Microbial Challenge Assay
Optima™ Steamer;
Escherichia coli.

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Efficacy of the Steamericas^{tm} Optima^{TM} Steamer for cleaning 3 surfaces challenged with *E. coli*

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EXECUTIVE SUMMARY

3 test surfaces (plastic, glass and stainless steel) were inoculated with the bacterium *E. coli*. After initial protocol development, the surfaces were treated with the Optima^{TM} Steam cleaner. The results indicate that the Optima^{TM} Steamer effectively eliminates or kills viable *E. coli* from plastic glass and stainless steel.

INTRODUCTION:

The Optima™ Steamer produced by SteAmericas™ Inc. is a portable steam-cleaning unit. The Optima™ Steam Cleaner superheats water, generating steam that is directed through the nozzle at the surface to be disinfected.

The purpose of this study is to determine if the Optima™ Steam cleaner can be used to disinfect 3 surfaces challenged with *Escherichia coli* (*E. coli*). The surfaces selected were: 1. Plastic (Polystyrene), 2. Metal (Restaurant grade Stainless Steel), and 3. Glass (16 oz. Glass Tumblers).

MATERIALS AND METHODS

Microbes:

Escherichia coli (E. coli) was purchased from: American Type Culture Collection (ATCC) 10801 University Boulevard, Manassas, VA 20110, USA. Upon receiving, the bacterium was cultured according to supplier instructions. Initial cultures were separated into master stocks (both agar slants and frozen glycerol stocks). In addition, working stocks were streaked on the appropriate agar medium such that single colonies could be obtained. Prior to the start of an experiment, a single colony was grown overnight in the appropriate medium and used to produce a working stock. Working stocks used were late log phase with confirmed viability between 10 million and 100 viable cells per milliliter.

Unless otherwise indicated, *E. coli* was maintained in LB medium at 37°C with agitation.

Test Surfaces:

3 test surfaces were used:

1. Plastic (Polystyrene), Fisherbrand 60mm polystyrene Petri plates.
2. Metal (Restaurant grade Stainless Steel), "Metal By The Inch" Restaurant grade Stainless Steel, custom cut into 4inch by 4 inch square pieces. Edges slightly bent to fit into 150mm sterile petri dishes. 60mm circular inoculation target indicated with Sharpie pen.
3. Glass (16 oz. Glass Tumblers). "16 oz. Glass tumblers" Wal-Mart, Anaheim, CA. with inner surface diameter of approximately 55 mm.

Test surfaces were sterilized using a standard laboratory autoclave. After sterilization, test surfaces were challenged by addition of one milliliter of a late log phase *E. coli* culture (approximately 10^7–10^8 CFU/ml). After surface inoculation, the culture was spread such that entire target area was covered. The test surface was then incubated for a minimum of one hour. Following incubation, excess culture media was removed by aspiration.

Test Armature:

A test armature was constructed from galvanized ¾ inch pipes, pipe clamps and standard scientific clamps. Different clamps were employed to hold the 3 different surfaces to be tested. When necessary, clamps were sterilized before use. The test armature was housed within the vented laboratory space next to the Optima™ Steam cleaner. A large plastic tray and plastic backing were used to collect excess condensation and define the test area.

SANITIZATION PROCEDURES PROTOCOL DEVELOPMENT

Evaluation of Output Temperature:

A laboratory grade thermometer (Fisher Scientific) with temperature range -40°C to 120°C was clamped to the test armature. The boiler of the Optima^{TM} Steamer was pre-warmed for 15 minutes according the manufacturer instructions.* 3 operators conducted temperature studies. Operator one, wearing heat resistant gloves, face shield and laboratory coat held the steam gun, operator two wearing heat resistant gloves, face shield and laboratory coat held a metal ruler and Operator 3 held a stop watch. The 3 operators worked together to measure output temperature at different distances and durations. Representative data is shown below.

* The boiler turned on and off intermittently during operation. It was later pointed out by the manufacturer that super-heated steam occurs only when there is continuous boiler operation, which occurs only after a different mode of operation than employed here.

Evaluation of Distance, Duration and Nozzle Movement Matrix

Preliminary studies were conducted to determine the optimal nozzle movement, duration and distance. Successful studies are described below:

Preparation of plastic surface prior to testing:

1. *E. coli* culture (ATCC SOE#61727) was seeded in 150 mL of fresh LB liquid media from a previous liquid culture and incubated at 37°C for 2 days.
2. On the day of testing, the stock *E. coli* culture is diluted from 1 to 10^{-8} and plated on LB agar plates to confirm viability and initial culture density.
3. 1.5 mL of the stock culture was removed and added to 150 mL of fresh LB media (1:100 dilution).
4. 1 mL of the new diluted culture was then added to each 6 cm plates and incubated at room temperature for 1 hour prior to testing.
5. After 1-hour incubation, the liquid culture was poured out of the plates.

Duration (representative example):

1. Treated plates were divided into 10 different testing groups: naive control plate (which remained outside of testing area), 30s, 20s, 10s, 5s, 4s, 3s, 2s, 1s, and 0s groups (brought into test area and mounted on armature).
2. Plates were secured to test armature with a clamp. Plates in each group were treated with steam cleaning for the corresponding amount of time. Plates in the 0s group were clamped and opened but not treated with steam cleaning.
3. Test groups treated with steam, were treated using the following procedure (which was pre-determined in preliminary studies).
   The nozzle was held about 3-4cm away from the plastic surface and cleaned for corresponding amount of time. The cleaning pattern is as followed:
   a. 30s: 10s edge clockwise, 5s center zigzag horizontal, 5s center zigzag vertical, 10s edge counterclockwise.

b. 20s: 5s edge clockwise, 5s center zigzag horizontal, 5s center zigzag vertical, 5s edge counterclockwise.
c. 10s: 5s edge clockwise, 5s center zigzag horizontal.
d. 5s: 3s edge clockwise, 2s center zigzag horizontal.
e. 4s: 2s edge clockwise, 2s center zigzag horizontal.
f. 3s: 2s edge clockwise, 1s center zigzag horizontal.
g. 2s: 1s edge clockwise, 1s center zigzag horizontal.
h. 1s: 1s right at the center of plate.
i. 0s: open then close immediately, no steam cleaning.

4. After treatment, 5 mL of fresh LB liquid media is added to each plate and the plates are sealed by parafilm and placed at 37°C with rocking for 10 days.
5. After the 10-day incubation, 1 mL of liquid was removed and the OD 660 was measured using a spectrophotometer (BioMate 3 Thermo Spectronic) blanked with naïve LB medium.

Results

Duration (s)	Repeat A (OD1000)	Repeat B (OD1000)	Repeat C (OD1000)
0	1519	1421	2115
1	0	1601	1661
2	1706	5	1699
3	0	0	0
4	1352	2062*	1801*
5	884	1493	1
10	1	0	3
20	1578	0	3
30	2	0	1

*** Yellow clumping yeast not consistent with *E. coli***

Duration: Conclusion

Protocol A (30 seconds) *eliminated all microbes capable of growing in LB broth at 37°C. This finding was consistent with early findings.

- Note that based on the 20 minute *E. coli* replication rate, it is predicted that a 10 day incubation under these conditions will result in a OD1000 reading over 1000. Readings less than 10 are attributable to background and readings between 10-500 are attributable to contamination by slow growing molds (likely airborne spores).

Distance (Representative Example)

1. Test groups treated with steam were treated using the following procedure, which was pre-determined in preliminary studies. The nozzle was held from 0-30 cm away from the plates for 30 seconds as described above.

2. After treatment, 5 mL of fresh LB liquid media is added to each plate and the plates are sealed by parafilm and placed at 37°C with rocking for 10 days.

3. After the 6-day incubation, 1 mL of liquid was removed and the OD 660 was measured using a spectrophotometer (BioMate 3 Thermo Spectronic) blanked with naïve LB medium.

Results

Optical Density x 1,000 for *E. coli*

Distance (cm)	Group A OD1000	Group B OD1000	Group C OD1000
1	0	0	0
2	1	0	1
3	0	7 *	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
10	2,274 **	0	1
20	665	975	156 *
30	1,436	1,408	1,299
Control	1,406	1,374	1,436

Reading for optical density was taken 10 days post Optima™ Steamer

*** Mold was present on plate**
**** Unusual coloring (Darker)**

Distance: Conclusion

A nozzle distance closer than 10 cm from the surface eliminates or kills the challenge organism.

Protocol Definition:

Validation and additional optimization studies were conducted with at least 3 repeats at a nozzle distances ranging from 3-9 cm (typically 5 cm) and duration of 30 seconds. All other conditions were as described above. Several generations of validation studies were conducted by different operators. In some cases both Optical Density and plating efficiency were evaluated. Successful protocols and results are described below.

Procedure (Plastic)

Preparation of plastic surface prior to testing:

1. *E. coli* (ATCC SOE#61727) was seeded in 150 mL of fresh LB liquid media from a previous liquid culture and incubated at 37°C for 2 days.
2. On the day of testing, the stock *E. coli* culture was diluted from 1 to 10^{-8} and plated on LB plate to assess initial culture density.
3. Then 1.5 mL of the stock culture was removed and added to 150 mL of fresh LB media (1:100 dilution).
4. 1 mL of the new diluted culture was added to 6 cm plates Fisherbrand 60mm polystyrene Petri plates) and then incubated at room temperature for 1 hour prior to testing.
5. After 1-hour incubation, the liquid culture was aspirated from plates and plates were immediately used for cleaning studies.

Cleaning Plates with the Optima™ Steamer

1. Plastic petri plates were cleaned in the following manner (developed during preliminary studies): The nozzle was held about 2-6 cm away from the plastic surface and cleaned for 30 seconds. The cleaning pattern was as follows: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction.
2. After treatment, 7 mL of fresh LB liquid media was added to each plate and the plates were placed on rocker. In addition, on the day of testing, the stock *E. coli* culture is diluted from 1 to 10^{-8} and plated on LB plate to confirm the initial culture density and viability.
3. After 1hr of shaking, 50 µL of the following dilutions from each plate was plated on LB plates: original, 1:10, 1:100 dilutions (in liquid LB).
4. LB plates were incubated at room temp for 2 days and resulting colonies were counted. The rest of the original 7 mL liquid culture was then sealed with parafilm and incubated at 37°C for 10 days. Following the 10-day incubation, the OD1000 was determined (as described above).

Results (Plastic).

Plate Name	LB Plate Original	LB Plate 10^{-1}	LB Plate 10^{-2}	OD1000
C0	Lawn	Lawn	Lawn	1725
C1	Lawn	Lawn	Lawn	2201
C2	Lawn	Lawn	Lawn	1655
C3	Lawn	Lawn	Lawn	1881
C4	Lawn	Lawn	Lawn	1555
SA1	No growth	No growth	No growth	0
SA2	No growth	No growth	No growth	5
SA3	No growth	No growth	No growth	0
SA4	No growth	No growth	No growth	0

C = control, SA = SteAmerica Optima™ Steamer, Lawn = Too numerous to count, No Growth = no colonies present.

Conclusion (plastic)

This protocol effectively removed or killed *E. coli* that was on the plastic surface.

Procedure (Glass)

Preparation of the glass surface prior to testing:

6. *E. coli* (ATCC SOE#61727) was seeded in 150 mL of fresh LB liquid media from a previous liquid culture and incubated at 37°C for 2 days.
7. On the day of testing, the stock *E. coli* culture was diluted from 1 to 10^{-8} and plated on LB plate to assess initial culture density.
8. 5 mL of the new diluted culture was added to sterile glass tumblers (16oz Walmart Brand) (Previously covered with tinfoil and sterilized in an Napco 900 Autoclave for 35 minutes, according to manufactures suggestions).
9. After 1-hour incubation, the liquid culture was aspirated from glasses and glasses were immediately used for cleaning studies.

Cleaning with the Optima™ Steam cleaner

5. Inoculated glass tumblers cleaned as follows (developed during preliminary studies): The nozzle was held about 5cm away from bottom or in a second condition at the lip of the glass. The surfaces were cleaned for 30 seconds. The cleaning pattern was as follows: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction. In some cases the exact pattern differed due to interference by a jet of steam emitted from the glass.

6. After treatment, 7 mL of fresh LB liquid media was added to each glass and the glasses were covered with tinfoil and then saran wrap to prevent evaporation. In addition, on the day of testing, the stock *E. coli* culture is diluted from 1 to 10^{-8} and plated on LB plate to assess initial culture density.
7. The glasses were incubated at 37°C for 10 days. Following the 10-day incubation, the OD1000 was determined (as described above).

Results (Glass)

Distance	A OD1000	B OD1000	C OD1000	D OD1000	E OD1000
5 cm from bottom	32	0	2	0	0
Lip	992	1	2	47	0
Control	1545	1632	1522	1871	1343

Conclusion (Glass)

Under this set of conditions, the Optima™ Steam cleaning effectively removed or killed all of the challenge organisms.

Procedure (Stainless Steel)

Preparation of the stainless steel surface prior to testing:

1. A 60 mm diameter circle was drawn in the center of the stainless steel plates. Plates were then covered in tinfoil and sterilized in the autoclave for 35 minutes following manufactures instructions.

2. *E. coli* (ATCC SOE#61727) was seeded in 150 mL of fresh LB liquid media from a previous liquid culture and incubated at 37°C for 2 days.

3. On the day of testing, the stock *E. coli* culture was diluted from 1 to 10^{-8} and plated on LB plate to assess initial culture density.

4. 1 mL of the culture was added to the center of the plate (within circle area).

5. After 1-hour incubation, the liquid culture was aspirated from surface and plates were immediately used for cleaning studies.

Cleaning Plates with the Optima™ Steamer

6. Stainless steel plates were cleaned in the following manner (developed during preliminary studies): The nozzle was held about 2-6 cm away from the surface and cleaned for 30 seconds. The cleaning pattern was as follows: 10 seconds around the edge in the clock-wise direction, 5 seconds in horizontal zigzag pattern, 5 seconds in vertical zigzag pattern and the last 10 seconds around the edge in the counter clockwise direction.

An additional 30 seconds of cleaning was used to clean the area around the test circle and an additional 30 seconds of cleaning was used to clean the back of the plates. Preliminary studies indicated that this extra cleaning was necessary to eliminate all microbial growth.

7. After treatment, plates were place in 150 cm sterile petri dishes and 40 mL of fresh LB liquid media was added to each plate and the plates were placed on rocker. In addition, on the day of testing, the stock *E. coli* culture is diluted from 1 to 10^{-8} and plated on LB plate to assess initial culture density.
8. Plates were put into plastic "zip-lock" bags and incubated for 10 days. Following the 10-day incubation, the OD1000 was determined (as described above).

Results (Stainless)

Condition	A OD1000	B OD1000	C OD1000	D OD1000	E OD1000	F OD1000
SA	0	2	665	0	1	0
Control	2012	1858	1788	1892	1477	1882

SA = SteAmerica Optima™ Steamer

Conclusion (Stainless)

Under this set of conditions, the Optima™ Steam cleaning effectively removed or killed all of the challenge organisms.

Summary of Results and Conclusion

Under this set of conditions, the Optima™ Steamer effectively removed or killed all of the challenge organism from plastic, glass and stainless steel surfaces.