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# Release of bacteria during the purge cycles of steam-jacketed sterilisers

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Abstract: The design of the steam-jacketed steriliser includes an exterior air-gap fixture through which purged chamber aerosols potentially could escape into the ambient environment. Studies of the purge cycle in two steriliser models tested the potential release of a genetically marked *Enterococcus faecalis*, together with *Bacillus stearothermophilus* spores introduced as exposed cultures. Direct plate counts, broth enrichment and polymerase chain reaction analysis were used to confirm any released organisms trapped in an all-glass impinger. From the retrieval of both bacterial strains, an estimated $10^3$ organisms can be released from uncontained bacterial loads of $10^{11}$ *E. faecalis* and $10^7$ *B. stearothermophilus*, even from properly functioning autoclaves. The release of an opportunistic pathogen from steriliser purge exhausts emphasises the importance of proper steriliser location, ventilation, containment of heavily contaminated loads, and adequate steriliser maintenance.

*Key words*: Autoclave. Steam sterilization. Waste management.

## Introduction

Since its inception in the 1930s, the basic design of the modern steam-jacketed (double-walled) steriliser has remained relatively unchanged. Common to all of the models is a feature that reduces the amount of air present prior to the actual sterilisation process. Air is associated with long heat-up times and is generally believed to impede effective sterilisation by interfering with the steam contact of surfaces to be sterilised.[1] Consequently, air is routinely removed from the steriliser chamber via a purge cycle, which employs either a vacuum or steam flush.

In the gravity steriliser, steam entering from the rear displaces and pushes air forward, towards and through a purge vent located in the lower front of the chamber. This air/steam mixture is flushed through a series of exit pipes and is discharged ultimately into the general waste. A thermostatic valve controls this flow, and, at some point beyond this valve, there is an air gap through which the vented air/steam mixture may escape into the surrounding environment (Figure 1). This forced purge of air from the interior, coupled with the air gap located outside the chamber, provides the potential for aerosol formation and venting of uncontained microorganisms from within the autoclave chamber during the purge process.

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## Materials and methods

### *Preliminary studies*

Pilot studies were performed in an AMSCO Model 3021 gravity steriliser (American Sterilizer Company, Erie, PA, USA) using co-inoculations of spores ($10^7$) of *Bacillus stearothermophilus* (ATCC 7953, North American Science Associates, Inc., Northwood, OH, USA) and one of the following clinical isolates: *Escherichia coli, Enterobacter agglomerans, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis* or *Acinetobacter anitratus* (gifts of Linda Perry, New England Medical Center Hospital, Boston, MA, USA). To prevent contamination of the test site area, strains were grown and prepared for inoculation in a laboratory well removed from the actual testing site.

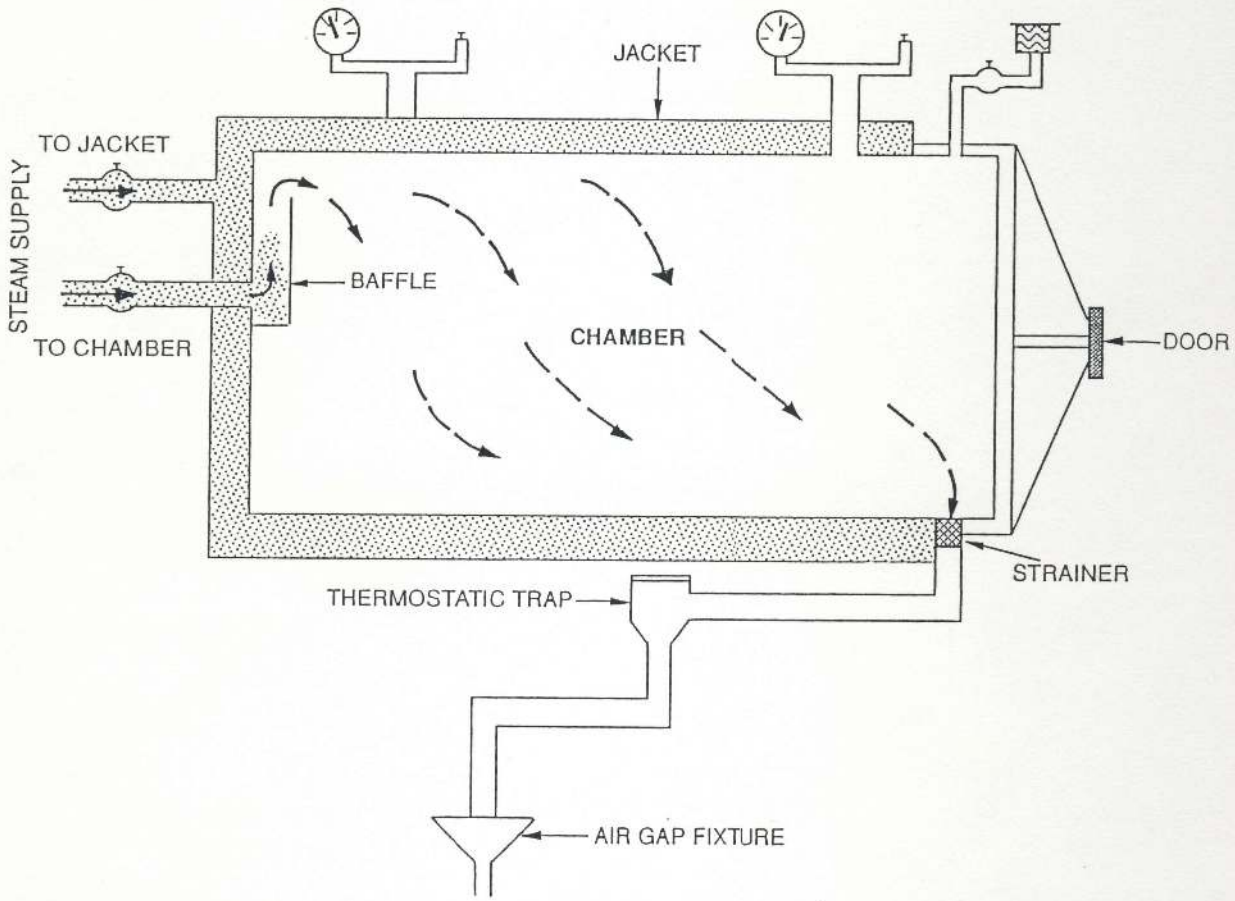


Fig. 1. Longitudinal cross-section of the steam-jacketed steriliser showing air/steam flow in the chamber and discharge through the thermostatic control valve (steam trap) and air-gap fixture (site of aerosol assay). Adapted from reference 20 and reproduced with permission.

The organisms were placed in a central location in the chamber as: i) bacterial lawns or two-dimensionally streaked colonies on exposed agar surfaces (four to seven plates); ii) liquid suspensions ($10^9$–$10^{10}$ cells total) exposed on glass surfaces; or iii) liquid suspensions soaked into cotton cloth. Air purged from the chamber during the purge cycle was sampled through an intake hose suspended in the air-gap fixture and attached to an Andersen Microbial Air Samplers (Graseby Andersen Samplers, Inc., Atlanta, GA, USA),[2] where it was impacted onto appropriate agar plates, according to the manufacturer's instructions. The positive pressure of the forced purge eliminated the possibility of inadvertently sampling ambient air.

From three/four trials of each organism, *S. aureus* and *E. faecalis* were each retrieved once (<10 colony-forming units [cfu] per autoclave purge), but these could only be identified tentatively as the inoculated strain because genetic confirmation studies were not performed. In a second autoclave (Consolidated Stills and Sterilizers, Boston, MA, USA; model SSR3A), a single trial of *Mycobacterium smegmatis* yielded positive, but genetically unconfirmed, results. The *B. stearothermophilus* control strain was retrieved ($\leq 10^2$ cfu per autoclave purge) from approximately half of all assays.

### *Modifications*

The sensitivity and specificity of these pilot assays were low, and complicated by excessive condensation and potential trauma from impacting the strains onto agar surfaces. Therefore, the methodology was modified to enhance retrieval. We focused on *E. faecalis* because of its significance as a serious hospital pathogen and its relative hardiness, particularly its resistance to heat.[3–6]

The test cultures comprised an equal mixture of heat-activated and nonactivated spore suspensions[7] of

*B. stearothermophilus* and *E. faecalis* strain JH2•2X bearing the chromosomal *tet*(M) resistance determinant TN*1545* (a gift of Marilyn Roberts, Seattle, WA, USA). This *tet*(M) genetic marker was included to confirm the presence of the inoculated organism in any samples.

Enterococci grown to saturation in brain–heart infusion (BHI) broth (BBL, Cockeysville, MD, USA) and concentrated by centrifugation, or grown as bacterial lawns on BHI agar, were placed in the autoclaves under three experimental conditions, representing different methods and increasing degrees of exposure.

*Condition 1:* Exposed bacterial lawns of *E. faecalis* (nine to 11 confluent agar plates [approximately $10^{12}$ cells]) and liquid suspensions of the *B. stearothermophilus* spores (approximately $10^7$ total, spread in open glass petri dishes) were placed in a central location in the chamber.

*Condition II* (performed in autoclave A [see below] only): Spread glass surfaces (12 open petri dishes) and soaked paper (two Kimwipes® [Kimberly–Clarke, Roswell, GA, USA] or paper towels) were co-inoculated with both strains ($10^9$–$10^{10}$ *E. faecalis* and $10^7$ *B. stearothermophilus*).

*Condition III:* Tissues (two Kimwipes®) soaked with approximately $10^7$ *B. stearothermophilus* spores and approximately $10^{10}$ *E. faecalis* cfu, suspended on a test tube rack located directly over the purge vent (Table 1, Experiment 1). This was repeated (Table 1, Experiment 2) using five such racks with approximately $10^6$ spores and a 10-fold greater inoculum of *E. faecalis* ($0.9–9.0 \times 10^{11}$ total cells).

Two gravity steam-jacketed sterilisers (American Sterilizer Company) were used. A modern computerised model, the Eagle 3000 (autoclave A; see Table 1), had a timed purge cycle of one minute. The second unit, Model 72A-AS-O1T626G7R-2A (autoclave B; Table 1), was approximately 30 years old, and was operated manually for these experiments. Its purge cycle was between one and two minutes (i.e. the time required to exceed 100°C).

The purge exhaust was collected at the rear of the autoclave unit by means of a vacuum pump attached to an autoclaved all-glass impinger (AGI-30, Ace Glass Co. Vineland, NJ, USA)[8] suspended in ice. Sterilised Tygon tubing, attached to the inlet port of the AGI-30, was suspended in the purge exhaust emanating from the juncture of the air-gap fixture (Fig. 1) and the drain pipe. The exhaust was trapped by high-velocity impingement into BHI (10 mL), serving as a collection medium. Following each assay, the attached intake hose and sampling port were flushed with buffered saline (5 mL), which was then pooled with the collection medium prior to processing.

Optimally, an estimated 4–5% of the vented chamber volume was sampled in the collection medium in the 1–2 min purge cycles. Culturable cells were quantitated by direct plating (0.5 mL) onto BHI agar and incubated at 37°C for *E. faecalis*, and onto trypticase soy agar (BBL) and incubated at 65°C for *B. stearothermophilus*. Likewise, qualitative assessments were performed by incubating collection broth (5 mL) at the two temperatures to detect low numbers of viable cells.

For polymerase chain reaction (PCR) analysis, the remaining collection medium was filtered through a 0.45 μm teflon-coated membrane filter (Gelman Sciences 66147, Ann Arbor, MI, USA). The filter was washed by vigorous vortex mixing into Tween 20 lysis buffer[9] (50 mL), incubated overnight at 65°C and boiled for 10 min. PCR for the *tet*(M) sequence was performed with primer sequences M3 and M4 (GCG accession number X04388), according to the protocol provided by the manufacturer of the *Thermus aquaticus* (*Taq*) DNA polymerase (Gibco BRL, Gaithersburg, MD, USA). PCR was performed in a DNA thermocycler (Perkin–Elmer Cetus, Norwalk, CT, USA) and comprised four minutes at 94°C, then 30 cycles, each comprising 94°C for 45 sec, 55°C for 60 sec, 72°C for 60 sec, with a final incubation of 10 min at 72°C.

Table 1. Recovery of *B. stearothermophilus* and *E. faecalis* from purged steriliser chambers

| Experiment[a] | Autoclave[b] | No. assays | No. positive assays[c] | |
|---|---|---|---|---|
| | | | Bacillus | Enterococcus |
| 1 | A | 5 | 1 | 2 |
| | B | 6 | 4 | 3 |
| | Totals | 11 | 5[d] | 5[e] |
| 2 | A | 4 | 1 | 3 |
| | B | 7 | 3 | 5 |
| | Totals | 11 | 4[f] | 8[g] |

[a]1: Co-suspensions ($10^{10}$ *E. faecalis* cfu and $10^7$ *B. stearothermophilus* spores soaked into two Kimwipes® suspended on test tube rack; 2: As in experiment 1, using $0.9–9.0 \times 10^{11}$ *E. faecalis* cfu and $10^6$ *B. stearothermophilus* spores on 10 Kimwipes®.

[b]A = AMSCO Eagle 3000 (chamber dimensions: 99 cm × 50 cm × 51 cm); B = AMSCO Model 72A-AS-01T66G7R-2A (chamber dimensions: 109 cm × 60 cm × 91 cm).

[c]As determined by one or more of the qualitative and quantitative assays used.

[d]Two by broth enrichment only; three quantitative positives showed $0.3–4 \times 10^2$ total cfu retrieved per assay.

[e]Qualitative recovery only: two by broth enrichment, three by PCR.

[f]All positive by broth enrichment and plate counts, ranging from $0.6–9 \times 10^1$.

[g]Two qualitative recoveries (one by broth enrichment, one by PCR); six quantitative recoveries ranging from $0.03–1.5 \times 10^2$ cfu per assay.

Control cells treated simultaneously showed a minimum detection level of 20 cells after two amplifications of 30 cycles each. A sample spiked with a 1 in 10 dilution of purified enterococcus chromosomal DNA containing *tet*(M) served as a control for the presence of substances inhibitory to PCR amplification.

All samples not showing the 1116 bp *tet*(M) product on agarose gel electrophoresis were reamplified for an additional 30 cycles before being considered negative. Confirmation of J2•2X *E. faecalis* containing *tet*(M) was performed by Southern blot analysis of chromosomal DNA. Chromosomal DNA from one isolate from each positive experiment was extracted[10] and digested with *Eco*RI endonuclease. Digestion patterns were compared with both identical and nonidentical enterococcal isolates as controls. Southern hybridisation[11] was performed using a radioactive phosphorus ($^{32}$P)-labelled PCR product of *tet*(M), made with a random-primed DNA labelling kit (Boeringer Mannheim, Indianapolis, IN, USA).

## Results

Under minimal exposure conditions (i.e. conditions I and II), *E. faecalis* and *B. stearothermophilus* were retrieved from the sampling of purge exhaust from both sterilisers: in five trials under condition I, *E. faecalis* was retrieved once (by PCR), while no *B. stearothermophilus* was cultured; in eight trials under condition II, *B. stearothermophilus* was cultured once, but no *E. faecalis* was isolated.

A number of assays were then performed in two experiments under condition III, using greater exposure and bacterial load (Table 1, Experiments 1 and 2). Inoculated Kimwipes® and paper were suspended on racks directly over the purge vent. The enterococcus was retrieved in 45% of trials with inocula of $10^{10}$ cells (Experiment 1) and 73% of trials with inocula of at least $10^{11}$ cells (Experiment 2). For assays in Experiment 2, the entire collection medium was concentrated by filtration prior to quantitative and qualitative assays. All washed filters were subsequently incubated in BHI broth to grow any residual cells.

Direct quantitative plating of the collection medium retrieved relatively low numbers of *E. faecalis* ($\leq 1.5 \times 10^2$ cfu per autoclave purge) only in Experiment 2, where the collection medium was concentrated prior to plating. *B. stearothermophilus* was retrieved from 41% of assays in which $10^6$–$10^7$ spores were exposed on paper (nine of 22 assays; Table 1). Retrievals ranged from $0.6$–$4 \times 10^2$ cfu in the collection media of each purge.

Approximately 250 L of air are expelled from a one-minute purge of the Eagle 3000 (autoclave A), and 600 L from a two-minute purge of the older model (autoclave B). At a sampling rate of 12.5 L/min, at best the AGI-30 can sample only 5% of the expelled volume during that time period. Therefore, with retrieval of up to $10^2$ cfu obtained from testing an uncontained bacterial load of $10^{11}$ enterococci, we estimate that up to $10^3$ culturable cells could be dispersed into the surrounding atmosphere at each purge cycle. It is well documented that direct plate counts significantly underestimate the total number of bacteria in an aerosol,[12,13] and therefore the total number of bacteria expelled could be much higher.

The detection of enterococcus by PCR alone on four occasions indicated that these cells were either in a stressed, nonculturable state or were dead. As designed, the experiments could not differentiate between these states.

## Discussion

This study confirmed that the air-gap fixture of steam jacketed sterilisers is a potential source for release of pathogens into the ambient environment. The extent of this release will depend on the length of the purge cycle, which may range widely among instruments of varying age, and the condition of the thermostatic control valve. Any residue build-up or trapped airborne particles that interfere with the proper functioning of this valve may delay sealing of the chamber, and, consequently increase the purge time, permitting the release of more bacteria from the chamber. Many decades-old sterilisers are still in use and may be subject to such malfunctions, without the knowledge of the operator.

The retrieval of test bacteria, whether culturable, viable but nonculturable, or nonviable, indicates escape of cells from inside the autoclave chamber during the purge cycle. *B. stearothermophilus* is routinely used as an indicator organism, solely to monitor autoclave function. The lower retrieval rate of this heat-stable organism, compared with that of the more fragile enterococcus, was thought to be due to its lower inoculum size, but may have resulted from the poor plating efficiency of its spores.[14] As a non-pathogen that reproduces only at temperatures far above body temperature, this organism poses no health risk; however, it does serve as a useful indicator of escape from the steriliser interior. Another opportunistic pathogen of this genus, *B. cereus*, has been shown to survive the elevated temperatures of a hospital laundry system.[15]

The significance of retrieving small numbers of *E. faecalis* in an aerosol is less well defined. *E. faecalis* was selected as an example of a hardy, problematic hospital pathogen that could be tested with relative ease; however, it is unclear whether or not an aerosol biobur-

den created by this organism, or other potential pathogens, exceeds in any significant way the aerosols produced by the original process of packaging and introduction of contaminated goods into the autoclave chamber. Infectivity is dependent upon the number of infecting organisms present, the state of the bacterial cell, and the health of the recipient host.

In addition, the significance of retrieving enterococcus in a viable but nonculturable state is not well defined. Cells may convert from one state to another, and a number of environmental conditions may trigger these transitions.[16,17] Nonculturable cells have been shown to retain their infectiousness when ingested by humans.[18]

The results presented here challenge the commonly held assumption that autoclaves are completely contained and, once loaded and sealed, present no additional bacterial burden to the external environment. The experiments were designed in steps, leading to a 'worst case' scenario in which bacteria were completely uncontained and exposed to the possibility of aerosol formation. Theoretically, as only a small volume of the autoclave was occupied (A: 0.4%, B: 0.8%), a severely contaminated and exposed load that filled the entire autoclave could release 100- to 200-fold greater quantities of organisms than retrieved here. Under advised sterilisation procedures, such exposure would not be expected as it would not represent standard operating conditions. Nonetheless, the quantity and condition of the materials introduced, and the care with which autoclaves are operated, may vary enormously, depending upon the packaging of the materials and the knowledge and skills of the operator. This is particularly relevant in countries or individual facilities where proper autoclave evaluation and maintenance practices are substandard.

At the very minimum, this study reinforces the need for careful consideration of the proper location of autoclaves. The release of organisms in areas where the bacterial burden is expected to be low or non-existent is of concern,[19] particularly the venting of double-ended autoclaves in a 'clean area' that is controlled for entry of pathogens. In this study, the numbers of bacteria retrieved during release were small and not considered of risk to either operator or the environment; however, their retrieval illustrated a potential route of entry or re-entry of pathogens into an environment. Moreover, it indicates that thermostatic valve function, as well as steriliser operation, should be monitored and evaluated periodically, as larger numbers of bacteria could be released during longer purge cycles, particularly when processing large uncontained loads.

Although these experiments were performed in two autoclaves only, the similarity in basic design of nearly all steam-jacketed sterilisers suggests that most of those in use today would be subject to a similar discharge from the purge exhaust. Furthermore, release may be enhanced in autoclaves in which air removal is assisted by a vacuum purge. The problem could be alleviated by the design and introduction of a filter to trap bacteria, thereby preventing escape into the surrounding environment.

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