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Microbac Laboratories, Inc.

Venice Division

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Research Project for Zimek Technologies, LLC.

Ambulance Test #01

10 minute treatment time with the new Zimek machine

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Initiation of Project: December 21, 2006

Report Date: December 27, 2006

Report Date (final): January 2, 2007

Background Information:

Mr. David Sparks, Executive Vice President of Research and Development for Zimek Technologies Management Company, contacted Microbac Laboratories, with an interest in determining, scientifically, the effectiveness of the micro environmental sterilizing machine produced by Zimek Technologies, LLC ("Zimek") which produces negatively charged micro particles in delivering the microbicidal Zimek QD disinfectant wherever air flows. Zimek Technologies Management Company is the Manager of Zimek Technologies, LLC.

The initial study, using a Plexiglas chamber, was done in the Microbac Laboratory February 21, 2006 and was reported March 1, 2006. The initial field test was done April 21, 2006 at the Chain of Lakes Middle School; 8700 Conroy Road; Orlando, Florida 32835.

Conditions of Field Test:

The purpose of this second field test is to demonstrate the effectiveness of the newly developed Z-4060 "high efficiency" Zimek micro particle treatment system under actual ambulance treatment conditions. The site was the Health Central Ambulance Station at the corner of McGuire Boulevard and Old Winter Garden Road north of Universal Studios in Orlando, Florida. The test was done outside the ambulance station using an older ambulance #205 with approximately 570 cubic feet in the treatment compartment.

The weather was a sunny 78 degrees F^o with no breeze. No changes were made inside the ambulance which is operated daily.

Ambulance #205 was manufactured and put into service in 2002. It was reported by the supervisor of Health Central Paramedics that Ambulance #205 averages 8 patients per day and was in service an average of 350 days per year.

Summary of Test Procedures:

Test A) Microbac Laboratories took 22 swab samples in representative areas using swabs containing 10 mL of Lethen Broth¹. One inch square pieces of numbered masking tape were used to mark each location. A swab sample of 25 cm² was taken to the left of each piece of numbered tape 30 minutes prior to the Zimek treatment. Following the Zimek treatment, a swab sample of 25 cm² was taken to the right of each piece of numbered tape. The swab samples were placed in a cooler and immediately returned to the Microbac Laboratories in Venice, Florida. Analysis of the swab samples began nine hours after the samples were taken.

The laboratory analysis compared the levels of pre-existing bacterial contaminants to the levels after the Zimek treatment. Each swab was analyzed for:

- 1) Heterotrophic Plate Count (total bacterial level).
- 2) Staphylococcus Count (including Staphylococcus aureus).
- 3) Coliform Plate Counts (including Escherichia coli).

Test B) Microbac Laboratories taped two low level bacterial cultures each of Staphylococcus aureus and Listeria innocua in small petri dishes to horizontal attendant seats, vertical windows and upside-down on the ceiling to test the effectiveness of the Zimek treatment. Following the Zimek treatment, the petri dishes were covered, taped shut and placed in sterile, sealed bags inside the cooler with the swab samples. Analysis of these living cultures began 11 hours after the Zimek treatment. Untreated controls were transported with the treated petri dish cultures to obtain initial bacterial counts before the Zimek treatment.

Zimek Treatment:

A flexible hose, six inches in diameter, was run from the new “high efficiency” Zimek sterilizing machine to the passenger window of the ambulance and sealed in place with plastic sheeting. The rear door of the ambulance was opened one inch to allow for flow of the Zimek treatment through the ambulance. The original planned treatment time of 20 minutes was shortened to 10 minutes as a mechanical breakdown on another ambulance required that ambulance #205 be put back into service as soon as possible.

After the additional 2 minute treatment evacuation period, the petri dishes were removed and the post-treatment swab samples were taken.

Procedure for analyzing swab samples:

Twenty-two swab samples were taken before treatment and twenty-two swab samples were taken after treatment. These forty-four swabs were transported back to the Microbac Laboratory in Venice, Florida in a cooler. The temperature of the swabs upon return to the laboratory December 21, 2006 was 8° C. Each swab was placed on the vortexor for seven seconds to thoroughly remove the bacteria from the swab into the 10 mL of Lethen Broth contained in the swab tube.¹

- 1) One mL of the Lethen Broth was removed under sterile conditions and pipetted onto a Heterotrophic, Aerobic Petrifilm Count plate (ID #VEN0019-308-3) The forty-four plates were incubated with positive and negative controls for 48 hours at 35° C. Colony counts were then taken and recorded in the laboratory notebook.
- 2) One mL of the Lethen Broth was removed under sterile conditions and pipetted onto a Staphylococcus Express Petrifilm Count plate (ID #VEN0019-253). The forty-four plates were incubated with positive and negative controls for 24 hours at 35° C. Counts were then taken of the Staphylococcus colonies and recorded in the laboratory notebook. The Staphylococcus film was then placed in those plates with positive counts. After an additional three hour incubation period at 35° C, counts were made of those Staphylococcus colonies surrounded by pink rings (indicative of Staphylococcus aureus).
- 3) One mL of the Lethen Broth was removed under sterile conditions and pipetted onto an E.coli/Coliform Petrifilm Count plate (ID #VEN0019-308-1). The forty-four plates were incubated with positive and negative controls for 24 hours at 35° C. Counts were made of the red colonies possessing gas bubbles and recorded in the laboratory notebook. Those plates that possessed coliform colonies were incubated an additional 24 hours to determine if any of the coliform colonies may have been Escherichia coli as indicated by blue colonies with gas bubbles.
- 4) Three mL of the Lethen Broth was removed under sterile conditions and pipetted into a test tube containing 27 mL of Listeria Enrichment Broth (ID #0019MIC-648). These forty-four test tubes were incubated at 30° C for 24 hours. Each culture was streaked onto a freshly prepared plate of Rapid Listeria media (ID #VEN0019-331) and incubated for 48 hours at 35° C. Counts were taken of any colony growth which would have indicated the presence of any one of the six Listeria species.

As each sample stems from one-tenth of the bacteria in each swab, the laboratory counts are multiplied by ten in the report to represent the true count per swab. A count of 24 results in a reported value of 240 CFU/swab. A count of zero results in a reported value of <10 CFU/swab. Standard approved procedures established by AOAC and FDA/BAM were used in the collection of bacteria and in their dilution onto Petrifilm plates.^{2&3}

Preparation of the low level bacteria samples in petri dishes:

The bacteria cultures employed in “Test B” were obtained from the Microbac Laboratory-Venice Division stock cultures.

Listeria innocua ATCC #33090

Staphylococcus aureus ATCC #6538

- 1) A loop of a 24 hour working *Listeria innocua* culture was streaked into eight small 60 mm petri dishes containing 2 mL of Plate Count Agar (PCA). Each dish was kept at room temperature for 15 hours before being placed into the ambulance.
- 2) A loop of a 24 hour working *Staphylococcus aureus* culture was placed into a 99 mL Butterfield dilution blank. This dilution was shaken for seven seconds in a one foot arc to thoroughly disburse the bacteria. One loop of this highly diluted culture was streaked into eight small 60 mm petri dishes containing 2 mL of Plate Count Agar. Each dish was kept at room temperature for 15 hours before being placed into the ambulance.

Procedure for analyzing bacteria in the small petri dishes:

The viable and non-viable bacteria were washed from the surface of each PCA petri dish with 1 mL of Butterfield Buffer into a 9 mL test tube of Butterfield buffer. Each test tube was shaken for seven seconds in a one foot arc to thoroughly disburse the bacteria. One mL was removed from each test tube dilution and placed onto a Heterotrophic, Aerobic Petrifilm Count plate. The untreated controls had additional dilutions in Butterfield Blanks to determine the bacterial count before the Zimek treatment. The Petrifilm plates were incubated at 35° C for 48 hours. The colony counts allowed for comparison of viable bacteria in each petri dish before and after the Zimek treatment for *Listeria* and *Staphylococcus* in the horizontal, vertical and ceiling positions.

As each sample stems from one-tenth of the bacteria in each swab, the laboratory counts are multiplied by ten in the report to represent the true count per swab. A count of 24 results in a reported value of 240 CFU/swab. A count of zero results in a reported value of <10 CFU/swab. Standard approved procedures established by AOAC and FDA/BAM were used in the collection of bacteria and in their dilution onto Petrifilm plates.^{2&3}



Results of swab test – Total Plate Count (CFU/swab).

	<u>Before Treatment</u>	<u>After Treatment</u>
1. Left hand grip-side door.	120	<10
2. Right hand grip-side door.	20	<10
3. Hatch handle.	10	<10
4. Floor-Front.	1,100	<10
5. Seat Belt Buckle and porous strap.	580	150
6. Steering Wheel.	20	<10
7. 2-way radio microphone.	30	<10
8. Plastic seat top.	30	<10
9. Sliding glass door.	<10	<10
10. Storage shelf.	10	<10
11. Porous canvas medical bag.	520	170
12. Shift lever.	160	<10
13. Driver’s porous fabric seat.	900	340
14. Front upper air vent louver.	820	<10
15. Sliding glass door.	<10	<10
16. Light switch panel.	20	<10
17. Porous rubber shelf liner behind sliding glass door.	380	180
18. Gurney rail.	640	280
19. Plastic seat top.	980	70
20. Floor-rear.	1,700	260
21. Porous pillow on gurney.	200	80
22. Front lower air vent louver.	150	30

Results of Swab Test- *Staphylococcus* Plate Count (CFU/swab).

	<u>Before Treatment</u>	<u>After Treatment</u>
1. Left hand grip-side door.	10	<10
2. Right hand grip-side door.	<10	<10
3. Hatch handle.	<10	<10
4. Floor-Front.	20	<10
5. Seat Belt Buckle and porous strap.	30 (10)	<10 (<10)
6. Steering Wheel.	<10	<10
7. 2-way radio microphone.	<10	<10
8. Plastic seat top.	20	<10
9. Sliding glass door.	<10	<10
10. Storage shelf.	<10	<10
11. Porous canvas medical bag.	140 (40)	10 (<10)
12. Shift lever.	30	<10
13. Driver's porous fabric seat.	820 (300)	20 (<10)
14. Front upper air vent louver.	120 (20)	<10 (<10)
15. Sliding glass door.	<10	<10
16. Light switch panel.	<10	<10
17. Porous rubber shelf liner behind sliding glass door.	50	<10
19. Gurney rail.	100	<10
20. Plastic seat top	<10	<10
21. Floor-rear.	320 (10)	10 (<10)
22. Porous pillow on gurney.	170 (10)	<10 (<10)
23. Front lower air vent louver.	<10	<10

- Quantities in brackets represent *Staphylococcus aureus* counts.
- Staphylococci can cause many forms of infection. (1) *S aureus* causes superficial skin lesions (boils, styes) and localized abscesses in other sites. (2) *S aureus* causes deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections (furunculosis). (3) *S aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and, with *S epidermidis*, causes infections associated with indwelling medical devices. (4) *S aureus* causes food poisoning by releasing enterotoxins into food. (5) *S aureus* causes toxic shock syndrome by release of superantigens into the blood stream. (6) *S saprophiticus* causes urinary tract infections, especially in girls. (7) Other species of staphylococci (*S lugdunensis*, *S haemolyticus*, *S warneri*, *S schleiferi*, *S intermedius*) are infrequent pathogens.⁴
- The Staphylococcus genus includes thirty-one species. Most are completely harmless and reside normally on the skin and mucous membranes of humans. Staphylococcus epidermidis is a commensal of the skin but can cause severe infections in immune suppressed patients. Staphylococcus bacteria can survive on dry surfaces, increasing the chances of transmission. Methicillin resistant Staphylococcus aureus (MRSA)



has recently become a major cause of hospital acquired infections and is being recognized with increasing frequency in community acquired infections.⁵

Results of Swab Test- Coliform Plate Count (CFU/swab).

	<u>Before Treatment</u>	<u>After Treatment</u>
1. Left hand grip-side door.	<10	<10
2. Right hand grip-side door.	<10	<10
3. Hatch handle.	<10	<10
4. Floor-Front.	80	<10
5. Seat Belt Buckle and porous strap.	30	<10
6. Steering Wheel.	<10	<10
7. 2-way radio microphone.	<10	<10
8. Plastic seat top.	<10	<10
9. Sliding glass door.	<10	<10
10. Storage shelf.	<10	<10
11. Porous canvas medical bag.	<10	<10
12. Shift lever.	<10	<10
13. Driver's porous fabric seat.	<10	<10
14. Front upper air vent louver.	<10	<10
15. Sliding glass door.	<10	<10
16. Light switch panel.	<10	<10
17. Porous rubber shelf liner behind sliding glass door.	<10	<10
18. Gurney rail.	10	<10
19. Plastic seat top.	<10	<10
20. Floor-rear.	<10	<10
21. Porous pillow on gurney.	<10	<10
22. Front lower air vent louver.	<10	<10

- The three plates that contained Coliform bacteria did not contain any Escherichia coli.

The total coliform group comprises aerobic and facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that produce acid and gas from lactose during metabolic fermentation. Coliform bacteria originate as organisms in soil or vegetation and in the intestinal tract of warm-blooded animals (fecal coli). This group of bacteria has long been an indicator of the contamination of water and possible presence of intestinal parasites and pathogens. The coliform bacteria are relatively simple to identify, are present in much larger numbers than the more dangerous pathogens, and react to the natural environment and treatment processes in a manner and degree similar to pathogens. Thus by observing coliform bacteria, the increase or decrease of many pathogenic bacteria can be estimated.



Results of Bacteria Tests on Small (60 mm) Petri Dish Cultures (CFU/ petri dish):

	<u>Before Treatment</u>	<u>After Treatment</u>
<u><i>Listeria innocua</i></u>		
Flat Surface #1	4,500	10
Flat Surface #2	4,500	<10
Vertical Surface #1	4,500	<10
Vertical Surface #2	4,500	<10
Ceiling Surface #1	4,500	<10
Ceiling Surface #2	4,500	10
<u><i>Staphylococcus aureus</i></u>		
Flat Surface #1	5,800	40
Flat Surface #2	5,800	60
Vertical Surface #1	5,800	50
Vertical Surface #2	5,800	70
Ceiling Surface #1	5,800	110
Ceiling Surface #2	5,800	90

Reference Material:

1. Rediswabs were obtained from Biotrace International. Each contained 10 mL of Lethen Broth. ID #VEN0019-306; Lot #RS06131; Expiration date 04-06-2008; ISO 9001-2000 Certified.
2. American Association of Analytical Chemists (AOAC) Official Method 966.23.
3. Federal Department of Agriculture/Bacteriological Analytical Manual (FDA/BAM) Chapter 3.
4. www.gsbs.utmb.edu/microbook University of Texas Medical Branch.
5. The Wikipedia Encyclopedia.
6. www.wellowner.org.

Respectfully submitted by:

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