of extracted teeth with a culture of *Bacillus stearothermophilus* before disinfection.<sup>7</sup> All of these protocols incubated the teeth at room temperature for one week and also included autoclaving as one of their methods. However, none of these protocols incorporated a rinsing step to militate the direct transfer of disinfectant into the subsequent detection culture. All three of these experiments showed that 10 percent formalin and autoclaving sterilized all of the extracted teeth. Those studies that employed thymol agreed with our findings, in that thymol was unable to sterilize any of the samples. None of the other disinfectants proved capable of sterilizing 100 percent of the samples with the exception of 5.25 percent NaOCl in the Lodayekar et al. study;<sup>6</sup> however, the Dominici et al.<sup>7</sup> study and our own disagreed with this finding. A perceived weakness of these and other protocols that have evaluated disinfectants against extracted teeth is the inherent variations in the microbial composition, microbial load, and biofilm matrix composition between individuals. It was considered prudent to reduce the perceived variability in microbial load between different extracted teeth by forming an in vitro biofilm of salivary bacteria on the tooth's surface under reproducible conditions.

The manufacturers of Virkon recommend that the solution should be replaced every seven days. A 1 percent solution of Virkon was prepared seven days in advance so that it would be at the extreme end of its recommended shelf life at the initiation of the experiment and therefore beyond it after a further seven days. However, the results showed that there was no appreciable difference in the efficacy of "fresh" or "old" Virkon in this model. Teeth that had been stored in 5 percent Virkon developed a deep pink discoloration, but this faded during the subsequent

immersion in BHI broth. Although Virkon is only intended for use at a concentration of 1 percent, its intermediate success at this concentration warranted further investigation at higher concentrations.

BHI broths containing teeth that had been previously immersed in Microsol3 had a tendency to cast a slight turbidity into the liquid following incubation. In all cases, the slight turbidity was found to be aseptic. A possible explanation for this turbidity is that Microsol3 had a disruptive effect upon the biofilm matrix during the disinfection phase and that these components were subsequently released into the BHI broth when agitated during incubation. Further studies would be required to ascertain the antimicrobial efficacy of the updated product, Microsol3<sup>+</sup> (Anachem). NaOCl proved ineffective in these experiments, notwithstanding the fact that a 1 percent solution has been previously shown to be capable of sterilizing biofilms of *Enterococcus faecalis* within sixty seconds.<sup>15</sup> This is possibly due to hypochlorous acid being neutralized by organic components within the tooth or biofilm matrix.<sup>16</sup>

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## Conclusions

No viable bacteria were detected in extracted teeth following immersion in Gigasept PA for seven days at 4° C. This was first demonstrated in an in vitro laboratory model using teeth laden with a biofilm of salivary bacteria and then subsequently using freshly extracted human teeth. Gigasept PA is a potentially safe alternative to formalin for sterilizing extracted teeth for educational use.

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