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Potential biological indicators for glutaraldehyde and formaldehyde sterilization processes

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Abstract The present study aimed to isolate, select, and evaluate bacterial isolates with potential for use as biological indicators for sterilization with glutaraldehyde and/or formaldehyde. A total of 340 local *Bacillus* isolates were screened for glutaraldehyde and/or formaldehyde resistance by determination of minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), and extinction time and were compared with *B. subtilis* (var. *niger*) ATCC 9372, the biological indicator for ethylene oxide sterilization, as reference. Of these, 85 isolates had glutaraldehyde MICs of 0.5% or higher, while 29 had formaldehyde MICs of 0.04% or higher. Of the 29 resistant isolates, 15 had MBCs of 0.05% or more. Extinction times were used to evaluate the bactericidal/sporicidal activity of glutaraldehyde. Eight had inactivation times of more than 5 h in 2% glutaraldehyde (pH 8), whereas 12 had inactivation times of more than 3 h in 1% formaldehyde, with one isolate in common. These 19 isolates were selected and evaluated as potential biological indicators for aldehydes by determination of the decimal reduction times (*D* values), compared with the reference strain. Eight glutaraldehyde-resistant isolates exhibited *D* values 2.0- to 3.5-fold higher than the reference strain (30 min.). Only five of 12 formaldehyde resistant isolates had *D* values higher than that of the reference strain. Using six resistant isolates, temperature coefficient values between 2.11 and 3.02 were obtained for 2% formaldehyde. Finally, 14 isolates were tested for potential pathogenicity and were identified to species level. All of the eight glutaraldehyde-resistant isolates, including the isolate with dual resistance, and three formaldehyde-resistant isolates were *B. licheniformis*, while two other formaldehyde-resistant isolates were *B. cereus*. Six of the selected *B. licheniformis* isolates are potential biological indicators for sterilization processes using aldehydes. Three can be suggested for glutaraldehyde only and three for both aldehydes.

Keywords Biological indicators · Formaldehyde · Glutaraldehyde · Sterilization

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Introduction

Different physical, chemical, and biological indicators are used to monitor sterilization cycles, but biological monitoring is the only means that integrates all sterilization parameters [5, 6]. Biological indicators are recognized as the closest to ideal monitors for sterilization processes [17] and they are equivalent or superior to physical measurements [37]. They are also the only efficient way to control many processes associated with sterilization [23].

Aldehydes are increasingly used as sterilizing agents. Glutaraldehyde is widely used in hospitals, particularly for heat-sensitive, flexible endoscopes [1]; and gaseous sterilization with formaldehyde (low-temperature steam and formaldehyde, LTSF) is replacing ethylene oxide for sterilization of heat-labile equipment, electric equipment, and objects made of heat-labile plastics in hospitals in Europe [27]. So far, however, there are no specific methods available for biological monitoring of these processes. *Bacillus stearothermophilus* spores, the biological indicator currently used in steam sterilization processes, is unreliable in LTSF [4, 12].

Spores of *B. subtilis* var. *niger*, used as a biological indicator in ethylene oxide sterilization are sensitive to LTSF [13] and no universally accepted or documented efficiency is available [25]. Therefore, there is no

currently available biological indicator that is specific and reliable for use in LTSF [20]. However, *B. stearothermophilus* preparations are used for monitoring LTSF in Europe and, in Sweden, *B. subtilis* spores are used [27].

However, despite the increasing use of glutaraldehyde as a liquid sterilant [1], there is no officially recommended biological indicator for this process.

The aim of the current work is to isolate and select aerobic spore-forming bacterial strains with uniformly high resistance to these aldehydes and to evaluate their potential as biological indicators in sterilization processes with these agents.

Materials and methods

Chemicals

Analytical grade glutaraldehyde (pentadial or glutaric dialdehyde) solution (25%) was obtained from Riedel–del Haen (Seelze, Germany). The sterilizing glutaraldehyde solution Cidex, containing 2.2–2.5% glutaraldehyde ready for activation by adding 0.6 g of sodium bicarbonate/l, was obtained from Johnson and Johnson Medical Limited, (Bracknell, UK). Formalin (37% formaldehyde solution stabilized with 10–15% methanol) was obtained from Fluka Chemie (Buchs, Switzerland). All other chemicals were analytical grade obtained from E Merck (Darmstadt, Germany).

Culture media

The media used included nutrient broth, nutrient agar (NA), trypticase soy broth (TSB; soy bean casein digest broth [35]), trypticase soy agar (TSA; soy bean casein digest agar [35]), Müller–Hinton broth (MHB) and agar [33], dextrose tryptone broth, fortified nutrient agar for optimum spore formation [8], and tryptose–phosphate broth (TPB) and agar [19]. All media were the products of Difco Laboratories (Detroit, Mich., USA). Media were sterilized by autoclaving at 121 °C for 15 min.

Diluents, buffers, and test cultures

Ringer's solution [11] and 0.1 M phosphate buffer (pH 7, pH 8 [28]) were used. *B. subtilis* (var. *niger*) ATCC 9372 spores loaded on strips of paper (2.2×10^6 spores/strip), used a as biological indicator for sterilization with ethylene oxide, were obtained from the Sterilator Company (Holland, Ohio, USA).

Isolation of *Bacillus* strains

Samples were collected from dust, in bench-top surface swabs from chemistry laboratories, and in soil samples. For soil samples, 2 g were suspended in 20 ml of sterile distilled water and shaken for 1 h. Then, 0.1-ml portions of the suspensions were used to inoculate two sets of enrichment broth medium containing 0.01%, 0.02%, or 0.05% formaldehyde and incubated at either 22 °C or 35 °C for 2 weeks.

Tubes showing growth were used to inoculate nutrient agar plates and were incubated at 37 °C for 48 h. Colonies of Gram-positive spore-forming rods were selected and used to inoculate nutrient agar slopes that were incubated at 37 °C. Cultures of pure isolates were stored at −70 °C. Subcultures were made weekly.

Standardized suspensions of the test bacteria and spores were prepared as follows: 5-ml aliquots of overnight cultures in TSB were used to inoculate TSA in Roux flasks that were incubated at 35 °C for either18 h (for bacteria) or 1 week (for spores). The cells were collected by centrifugation, washed twice, and resuspended in quarter-strength Ringer's (QSR) solution. The suspensions were diluted to contain from 5×10^5 colony-forming units (cfu)/ml to 5×10^6 cfu/ml, according to a previously prepared linear relationship between optical density and total counts.

Minimum inhibitory concentration

To assess the minimum inhibitory concentration (MIC), test-tubes holding 10 ml of liquid medium containing formaldehyde (0.015–0.06%) or glutaradehyde (0.15–0.5%), or controls containing plain medium were inoculated with 0.1 ml of the standardized suspension of a test organism to make the final microbial suspension between 2×10^4 cfu/ml and 2×10^6 cfu/ml [3]. Tubes were incubated at 35 °C for 24 h and 48 h and examined for signs of growth. MHB was used for glutaraldehyde instead of TPB [19], which gave unsatisfactory results in preliminary tests, whereas TSB was used for formaldehyde [33].

Minimum bactericidal concentration

To assess the minimum bactericidal concentration (MBC), Aliquots (50 µl) of the suspensions in MIC tubes showing no signs of growth after incubation were spread on the surface of NA plates containing the appropriate neutralizing agent, incubated at 35 °C for 48 h, and colonies were counted. Similar volumes of positive controls at zero time were also plated. The lowest concentration causing a reduction in the colony count by 99.9%, compared with the positive controls, was considered as the MBC [31].

For formaldehyde, 100 µl from MIC tubes showing no growth after 48 h incubation at 35 °C were subcultured into 10 ml of TSB containing 0.5% ammonium chloride and incubated at 35 °C for 48 h. The lowest concentration showing no growth after subculture and incubation was considered as the MBC.

Determination of extinction time

Spore suspensions ($1–2\times10^6$ cfu/ml) were made in filter-sterilized solutions of either 2% alkalinized glutaraldehyde [15] or 1% formaldehyde in 0.1 M phosphate buffer (pH 7 or pH 8). Reaction mixtures were maintained at 23 °C and 1-ml samples were taken at 1-h intervals (up to 9 h for glutaradehyde or 3 h for formaldehyde). Samples were immediately added to equal volumes of either 4% glycine HCl or 2% ammonium chloride in QSR solution for glutaraldehyde or formaldehyde, respectively. After 30 min, 1-ml volumes were subcultured into 10 ml of TSB and incubated at 35 °C for up to 1 week. Spore suspensions of *B. subtilis* ATCC 9372 were treated similarly and used as controls. Glutaraldehyde (3% or 4%) or formaldehyde (1.5%, 2%, 3%) was used for spores not killed within the test time (9 h for glutaraldehyde or 3 h for formaldehyde). Sampling time intervals were reduced to 20 min or 30 min for high formaldehyde concentrations.

Determination of *D* value

Spore suspensions ($1–2\times10^6$ cfu/ml) were made in either 2% alkalinized glutaraldehyde solution [16] or 2% formaldehyde in 0.1 M phosphate buffer (pH 7). Reaction mixtures were maintained at 23 °C and samples were taken at zero time, after 20, 40, or 60 min, and then at 1–h intervals up to 6 h for viable counting after inactivation of the antimicrobial activity.

For inactivation of the antimicrobial activity, treated spores were equilibrated with either 2% glycine HCl or 1% ammonium chloride in QSR solution for 30 min, for glutaraldehyde or formaldehyde, respectively, before plating them on TSA.

Equivalent spore suspensions in buffer without glutaraldehyde or formaldehyde were treated similarly and used as controls. Logarithms of the means of three counts for each sample were plotted versus time. *D* values were calculated from survival curves, according to Soper and Davies [32].

The temperature coefficient was calculated after graphic representation and determination of the *D* values at 23 °C and 33 °C [32].

To exclude potential pathogenicity of the candidate isolates, groups of five rabbits for each isolate were injected intraperitoneally with 1 ml of either a live or heat-killed spore suspension containing approximately 1×10^6 cfu/ml and the rabbits were observed for 2 weeks for signs of illness or fatality.

Physiological and biochemical tests for identification of *Bacillus* species were performed according to Claus and Berkeley [10]. The identities of the isolates were confirmed using the API 50 CHB system. *Bacillus* isolates were subcultured on slants of fortified NA, incubated for 1 week at 35 °C for maximum spore production, protected from drying, and stored at 2–10 °C for 1 year [10].

Results

Determination of MIC

The reference strain had MICs of 0.35% and 0.01% for glutaraldehyde and formaldehyde, respectively. Of the 340 isolates, 255 were inhibited by 0.4% glutaraldehyde; and the remaining 85 isolates comprised 52 with MICs of 0.5%, 19 with MICs of 0.6%, and one with a MIC of >0.6%.

For formaldehyde, 311 isolates were inhibited by 0.03% formaldehyde, 23 had MICs of 0.04%, six had MICs of 0.05% or more, and one isolate had a MIC of >0.06%.

MICs of >0.4% and >0.03% were taken as parameters for resistance to glutaraldehyde and formaldehyde, respectively. The number of isolates showing resistance to formaldehyde only, glutaraldehyde only, and both aldehydes were 20, 116, and 70, respectively.

Resistance to bactericides and sporicides

MBCs of formaldehyde were determined for the 29 isolates showing relatively high resistance to formaldehyde (MICs >0.03%). The reference strain and 14 of the isolates tested had a MBC of 0.04%. Five had MBCs of 0.05% and the remaining ten isolates had MBCs of >0.05%. For glutaraldehyde, MBC results were irreproducible.

For evaluation of the bactericidal/sporicidal activity, the extinction times were determined. The 85 glutaraldehyde-resistant isolates (MICs >0.4%) and the 29 formaldehyde-resistant isolates (MICs >0.03%) were used.

For glutaraldehyde, results of those isolates with a death time of 3 h or more in 2% solution are shown in Table 1.The results for formaldehyde-resistant isolates are shown in Table 2.

Table 1 Death time of resistant spores treated with glutaraldehyde. Subculturing was in trypticase soy broth (TSB), incubated for 1 week at 35 °C. Those isolates showing a death time less than 1 h with 2% gluraraldehyde are not listed

Isolate number	Death time (h) in glutaraldehyde		
	2%	3%	4%
29	>5	>4	2
34	3	2	<1
39	3	2	<1
43	>5	>4	<1
72	3	2	<1
85	>5	4	2
90	5	3	<1
99	5	3	<1
147	4	3	<1
150	4	2	<1
155	4	3	<1
157	5	2	<1
159	5	2	<1
160	>5	>4	<1
161	3	2	–
162	>5	4	2
165	5	3	<1
167	4	3	<1
175	5	3	<1
182	5	3	<1
201	4	3	<1
205	4	3	<1
206	5	2	<1
221	>5	4	<1
228	4	2	<1
234	3	2	<1
242	3	2	<1
251	>5	3	<1
269	4	2	<1
271	>5	4	<1
Bacillus subtilis ATCC 9372	3	>1	–

Twenty isolates showing resistance to either aldehyde or both were subjected to a *D* value determination for the respective aldehyde.

Alkalinized 2%glutaraldehyde (pH 8) and buffered 2% formaldehyde (pH 7) solutions were used. The *D* values (Table 3) were obtained from the survival curves.

Temperature coefficients for 2% formaldehyde solution using six selected formaldehyde-resistant spore preparations and the reference spores were calculated from the *D* values (Table 4).

Spore suspensions of isolates showing *D* values greater than those of the reference strain were inoculated intraperitoneally into healthy rabbits at a dose of 10^6 cfu/ml of saline. The rabbits showed no signs of illness during the 2-week period of observation.

Identification of the selected isolates

Potentially useful isolates were identified to species level. For formaldehyde-resistant isolates, those having *D* values higher than the reference strain (isolates 43, 58, 215, 228, 325) and those

Table 2 Death time of resistant spores treated with formaldehyde at different concentrations. Subculturing was in TSB, incubated for 2 weeks at 35 °C

Isolate number	Death time (min) in formaldehyde		
	1%	1.5%	2%
8	< 60	< 45	< 20
10	< 60	< 45	< 20
22	< 60	< 45	< 20
24	< 60	< 45	< 20
33	< 60	< 45	< 20
42	< 60	< 45	< 20
43	> 180	> 90	> 40
44	< 60	< 45	< 20
58	> 180	90	> 40
65	> 180	90	> 40
68	> 180	> 90	> 40
70	> 180	> 90	> 40
74	< 60	< 45	< 20
105	< 60	< 45	< 20
111	> 180	60	< 20
131	120	< 45	< 20
144	< 60	< 45	< 20
154	> 180	60	< 20
156	120	< 45	< 20
176	120	< 45	< 20
185	< 60	< 45	< 20
190	< 60	< 45	< 20
202	> 180	90	< 20
211	> 180	60	40
215	> 180	> 90	> 40
228	> 180	> 90	< 20
229	< 60	< 45	< 20
281	120	60	40
325	> 180	> 90	> 40

Table 3 *D* values [32] for selected isolates and reference standard strain treated with either 2% glutaraldehyde (pH 8) or 2% formaldehyde (pH 7). *ND* Not determined

Isolate number	*D*value (min)	
	Glutaraldehyde	Formaldehyde
29	77.5	ND
43	96	175
58	ND	69
65	ND	36
68	ND	37
70	ND	41
85	98	ND
111	ND	50
154	ND	22
160	66	ND
162	83	ND
202	ND	44
211	ND	35
215	ND	86
221	67	ND
228	ND	87
251	110	ND
271	78	ND
325	ND	120
B. subtilis (var. *niger*)ATCC 9372	30	54

showing high resistance as demonstrated by MIC, MBC, and extinction time data (isolates 202, 211) were selected.

Table 4 Temperature coefficients [32] of formaldehyde using some resistant isolates and the reference strain

Isolate number	Temperature coefficient	
	Q_{10}	θ
43	3.02	1.1168
202	2.53	1.0972
211	2.74	1.1062
215	2.11	1.0445
228	2.27	1.0850
325	2.42	1.0924
B. subtilis (var.*niger*) ATCC 9372	2.16	1.0801

For glutaraldehyde, those isolates having *D* values higher than the reference strain (isolates 29, 43, 85,160, 162, 221, 251, 271) were used. Identification was based on cultural characteristics, microscopic features, spore shape and position, and on biochemical and physiological features [10]. The identities of the isolates were also confirmed by the API 50 CHB system and results are presented in Table 5.

Discussion

Although the use of glutaraldehyde and formaldehyde is increasing in sterilization practices, there are no specific biological indicators for monitoring these processes. An ideal biological indicator should be an aerobic, nonpathogenic, resistant spore [20]. The indicator must also show an efficient recovery after exposure to the sterilization process [24].

High-resistance stability characteristics, a linear semilogarithmic survivor curve and a high growth index are required for a biological indicator [36]. Determination of MICs for glutaraldehyde presented a problem because of the high reactivity of the chemical [16, 29, 30]. According to Hill et al. [19], TPB was used for MIC determination for glutaraldehyde. However, in preliminary experiments, this medium showed a deep blackening after the addition of glutaraldehyde and the results were inconsistent. Therefore, MHB was used instead with glutaraldehyde.

Preliminary tests (Fig. 1) revealed that inclusion of the neutralizing agents glycine or ammonium chloride in the recovery medium reduced the chances of recovery of injured spores, compared with media without them (data not shown). Similar observations were previously reported [9, 22]. Treatment with the neutralizing agents before culture gave better results.

Because of the lack of reproducibility of MBC results, especially with glutaraldehyde, other techniques for evaluation of bactericidal activity were sought. The suspension test for determination of the extinction time was the reliable alternative. The MBC and extinction time data enabled further selection of useful isolates. Thus, the eight isolates surviving 5 h exposure to 2%

Table 5 Identification of resistant isolates, according to the API 50 CHBsystem

Isolate number	Significant taxon		Next choice(s)	
	Species	% Identity	Species	% Identity
29	*B. licheniformis*	99.2	*B. subtilis*	0.6
43	*B. licheniformis*	97.7	*B. subtilis*	2.2
58	*B. cereus*	99.1	*B. laterosporus*	0.4
85	*B. licheniformis*	94.3	*B. subtilis*	5.6
			B. amyloliquefaciens	0.1
160	*B. licheniformis*	99.9	*B. marcerans*	0.1
162	*B. licheniformis*	98.8	*B. marcerans*	1.0
202	*B. circulans*	99.9	*B. marcerans*	0.1
211	*B. circulans*	99.9	*B. polymyxa*	0.1
215	*B. cereus*	86.6	*B. mycoides*	13.3
			B. anthacis	0.1
221	*B. licheniformis*	99.2	*B. subtilis*	0.6
228	*B. licheniformis*	99.2	*B. subtilis*	0.6
251	*B. licheniformis*	99.0	*B. subtilis*	0.8
271	*B. licheniformis*	99.9	*B. subtilis*	0.1
325	*B. licheniformis*	99.9	*B. subtilis*	0.1

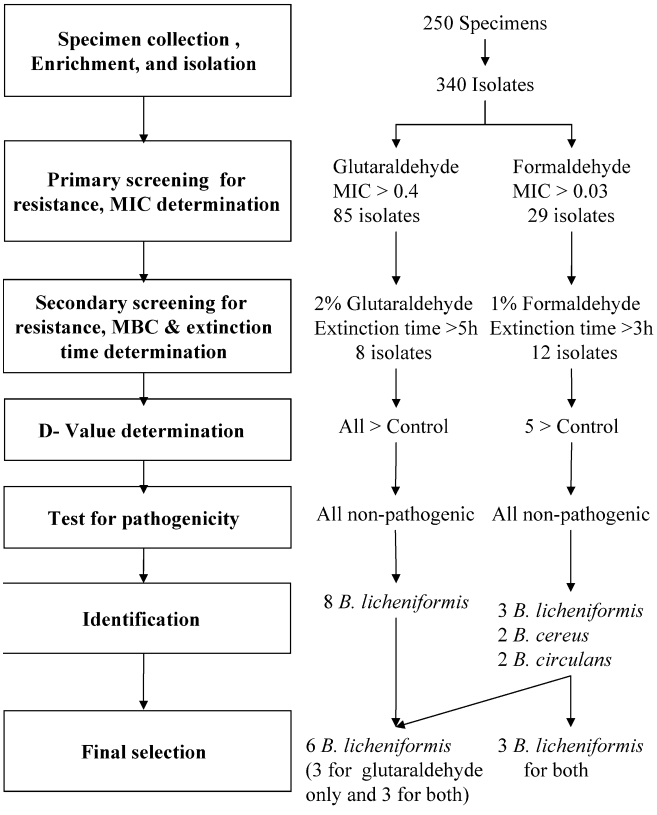

Fig. 1 Flow chart depicting the protocol used for isolation and selection of potential biological indicators. *B. Bacillus*

glutaraldehyde and the 12 resisting treatment with 1% formaldehyde for 3 h were selected for *D* value determination. The *D* value reflects the level of resistance and is a good criterion for the performance of the biological indicator [7]. The eight glutaraldehyde resistant isolates showed *D* values 2.0- to 3.5-fold higher than that of the reference strain. Of the 12 selected formaldehyde-resistant isolates, only five showed *D* values greater than that of the reference strain. Logarithmic plots were linear for all isolates tested, except for isolates 70 and 111, which showed initial shoulders.

One criterion of a biological indicator is freedom from pathogenicity. Within 2 weeks following intraperitoneal injection of the spore suspensions into healthy rabbits, no signs of illnesses or abnormalities were observed, but further investigation using other experimental animals is required before ruling out the pathogenicity of these isolates.

Many demonstrated higher resistance to formaldehyde or glutaraldehyde than the biological indicator currently used for validation of chemical sterilization with formaldehyde. *D* values as high as 110 min were obtained with glutaradehyde for some isolates. The majority of the resistant isolates belong to one species, *B. licheniformis*. Two formaldehyde-resistant isolates were identified as *B. cereus*, which can cause food-poisoning in man and other animals [14, 26, 34]. They were excluded as potential biological indicators. The two *B. circulans* isolates were also excluded based on their inconsistent resistance and poor growth on ordinary culture media.

The selected *B. licheniformis* isolates meet the requirements of good biological indicators, since they are non-fastidious, non-pathogenic [2, 21], do not require special growth requirements, and have consistent *D* values. Strains of *B. subtilis* are used as biological indicators for dry-heat sterilization and chemical sterilization with ethylene oxide, while a strain of *B. pumilus* is used as a biological indicator for radiation sterilization [18]. These facts further support these isolates as candidates for use as biological indicators for sterilization processes with aldehydes.

Six of the *B. licheniformis* isolates can be nominated as biological indicators: isolates 43, 58, and 228 for both aldehydes and isolates 29, 85, and 251 for glutaraldehyde only. Isolate 43 may be recommended as a potential biological indicator for aldehydes. However, further

studies are required before considering these isolates as biological indicators. Such studies would include finding the ideal method of preparation of carrier, quantitation and calibration of such carriers, preservation of these carriers, and the ideal recovery conditions for spores on the carrier.

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