Clean Earth Technologies, LLC

Test Report

"Studies on Physical and Microbiological Characteristics of Selected Clinical Swabs"

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Submitted to

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1. Summary

Diagnostic sensitivity for a clinical test varies with the number of cells collected and released by swabs because the fiber used in the swab head is in direct contact with the organism to be recovered. An ideal swab system must have the ability to absorb organisms from the site of infection, to maintain the viability of organisms during transport and prior to culturing or detection system, and finally, to allow the release of organisms from the swab onto the appropriate media. The type of fibers comprising the swab and their physical structure is important because the fibers used in the swab head are in direct contact with the organism to be recovered from a given site. The construction of the swab is equally important because it affects the amount of diagnostic specimen recovered.

The purpose of this investigation is to determine the physical characteristics of the Nylon, Rayon, and Research Flocked swab as well as, the Macrofoam swab made by Puritan Medical Products. These properties are important to collect and release clinically significant bacteria were compared to the same properties of Nylon Flocked swab made by Copan Diagnostics Inc (Copan).

Water and protein absorption capacities of all swab types were measured that ranged from 17% to 21.5% and 13.6% to 19.6%, respectively. The Research Flocked swab exhibits the highest water and protein absorption capacities in the group. However, water absorption capacity varies significantly as a function of the swab tip materials, which were compared after being removed from the swab shafts. Swabs ranked in the order of increasing water absorption capacity are, Nylon Flocked (Copan), Rayon flocked, Nylon Flocked (Puritan), Research Flocked, and Macrofoam. The solvent (*e.g.*, water) absorption capacity of a swab enables it to be moistened; therefore adding a solubilization effect and also enhances the physical removal process of microorganisms or particulates from the swab.

A simulated qualitative study on the ability of swabs to collect and release microorganisms was done by dipping swabs in a suspension of polystyrene beads (1 μm size to represent bacteria) and examined under scanning electron microscope for the collection and release of beads before and after brief washing. The results of the study demonstrate the superior ability of Research Flocked to collect beads, although the fractional release of beads by the Research Flocked was comparable to or less than other swabs. On the other hand, the efficiency of bead collection for the Macrofoam swabs was about equal to the release of beads showing a more balanced effect.

The validity of the model study with polystyrene beads was verified experimentally by using suspensions of *Hemophilus influenzae*, *Neisseria gonorrhoeae, Staphylococcus aureus, Streptococcus pneuomoniae, S. pyogenes, Bacteriodes fragilis* and *Peptostreptococcus anaerobius* for the capture and release of bacteria by the swabs. Statistical analysis (ANOVA) of the data was performed by swab type and in different ways to analyze the results.

The experimental results reveal that the Research Flocked swab demonstrates the highest (69%) and significantly superior recovery of all bacteria. The Macrofoam swab occupies second place

with 62% recovery, and the Nylon Flocked swabs of Puritan and Copan recovered ~ 54% of all bacteria placing them at the lowest ranks. Further, the recovery of all Gram-negative bacteria was significantly higher (68% to 83%) compared to Gram-positive bacteria (37% to 57%) across the swab types. No significant difference in the recovery between aerobic and anaerobic bacteria was found among swabs.

In summary, the results of the study demonstrate the overall superiority of Puritan Medical Products' Research Flocked swab over other swabs and its potential to increase the diagnostic sensitivity of clinical tests by collecting and releasing significantly larger number of bacteria. Additionally, the commonly-used Macrofoam swab also demonstrated its value to collect and release bacteria by occupying the second place in the present study.

2. Introduction

Detection of antigens, nucleic acids, and isolation of microbes depend on pre-analytical devices used for specimen's collection. The standard method for the collection of cellular samples is the swab. Diagnostic sensitivity for a given clinical test varies with the number of cells collected and released by the sampling devices such as swabs emphasizing the need for proper specimen collection for reliable and accurate diagnosis. Traditionally, compact fibers such as rayon, Dacron or cotton are compressed and shaped to form the swab tip. It is estimated that only 10-15% of the organisms collected on traditional swabs, composed of spun fibers, can be recovered from cultures due to entrapment of organisms. With particularly small samples, this could mean the entire sample potentially remains within the swab tip, prohibiting subsequent sample analysis and resulting in the loss of both time and results. These are all critical aspects to be considered when choosing the most appropriate collection device. An ideal swab would be designed to collect many cells and allow for their release into media, while demonstrating conformity to the directive requirements for the intended use as specified by the manufacturer. This is essential because "not all swabs are created equal".

As the fibers used in the swab head are in direct contact with the organism to be recovered from a given site, the construction of the swab also plays an important role and enhances the amount of diagnostic specimen recovered. This is of paramount importance in view of the myriad of different pathological agents that mediate disease conditions; a need exists to efficiently collect a clinical sample for accurate diagnosis. Additionally, other important parameters often considered for swabs include the ability to preserve the target without affecting diagnostic method sensitivity while maintaining the viability of organisms. The purpose of this investigation is to determine the physicochemical characteristics, evaluate the performance of flocked swabs, namely nylon, rayon and research flocked swabs plus the macrofoam swab manufactured by Puritan Medical Products, and compare them to the flocked nylon swab of Copan Diagnostics Inc.

3. Materials and Methods

Swabs from freshly manufactured lot numbers were obtained from Puritan and Copan. Macrofoam (REF 25-1506 1PF), Research Flocked (PSR #301-09), Nylon Flocked (PSR #274-09 & 303-09), and Rayon Flocked (PSR #272-09 & 302-09) were supplied by Puritan Medical

Products, LLC., Guilford, ME. Nylon Flocked swabs (PSR #303-09; REF. 502CS01.US) of COPAN Italia S.p.A. were obtained through VWR International. Pre-poured Tryptic Soy Agar II containing 5% sheep blood (SBA) and Chocolate II Agar were from BBL™. Pre-reduced Anaerobic Sterilized (PRAS) Dilution Blanks were from Anaerobic Systems, Morgan Hill, CA. Anaerobic Indicator (BR0055B) and AnaeroGen were from Oxoid, UK. Bovine serum albumin (B4287) was obtained from Sigma-Aldrich. *Streptococcus pyogenes* ATCC 19615, *S. pneuomoniae* ATCC 6305, *Staphylococcus aureus* sub sp. *aureus* ATCC 25904, *Hemophilus influenza* ATCC 49247, *Neisseria gonorrhoeae* ATCC 43069*, Bacteriodes fragilis* ATCC 28285, and *Peptostreptococcus anaerobius* ATCC 27337 were obtained from the ATCC, Manasas, VA. McFarland standard (0.5) and 1% (w/v) Microbeads (1 μm) suspension were purchased from PML Microbiologicals, Wilsonville, OR and Polysciences, Warrington, PA, respectively. All chemicals used in the study were of reagent grade.

Composition of GC broth without supplements was 15 g of Bacto protease peptone (# 3), 4 g of K_2HPO_4, 1 g of $KH_2\ PO_4$, and 5 g of NaCl in 1 liter of distilled water (pH 7.2). GC broth was sterilized by autoclaving.

3.1 Absorbance studies Each pre-weighed swab was immersed in 1 ml of distilled water for 15 sec. Following the water absorption, the swab was removed, absorbance values were determined by weighing the wet swab to compute percent water absorption (N=3). To determine the protein absorption, a 22% bovine serum albumin solution was used instead of water and the test repeated with fresh swabs (N=3). To determine the water absorption of the swab head material (N=8), the swab tip material (~0.5 g) was removed from swab-shafts using a scalpel blade. A pre-weighed quantity of swab tip material was transferred into a 1.7 ml micro-centrifuge tube. Following the addition of 1 ml of distilled water, the tube was shaken at ambient temperature for 1 min to facilitate water absorption. The tube containing wet fibers was centrifuged for 5 min at 10,000 rpm and the unbound water removed. The mass of wet fiber was determined after removing moisture adhering to the tube side using a bibulous paper. This process was repeated twice and the percent water or protein absorption was computed.

3.2 Zeta potential measurements The tip material of the flocked swabs was removed using a scalpel blade. Size reduction of fibers to was done and they were suspended in various 0.01M sodium phosphate buffers (pHs 5, 6, 7 and 8). Zeta potential of fiber or polystyrene bead suspension was independently measured to understand the binding of beads to swab fibers. All measurements were done at 20^o C with a Malvern Zeta Sizer, model Nano ZS90 (Malvern Co., Worcestershire, UK) using a disposable zeta potential cell according to the manufacturer instructions. The Zeta potential of polystyrene beads (1 μm) was also measured in 0.01M phosphate buffers (pHs 5, 6, 7 and 8). In this experiment, electrical potential was measured by applying an electric field across the fiber or bead suspension allowing them to migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. Each zeta potential is an average of 15 independent measurements.

3.3 Scanning electron microscopy (SEM) measurements SEM images of swab tips were recorded to examine the physical structure of swab fibers at higher magnification. To study the ability of swabs to collect bacteria in a model system, swab tips were be placed in 1% polystyrene beads (1 μm) suspension (Microbead) for 15 sec, then removed, air-dried for 45 sec

and prepared for SEM. To measure the release of bacteria in the same model system, swab tips adhering the polystyrene beads as described above were placed in 2 ml of distilled water, vortexed at high speed for 10 sec, and dried (as below) before preparing for SEM examination.

To prepare for SEM, swabs harboring polystyrene beads were subjected to critical point drying under reduced helium pressure at 230 Pa for 20 min followed by sputtering a gold-palladium layer on it, using a DC plasma sputtering machine (Pathan *et al*., 2008). A Hitachi S-3200 Variable Pressure SEM, a high resolution thermionic SEM, which allows control of the specimen chamber vacuum level and the environment, was used for this purpose. Revolution SEM, an active digital imaging system was incorporated into the SEM to accurately control the beam and image acquisition for obtaining sharp digital images. Electron photomicrographs were obtained at various magnifications.

3.4 Culture studies Culture studies were conducted to evaluate the collection and release of various gram-positive and gram-negative bacteria. Well isolated colonies from each of the bacterial strains were streaked for isolation on SBA with the exception of *H. influenzae* and *N. gonorrhea*, which were streaked on chocolate agar. After incubation at 37°± 1° C for 18-24 h or 48 h (for *P. anaerobius*), several well-isolated colonies were selected and transferred using a nylon flocked swab pre-wet with 0.85% NaCl to a tube containing 5 ml of 0.85% sterile saline (pH 6.8-7.2) in a glass test tube. Bacterial cells suspension was obtained by vortexing the tube thoroughly for 15 sec. The 0.5 McFarland standard (1.5×10^8 CFU/ml) was agitated on a vortex mixer immediately prior to use. The turbidity of bacterial suspension was adjusted to that of 0.5 McFarland either by adding sterile saline or by adding more bacterial growth. The first $\log_{10}$ dilution was made in saline to obtain bacterial suspension containing ~10^7 CFU/ml. The inoculum of each ATCC test organisms was prepared immediately, prior the performance of swab absorption study.

The test swab was placed in 5 ml of bacterial suspension (~10^7 CFU/ml) in a glass test tube for 10 sec to allow absorption of liquid and facilitate the adherence of bacteria to the swab head. Due to the porous structure of the macrofoam, the swab head was placed in the bacterial suspension, and pressed against the tube wall to expel air bubbles. Bacterial adhesion to the swab was achieved by placing it in the tube for 10 sec after air expulsion. The swabs were removed from the bacterial suspension, held in air for 45 sec, transferred to the dilution medium, and vortexed for 15 sec to release the bacteria. This was followed by serially diluting the viable organisms at 1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000 in the dilution medium (Table 1). Quantitation of viable organisms was done by plating 100 µl samples in duplicates for each of the dilutions on the culture media (Table 1). The organisms were spread over the agar culture media with a plate spreader, and the plates were incubated under the culture conditions as shown in Table 1. Bacterial recovery was determined by counting the colonies recovered in each of the dilutions. The number of organisms (CFU/ml) recovered was expressed as an average for the duplicates at a given dilution (A). The number of viable organisms (CFU/ml) present in the bacterial suspension (~10^7 CFU/ml) was also determined after performing serial dilutions and enumeration of viable organisms at each dilution (B). Average water absorption capacity of the swab in milliliters (C) as well as the average dry weight of the swab (D) was used also in the computation of percent recovery. Appropriate negative controls were run in parallel. Ten swabs

of each swab type were used to determine the recovery of each organism and the total sample size (N) was 350.

$$\text{Recovery (\%)} = \frac{\text{Dilution Factor x A}}{\text{Dilution Factor x B x C x D}} \text{ x 100}$$

Table 1. Summary of culture media and conditions used in the study

Organism	Dilution Medium	Culture Medium	Culture Conditions
S. aureus *S. pneuomoniae* *S. pyogenes*	0.85% saline	SBA	37°± 1° C for 18-24 h, 5% CO_2
H. influenza *N. gonorrhea*	GC broth without supplements	Chocolate agar	37°± 1° C for 18-24 h, 5% CO_2
B. fragilis	PRAS Dilution Blanks	SBA	37°± 1° C for 18-24 h, anaerobic
P. anaerobius	PRAS Dilution Blanks	SBA	37°± 1° C for 48 h, anaerobic

All the operations described above were performed at ambient temperature unless otherwise specified. Due to the obligate anaerobic nature of *P. anaerobius,* all dilutions were performed at ~ 4°C to minimize the metabolic activity of bacteria. The whole procedure was completed within 30 min, to prevent the loss of the organism's viability in the inoculum prior to culturing. PRAS Dilution Blanks used as dilution medium for *P. anaerobius* contains chemicals (sodium thioglycolate and L-cysteine) to provide a reduced environment, maintain bacterial viability without significant multiplication, and meet the stringent viability requirements of obligate anaerobes. The blanks supplied in tubes with a screw cap and rubber septa (Hungate caps) packaged by the manufacturer under oxygen-free conditions prevents the formation of oxidized products prior to use. All bacteria except *B. fragilis* and *P. anaerobius* were cultured under 5% CO_2 as shown in Table 1. For culturing *B. fragilis* and *P. anaerobius,* AnaeroGen sachets were placed in an anaerobic jar according to manufacturer instructions to create an anaerobic environment, reducing oxygen level in the jar to < 1%, and maintaining CO_2 levels from 9% to 13%. The anaerobic condition was checked with an anaerobic indicator strip (BR0055B) prior to placing the cultures in the anaerobic jar according to manufacturer recommendations. The anaerobiosis is indicated by a visual change of the indicator from pink to white.

3.5 Quality control It is essential that all microbiological tests are quality controlled to guarantee the result is true and comparable to other studies conducted in a similar manner. The quality control assures meticulous performance of test organisms, kits, reagents, and culture media while also increasing the precision in reporting. Hence we used commercially available prepoured media, reagents, and kits that passed necessary QC by the manufacturer. All cultures were purchased from the ATCC that have been well characterized. Lyophilized cultures of the test organisms were prepared according to manufacturer's instructions, streaked on appropriate media to obtain isolated colonies, and sub-cultured at least twice. The identity of the organisms

was also confirmed by Gram staining and observation of the bacterial morphology under the microscope at 1,000X magnification. Necessary in-house QC measures were instituted throughout the study to maintain the study quality.

3.6 Data Analysis Statistical analysis of data collected in this study was done by using JMP-7 (SAS Institute, Cary, NC). One-way Analysis of Variance (ANOVA) was used to test the equality of several means at one time by using variance and to establish the test of significance. The *p*-values were computed by ANOVA and then the test of significance was applied ($\alpha = 0.05$). In this approach, the independent variable, Y = % recovery, X = swab, Gram reaction, or aerobe/anaerobe. The null hypothesis (H_0) is that all swabs have equal performance, all organisms recover at the same rate irrespective of their Gram reaction or aerobic/anaerobic nature. Statistical significance of difference between means were compared by Tukey-Krammer HSD (Honestly Significant Difference) test, an exact α-level test (as sample sizes are the same), and the traditional letter-coded report where means are not sharing the same letter are significantly different (Sall *et al*.,2007) reported.

Box (or Box-and-Whisker) plot, a histogram-like method of displaying the data was used to present results. These plots provide a graphical summary of analyses-horizontal lines show lower, median, and upper quartile values. Skewness is indicated if the median line is not centered in the box. Whiskers are the lines extending above and below the box. Outliers, data with values beyond the end of the whiskers, are more than 1.5 times the inter-quartile range, indicated by plus signs (Chambers, *et al*., 1983).

4. Results and Discussion

4.1 Water absorption by the whole swab The water absorption capacity of all swabs ranged from 17.1% to 21.5%. Among the group, the Research Flocked swab exhibited the maximum water absorption and its ability to retain water was significantly greater than the Rayon Flocked swab (Figure 1). Differences in water absorption among Macrofoam, Copan Nylon Flocked, and Puritan Nylon Flocked were minimal.

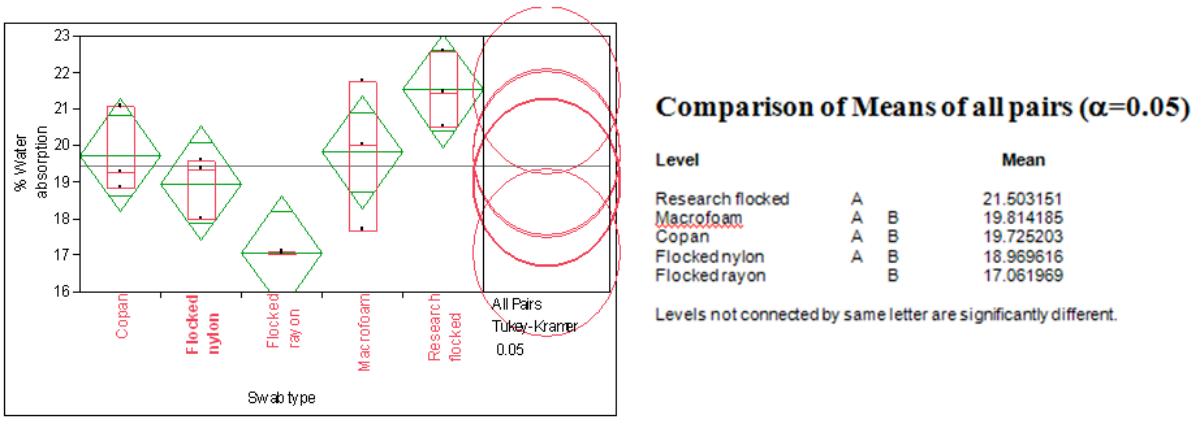


Figure 1. One-way ANOVA of water absorption capacity of swabs.

The water absorption measured in this experiment includes total (free and bound) water which is dependent on the chemical nature of fibers and surface coatings. It is known that two-dimensional structures made of synthetic fibers, for example polyamide or polyester fibers and filaments, have a poor absorptivity and they store low amounts of water. On the other hand, fiber materials rendered hydrophilic by applying onto them binders carrying hydrophilic groups (-OH or -COOH groups) or adding cellulose ethers which are water-insoluble (> 50% by weight), but have a high absorptivity (U.S. Pat. No. 3,965,091; U.S. Patent 4136218) results in retention of large amounts of bound water. Water absorption capacity of a swab plays an important role to extract microorganisms from the collection site by the capillary (or wick) action and prevents dehydration of organisms. From the absorption data, it is expected that the Research Flocked swab will have better extraction performance than the other swabs, especially the Rayon Flocked swab.

4.2 Protein absorption by the whole swab The protein absorption capacity of all swabs ranged from 13.6% to 19.6%. Among the group, the Research Flocked swab exhibited the maximum protein absorption and its ability to retain protein was significantly different from Rayon Flocked and Macrofoam swabs (Figure 2). Differences in protein absorption among Research Flocked and Rayon Flocked or Macrofoam swabs were significant (Figure 2). The data show that the Research Flocked swab is about 46% more protein absorptive than the Macrofoam swab.

Protein absorption by swabs is likely to contribute to increased bacterial adsorption when they are used to collect specimens from wounds, mucous membranes, blood, *etc* because body fluids contain water and variable amount of protein. Further, bacteria contain appendages such as pili which are comprised of proteins and used for adhesion, attachment to receptors, conjugation, *etc* (Forbes *et al*., 1998) may be easily adsorbed to the swab fibers. Protein and water absorption capacity of swabs can be improved by imparting hydrophilic functional groups (such as –OH, -COOH, $-NH_3$, *etc*).

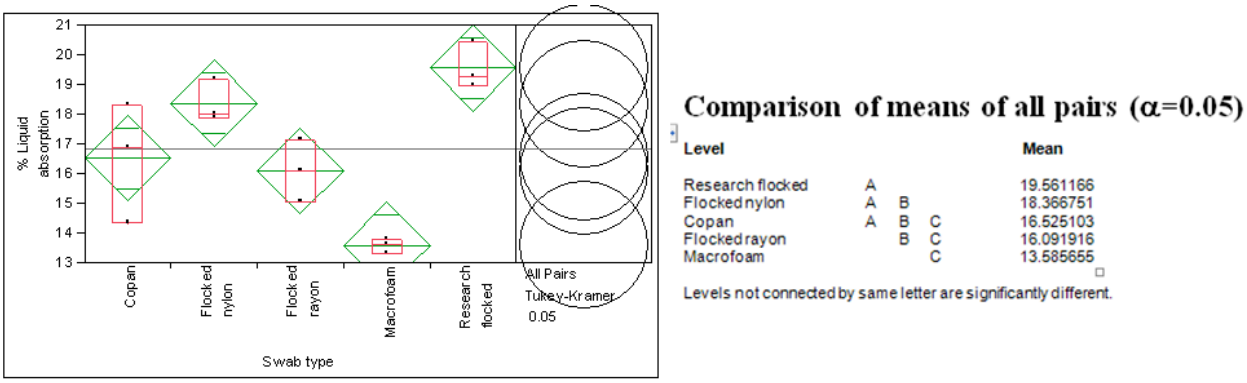


Figure 2. One-way ANOVA of protein absorption capacity of swabs.

4.3 Water absorption by the fibers or foam of the swab head In this experiment, fibers or foam were separated from the swab head to determine the water absorption as described in the Methods section. The method was used because the weight of fibers of all flocked swabs were a

very small fraction of weight of the swab (<1%), and therefore, water absorption of the whole swab (see Section 4.1) will not be a true reflection of the physical property. This is evidenced by differences in water absorption profiles between whole swabs and separated swab fibers or foam. For example, Research Flocked and Rayon Flocked ranked the highest and lowest, respectively with Nylon Flocked (Copan) in the middle when whole swabs were used. On the other hand, Macrofoam and Nylon Flocked (Copan) ranked the highest and lowest, respectively with Nylon Flocked (Puritan) in the middle when swab fibers or foam were used for measurements (Figure 3).

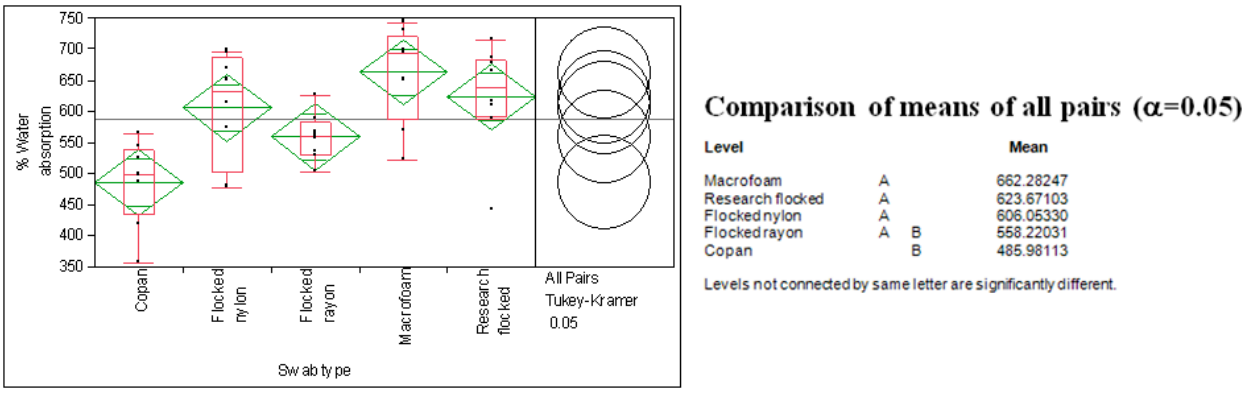


Figure 3. One-way ANOVA of water absorption capacity of fibers or foam of the swabs.

Table 2. Zeta potential of polystyrene beads and swab fibers used in the studies

Sample	Zeta Potential, mV (± SD)			
	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Polystyrene beads	-39.2 ± 3.8	-64.4 ± 1.2	-159 ± 1.3	-143 ± 1.6
Rayon Flocked	-11.7 ± 0.3	-14.4 ± 4.6	-2.6 ± 0.6	-5.5 ± 3
Nylon Flocked (Puritan)	-7.7 ± 0.3	-16.1 ± 2.3	-10.5 ± 1.8	-11.2 ± 2.9
Nylon Flocked (Copan)	-7.8 ± 1.5	-8.6 ± 1.8	-9.6 ± 2.7	-2.4 ± 1.2
Research Flocked	-17.6 ± 1	-17.7 ± 1	-9.9 ± 1.2	-13.4 ± 1.9

* mean of 15 measurements

Water absorption by swab fibers is determined by the chemical composition, microstructure, and surface polarity. These properties may influence other physical properties such as zeta potential by replacing electrolyte ions (Bismarck *et al*., 2002; Bellman *et al*., 2004). Further, water absorption of swab fibers might play an important role in protecting bacteria because desiccation is a leading cause of death of bacteria on swabs (Barry *et al*., 1972).

4.4 Zeta potential measurements Zeta potential (ξ), the electrical potential at the "shear plane" of flocked swab fibers was studied to understand the degree of the repulsion or attraction among fibers, as well as, between fibers and bacteria. Zeta potential describes the nature of the electrostatic potential near the surface of a particle and the value of ξ will be important in establishing whether the repulsive electrostatic potential barrier between neighboring particles is high enough to attract each other due to short-range attractive Van der Waals forces. An electrophoresis method was employed to determine the ξ of fibers by measuring the velocity of the particles in a D.C. electric field and the results are shown in Table 2.

Our results reveal that both flocked fibers and polyester beads have negative charge in the pH range tested. Increasing pH increases negative charge that can be attributed to deprotanation and ionization of –COOH groups. However, we observed lower values ξ values for rayon, nylon and Research flocked fibers of Puritan at pHs 7.0 and 8.0 than the same values at pH 6.0. On the other hand, nylon flocked fibers of Copan showed similar anomalous behavior of lower ξ values at pH 8.0. Lower ξ values may be attributed to water absorption and swelling of fibers at these pHs that can lower ξ (Bismarck *et al.*, 2002). It is also important to point out high SD which may be attributed to the larger sized fibers that might have distorted mobility during measurements.

The maximal ξ of the fiber surface, which generally occurs in the alkaline range, is a reflection of whether the surface is hydrophilic or hydrophobic. Fibers made of synthetic polymers show a hydrophobic surface where the adsorption of electrolyte ions causes the surface charge and the negative zeta potential (Espinosa-Jiménez,1993 ; Iyer and Jayaram, 2008). Bellman *et al* (2004) determined the ξ of various textile fibers, *viz*., cotton, polyamide, polyester, and polyacrylnitrile in the pH range of 2 to 10 and ζ plateau of those fibers was -12, -28, -48, and -55, respectively. In addition to the zeta potential, surface roughness and surface hydrophobicity are also important in adhesion of microorganisms to surfaces (Kang and Choi, 2005; Terada *et al*., 2006).

4.5 SEM measurements Capture and release of polystyrene beads was studied using a model system to simulate bacteria as described under Methods (Section 3.3). Swabs were dipped in a polystyrene bead suspension and SEM photomicrographs of swab heads were recorded before and after washing (Appendix 1). The two most significant observations demonstrated by the model system are the superior ability of Research Flocked to collect beads, although the fractional release of beads by the Research Flocked was comparable to or less than other swabs. On the other hand, the efficiency of bead collection for the Macrofoam swabs was about equal to the release of beads (Appendix 1). SEM photomicrographs also demonstrated important structural details of Research Flocked. At 200 X magnification, we were able to visualize several stalks of bundles of fibers of Research Flocked, each consisting of >25 fibers that open as a tuft at the end, resembling a flower. The length of each fiber of the tuft was estimated at ~ 15 μm to 40 μm. Based on this observation, we speculate that the stalk portion of Research Flocked swab consisting of bundles of fibers offers higher mechanical strength during the swabbing operation and the tuft of fibers at the end facilitate efficient collection of microorganisms.

4.5 Culture studies Specimen collection and transportation has been often overlooked or undervalued even though they have been critical components of a quality health care system. Careful selection and evaluation of swab systems under the conditions and challenges is important in order to understand product limitations and assist in improvements in specimen management and laboratory analysis. For specimen collection, aspirates of fluids and exudates or infected tissues from suspected/infected sites are superior to samples collected on swabs (Brook, 1987; Perrry, 1997). However, because of the ease of using swabs, swab specimens represent a significant percentage of diagnostic samples processed in clinical microbiology laboratories (Perry, 1997). Careful evaluation of swab transport devices for their ability to maintain viability of bacteria during transit to the laboratory is important to ensure delivery of quality specimens.

In the initial phases of the study, physical characteristics of swabs that could potentially influence the collection and release of bacteria were evaluated. In this part, we compared the ability of each swab to collect and release a group of 7 different types of bacteria. The results of the study reveal the number of viable bacteria released by each swab rather than the actual number of CFU present in the swab tip. It is important to note that the viability of all CFU in the swab cannot be guaranteed, some organisms may have become nonviable, preventing colony formation on the agar media even after release. The test procedures employed for determining bacterial viability were based upon the quality control methods described in Clinical Laboratory Standards Institute M40-A (CLSI, 2003) guidelines and can be compared to the performance characteristics of similar devices but not used in the present study.

The test organisms utilized in this study were those specifically prescribed in M40-A for establishing performance claims and quality control of swab transport systems and include a representative panel of aerobes, anaerobes, fragile/non-fragile organisms, and fastidious bacteria. We preferred to use the swab elution method instead of the roll-plate method. A limitation of the roll-plate method for bacterial viability performance testing is that it is not a quantitative method; it is, at best, a semi-quantitative method. Although the swab elution method does not reflect the standard protocol used in most clinical laboratories, it allows a quantitative measurement of the ability of a transport system to maintain viable organisms. Recovery of viable bacteria with various swabs under the test conditions were compared to each other. As the current study does not utilize a transport medium, our results cannot be compared to peer-reviewed published studies in which the researchers compared the performance of bacterial transport system (containing swab plus transport medium) and bacterial recovery (or viability) studied as a function of time and temperature of incubation.

4.6.1 Analysis using pooled data set The recovery of all bacteria from all swab types was combined before the data analysis. As explained under Methods (Section 3.6), one way ANOVA was performed using the approach, Y = % recovery, X = swab, Gram reaction, or aerobe/anaerobe.

4.6.1.1 By the swab type The recovery of all bacteria by swab type is shown in Figure 4. One-way ANOVA showed the highest and the lowest recovery of all viable bacteria by the Research Flocked (Flower) and Nylon Flocked (Copan), respectively with significant differences as shown in Figure 4. The Macrofoam swab ranked the second place. The recovery values

presented are important and reflect the overall performance of the swabs with clinically relevant bacteria.

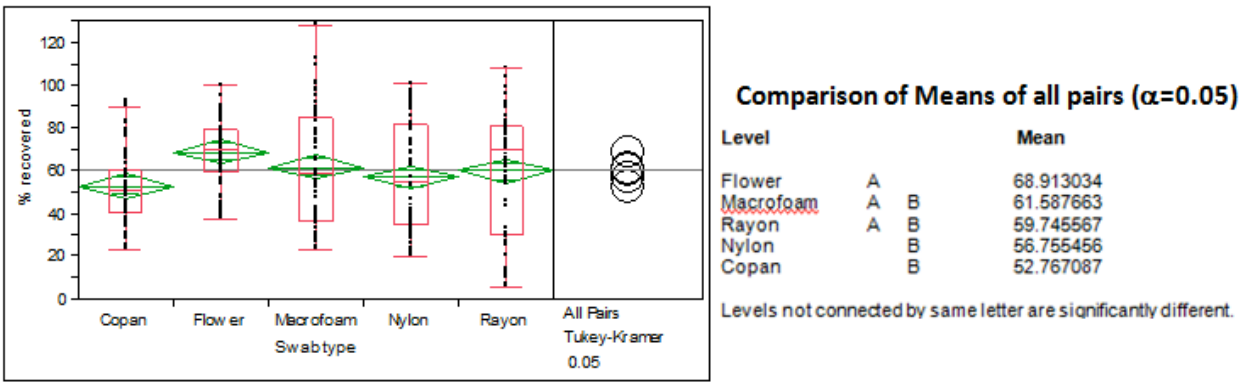


Figure 4. One-way ANOVA of recovery of bacteria-By swab type.

4.6.1.2 By the organism The pooled recovery data was analyzed to rank the recovery by each organism. The results revealed that the recovery of both *N. gonorrhoae* and *H. influenzae* was ~ 80% while the recovery of *S. pneumonia* and *S. pyogenes* was ~ 40% (Figure 5), showing a significant difference. Interestingly, the recovery of anaerobes was in between the recovery values of Gram-negative and Gram-positive bacteria. The note worthy feature of this study is the highest overall recovery of fastidious organisms (such as *N. gonorrhoae* and *H. influenzae)* and poor recovery of the less fastidious organisms *(*such as *S. aureus* and *S. pyogenes)*. Roelofsen *et al* (1999) compared the recovery of various bacteria with viscose swabs (Copan) to polyurethane swabs (BD) and the results revealed significantly higher recovery of various bacteria with polyurethane swabs than viscose swabs without transport medium at zero hours.

From the experiments of Collee *et al* (1974), it is evident that considerable number of bacteria can be expressed when a swab loaded with organisms is agitated in a sterile broth and they attributed the death of delicate organisms to the non-release as well as progressive death on the swab due to aerobic conditions. For example, certain bacterial pathogens did not survive on cotton swabs which was attributed to toxic components associated with these fibers (Barry *et al*., 1972). The recovery of by organism and by swab type showing the recovery of each organism by different swabs and the vice versa is discussed in Section 4.6.4.

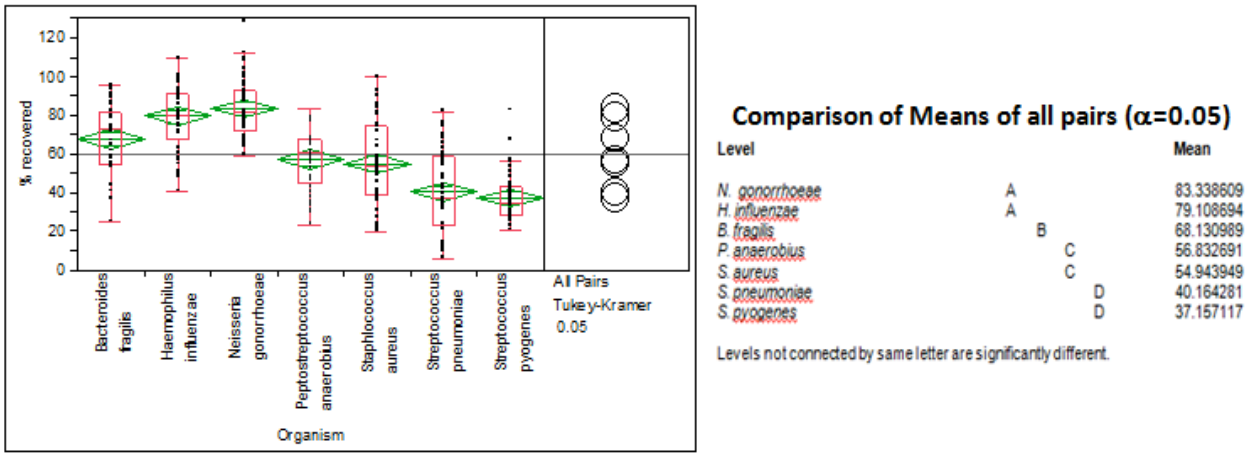


Figure 5. One-way ANOVA of recovery of bacteria-By oganism.

4.6.1.3 By the Gram-reaction The study consisted of three Gram-negative (*N.gonorrhoeae*, *H.influenzae*, and *B. fragilis*) bacteria and four Gram-positive (*P. anaerobius, S. aureus, S. pneumonia*, and *S. pyogenes*) bacteria. The recovery values showed a clearly significant delineation between Gram-negative and Gram-positive bacteria with the former exhibiting significantly higher recovery than the latter (Figure 6).

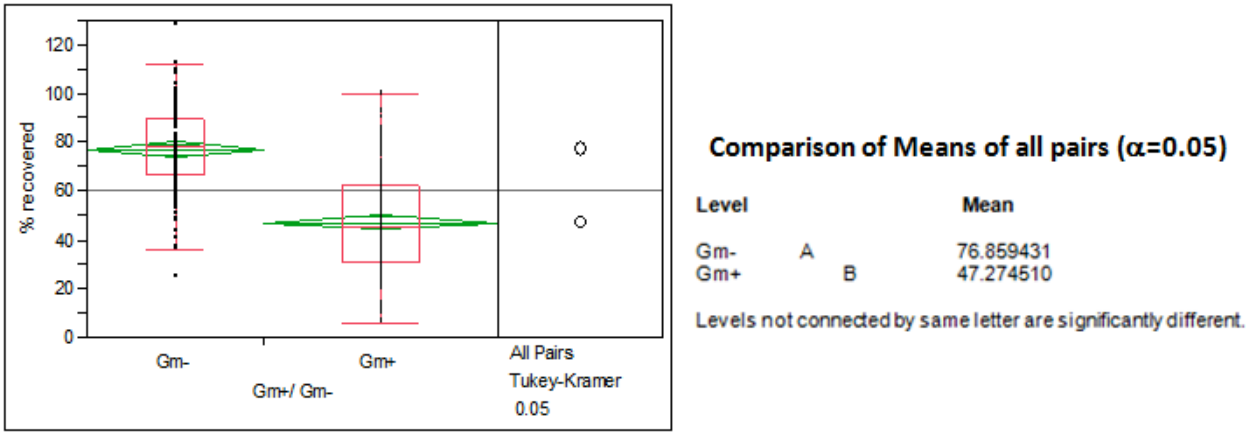


Figure 6. One-way ANOVA of recovery of bacteria-By Gram-reaction. (Gm+ and Gm- are Gram-positive and Gram-negative, respectively)

4.6.1.4 By Aerobiosis/anaerobiosis Except for *B. fragilis* and *P. anaerobius*, all organisms in the study were aerobes. No significant difference in the recovery of aerobes and anaerobes was evidenced in the current study (Figure 7) when comparing all swabs together.

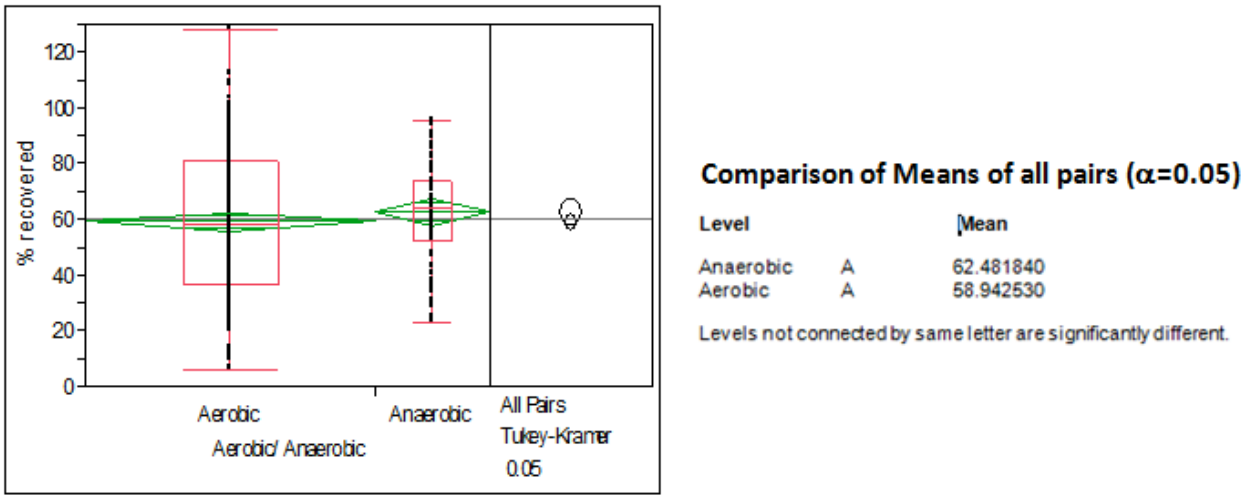


Figure 7. One-way ANOVA of recovery of bacteria-By aerobiosis/anaerobiosis.

4.6.2 Analysis by aerobiosis/anaerobiosis The entire set of recovery data was analyzed based on the ability of bacteria to grow under aerobic or anaerobic conditions.

4.6.2.1 Analysis by aerobiosis Among the test bacteria there were 5 aerobes and 2 anaerobes. The recoveries of aerobic bacteria were compared to each other by all swabs and the results are shown in Figure 8. The results shown in Figure 8 are similar to the results discussed in Figure 5. Further, analysis of recovery of all aerobes by swabs demonstrated the highest recovery with the Research Flocked swab (68%). No difference was observed in the recovery of aerobes (59.4%) by Macrofoam and Nylon Flocked (Copan).

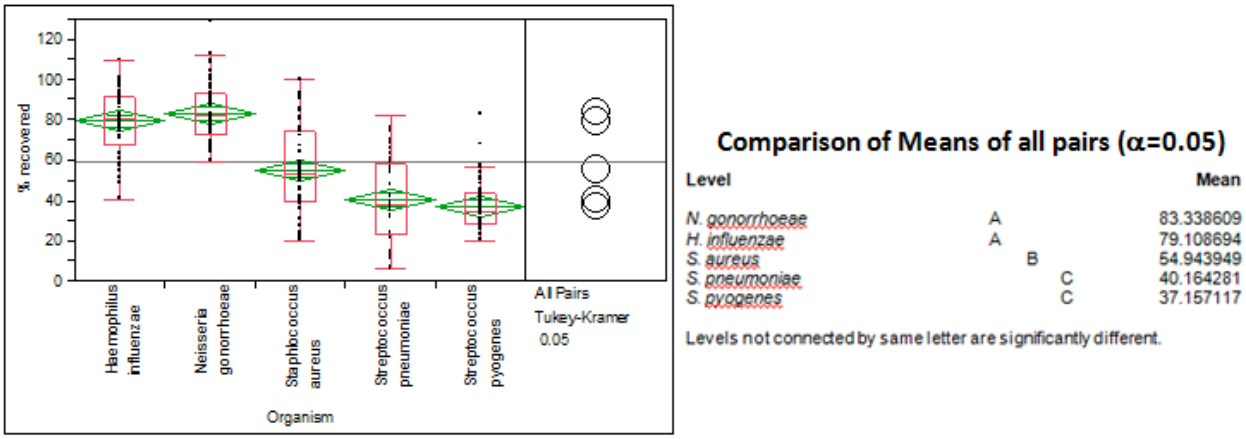
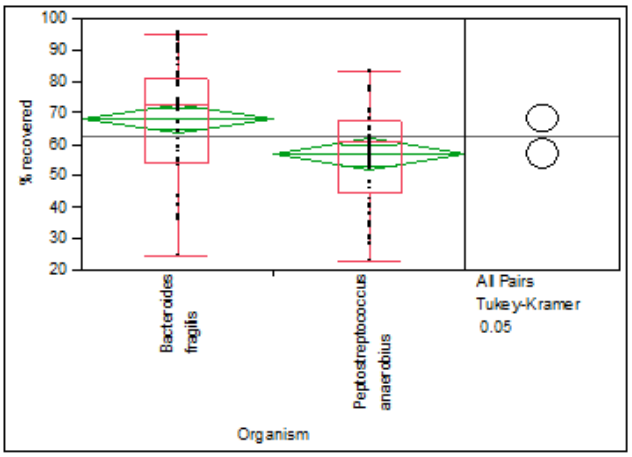


Figure 8. One-way ANOVA of recovery of aerobic bacteria.

4.6.2.2 Analysis by anaerobiosis The recoveries of the two anaerobic bacteria were compared to each other, and the results are shown in Figure 9. The results shown in Figure 9 are similar to the results discussed in Figure 5. Recovery of *B. fragilis* was higher than *P. anaerobius*, and the total recovery of each obligate anaerobe was significantly different from each other. Aero-tolerance by *B.fragilis* (Takeuchi *et al.*, 1999; Baughn and Malamy, 2004) might

have contributed to higher recovery of viable cells. The aero-intolerance by *P. anaerobius* (Rolfe *et al*., 1978; Carlsson *et al*., 1978) might have contributed to its lower recovery although utmost precautions were taken to maintain an anaerobic environment. In contrast to *B. fragilis*, the sensitivity of *Peptostreptococcus* sp to brief exposure of air was demonstrated by Barry *et al* (1972) with cotton and alginate swabs.



Comparison of Means of all pairs (α=0.05)

Level		Mean
B. fragilis	A	68.130989
P. anaerobius	B	56.832691

Levels not connected by same letter are significantly different.

Figure 9. One-way ANOVA of recovery of anaerobic bacteria.

4.6.3 Analysis by Gram reaction The entire recovery data was analyzed based on the Gram reaction of bacteria. As shown in Figure 6, the recovery of Gram-negative bacteria was higher than Gram-positive bacteria.

4.6.3.1 Recovery of Gram-negative bacteria-By organism Among the test organisms, were three Gram-negative bacteria. The results of recovery of each of the three Gram-negative bacteria are shown in Figure 10. The data are similar to the results discussed in Figure 5. Recovery of *B.fragilis* was significantly lower and different from the recovery of *N. gonorrhoeae* and *H. influenza.* There were no significant differences in recovery by *N. gonorrhoeae and H.influenzae.*

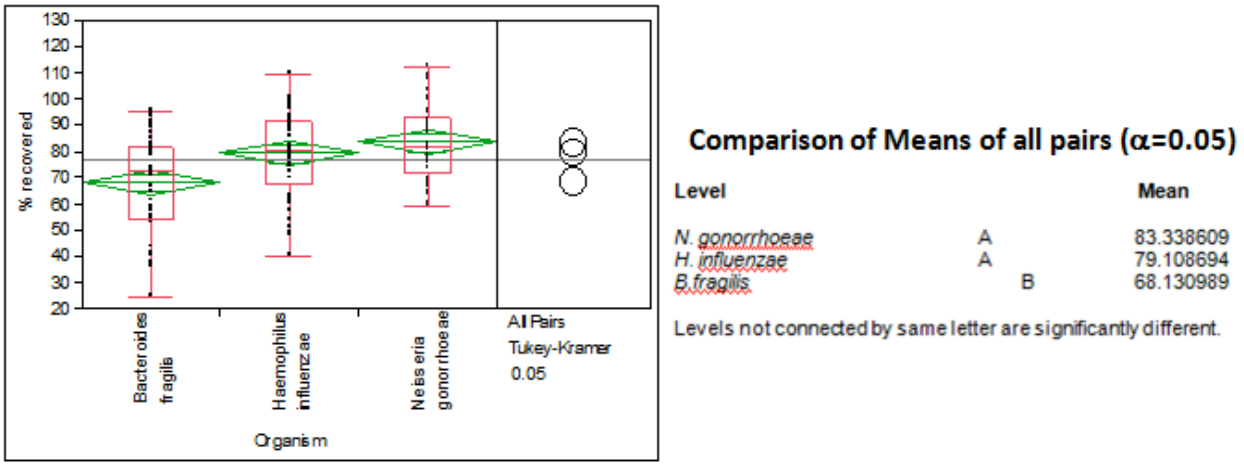


Figure 10. One-way ANOVA of recovery of Gram-negative bacteria-By organism.

4.6.3.2 Recovery of Gram-negative bacteria-By swab type Examination of the recovery of Gram-negative bacteria by various swabs (Figure 11) revealed the highest (86%) and the lowest (56%) recovery by Macrofoam and Nylon Flocked (Copan), respectively; the differences were statistically significant. The difference in recovery of all Gram-negative bacteria between any swab of Puritan and Nylon Flocked (Copan) was between 18% and 30%, under the test conditions suggesting the potential to use Puritan's swabs for selective applications. The difference in recovery between the highest and the lowest recovery of Puritan swabs was ~ 12%. It is interesting to note that Gram-negative bacterial infections represent one third of the microbiologically documented infections in febrile series of neutropenic patients (Glauser *et al*., 1997).

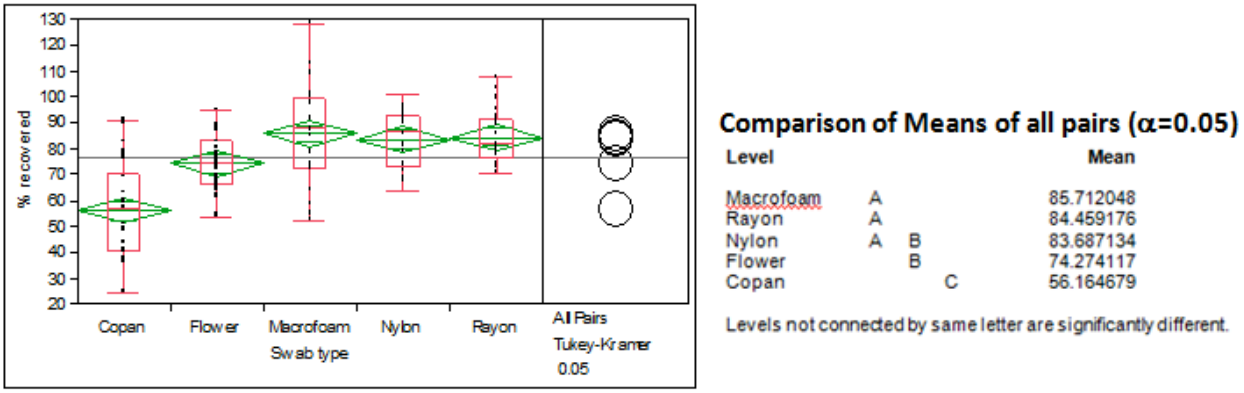
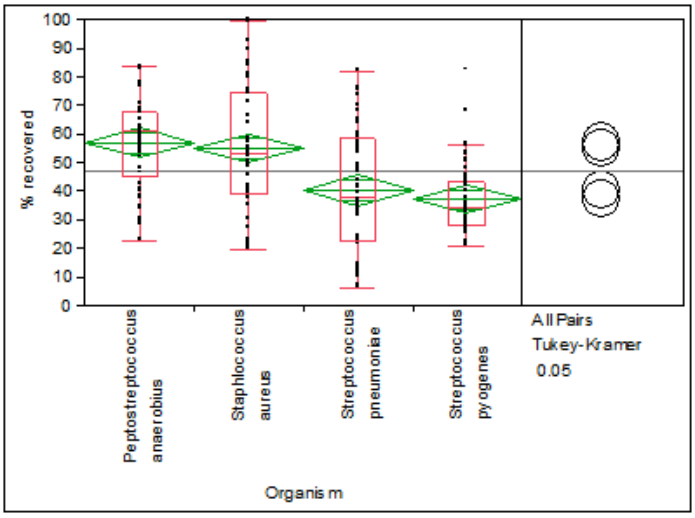


Figure 11. One-way ANOVA of recovery of Gram-negative bacteria-By swab.

4.6.3.3 Recovery of Gram-positive bacteria-By organism Examination of the recovery of Gram-positive bacteria by organism fell into two groups (Figure 12) which was also evident in Figure 5. Among Gram-positive bacteria, recovery of *P. anaerobius* and *S. aureues* was significantly higher (57%) than the recovery of *S. pneumoniae* and *S. pyogenes* (37%) combining all the swabs together.



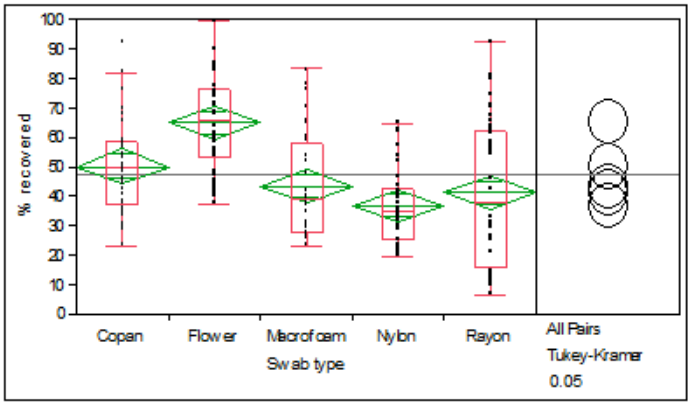
Comparison of Means of all pairs (α=0.05)

Level			Mean
P. anaerobius	A		56.832691
S. aureus	A		54.943949
S. pneumoniae		B	40.164281
S. pyogenes		B	37.157117

Levels not connected by same letter are significantly different.

Figure 12. One-way ANOVA of recovery of Gram-positive bacteria-By organism.

4.6.3.4 Recovery of Gram-positive bacteria-By swab type Examination of the recovery of Gram-positive bacteria by various swabs (Figure 13) revealed the highest (65%) and the lowest (37%) recovery by Research Flocked and Nylon Flocked swabs, respectively. Note worthy that recovery by Research Flocked swab is significantly different from the rest. The recovery of various Gram-positive bacteria by Nylon Flocked (Copan) was not significantly higher than recoveries obtained with Macrofoam and Rayon Flocked swabs.



Comparison of Means of all pairs (α=0.05)

Level				Mean
Flower	A			64.892223
Copan		B		50.218894
Macrofoam		B	C	43.494374
Rayon		B	C	41.210361
Nylon			C	36.556697

Levels not connected by same letter are significantly different.

Figure 13. One-Way ANOVA of recovery of Gram-positive bacteria-By swab.

4.6.4. Recovery-By each organism and swab type Finally, the recovery was analyzed by organism and by swab type so that swabs exhibiting higher recovery of viable organisms can be recommended for specific applications (Figures 14 & 15). From Figure 14, it can be concluded that the swabs exhibiting the highest and the lowest recovery of *H. influenzae*, *N. gonorrrhoeae*,and *P. anaerobius* were Macrofoam and Nylon Flocked (Copan), respectively. Additionally, Nylon Flocked (Puritan), Research Flocked, and Rayon Flocked were superior to Nylon Flocked (Copan) in recovering *B. fragilis* (Figure 14A).

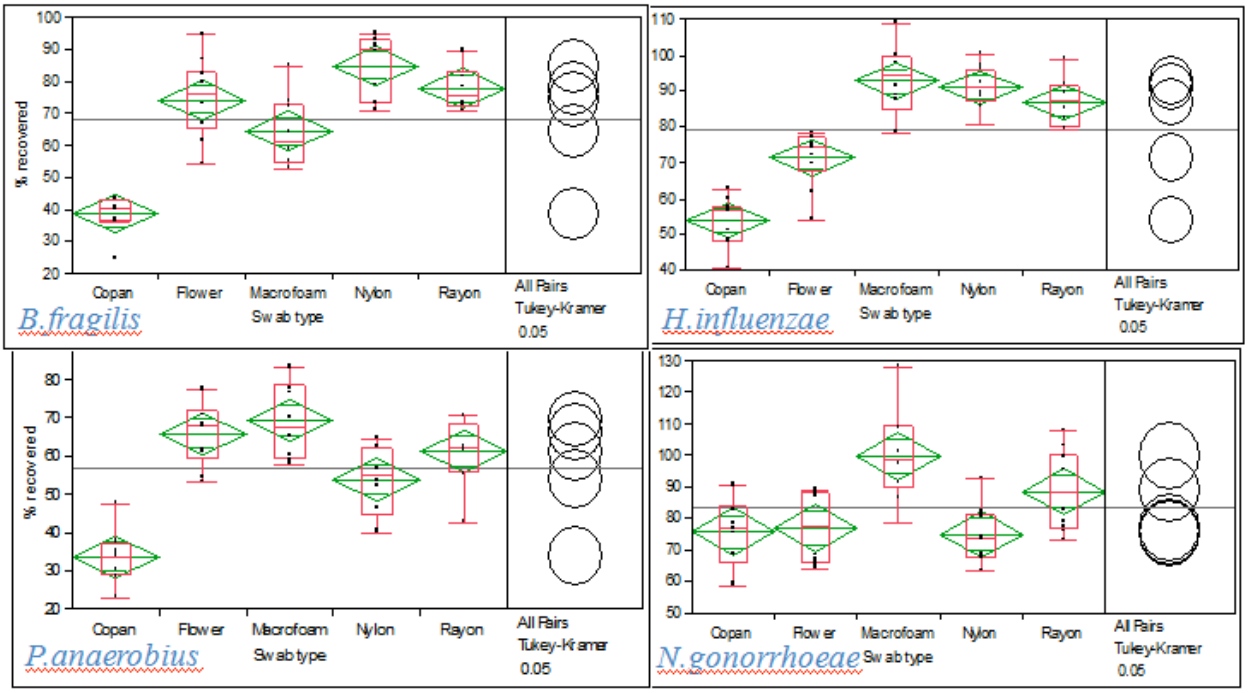


Figure 14A. One-way ANOVA of recovery of each bacteria.

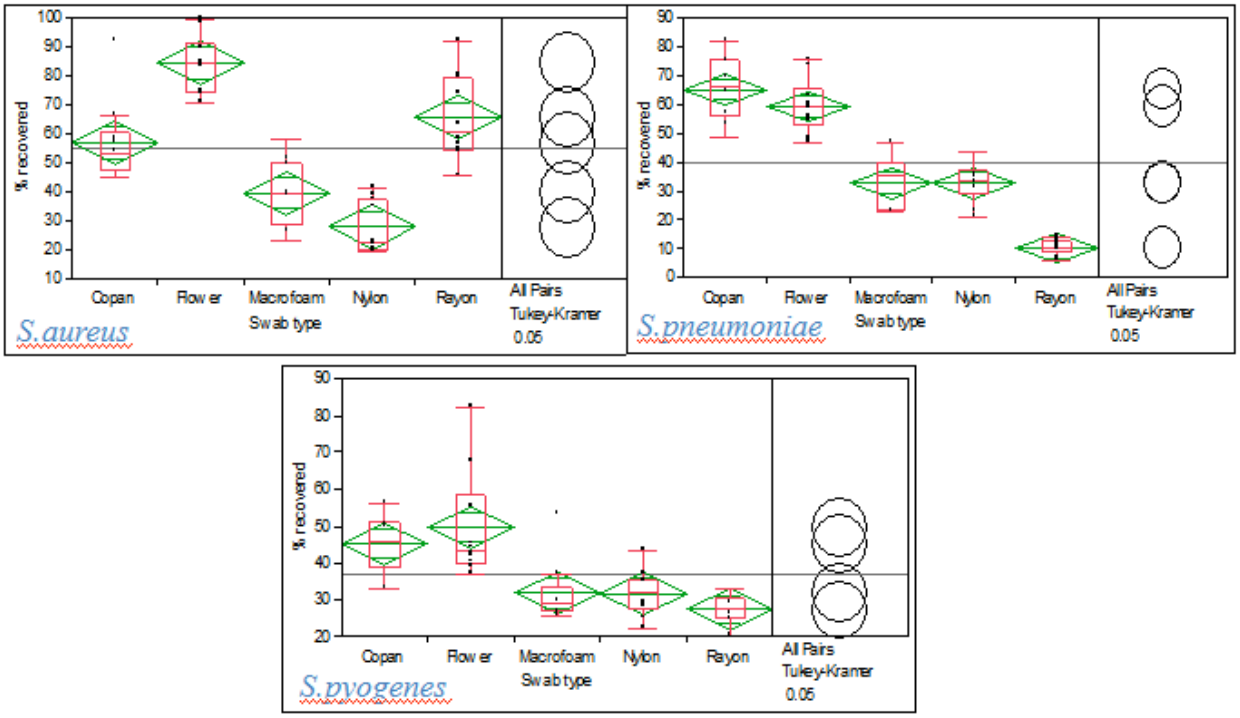


Figure 14B. One-way ANOVA of recovery of each bacteria.

The Research Flocked recovered the largest number of viable *S. aureus* and *S. pyogenes* (**Figure 15A**). The recovery of *S. pneumoniae* was the highest with Nylon Flocked (Copan). It is interesting to note consistently lower recovery (<50%) of *S. pneumoniae* and *S. pyogenes* with all swabs used in the study (**Figure 15A & B**). One of the interesting features of this study is the higher recovery of fastidious organisms, *H. influenzae*, *N. gonorrrhoeae* but low recovery of the other fastidious organism, *S. pneumoniae*. It can be argued that some of the observed differences in recovery of organisms from the test swab is attributable to a direct toxic effect of one of the components of the swab.

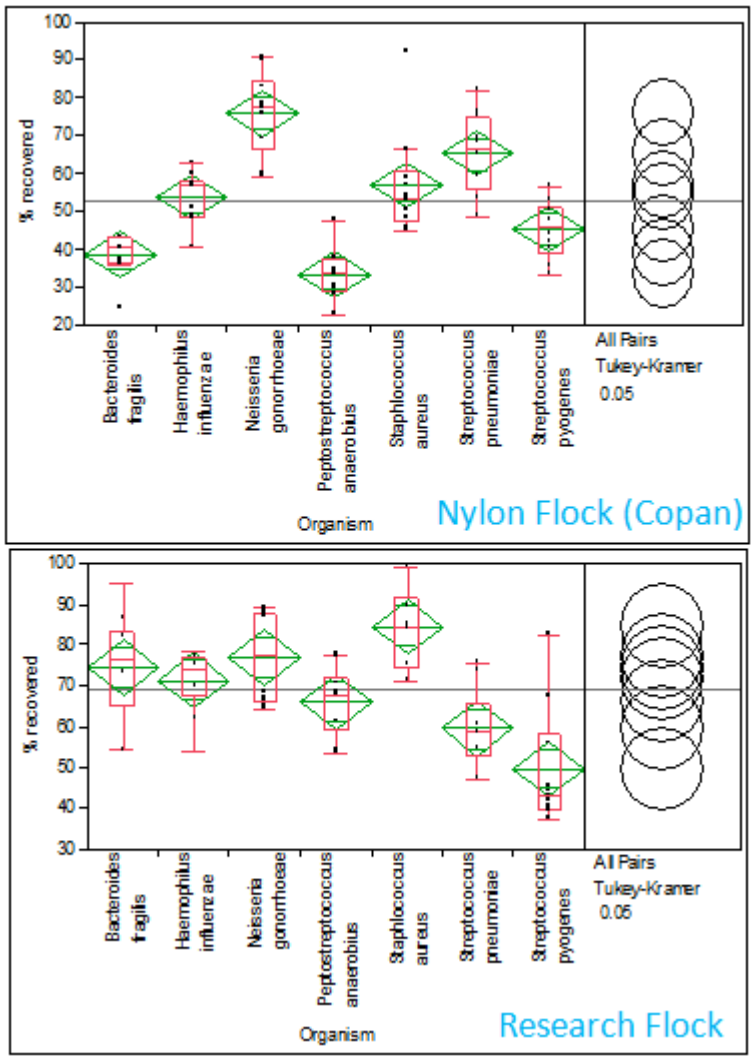


Figure 15A. One-way ANOVA of recovery of each bacteria-By swab.

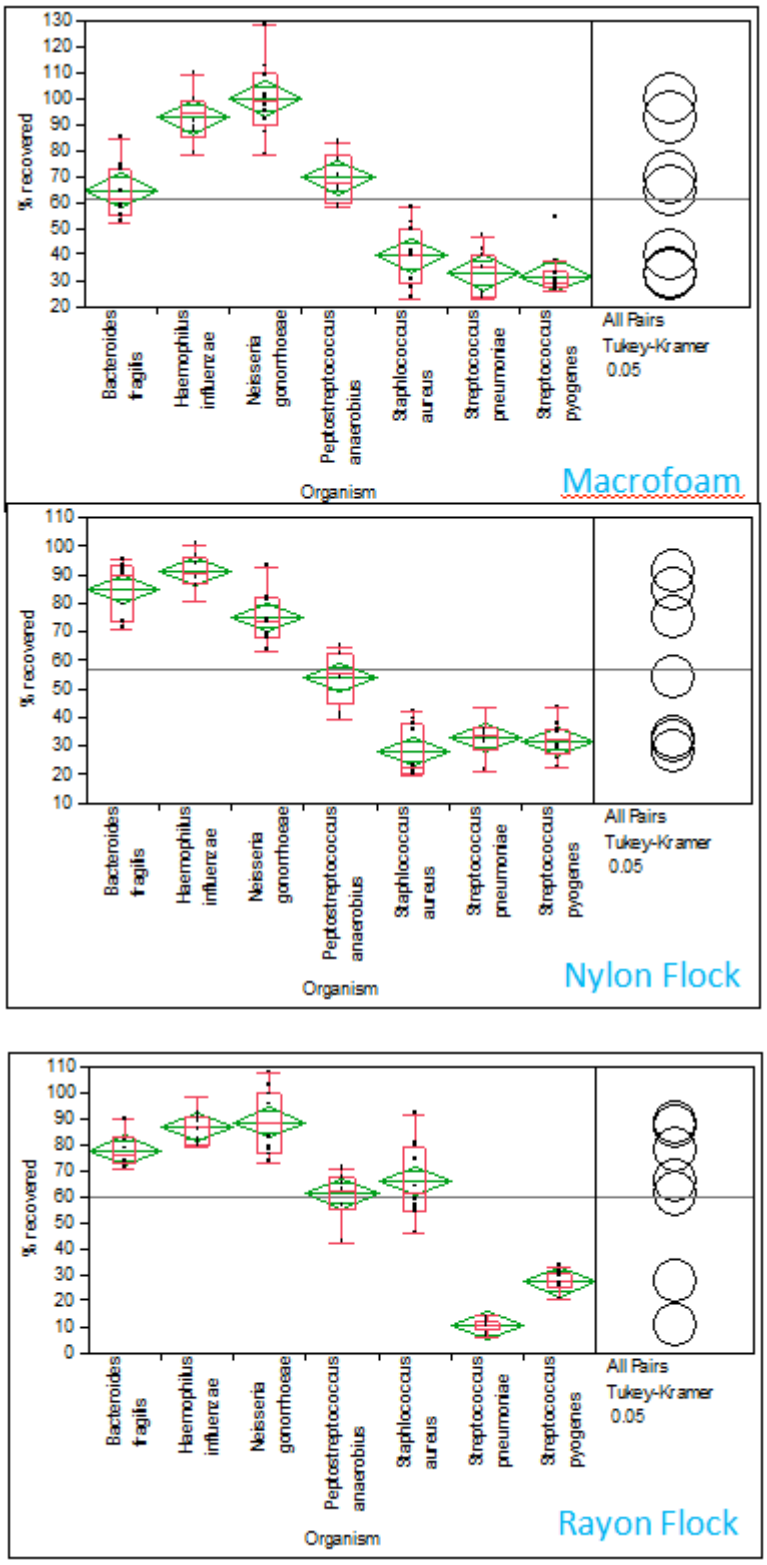


Figure 15B. One-way ANOVA of recovery of each bacteria-By swab..

5. Conclusions

Under most conditions of routine clinical practice, a swab carrying pathogenic microorganisms is used to provide sufficient information to direct antimicrobial therapy. A comparative study of this nature as described here cannot be performed with actual clinical specimens without introducing uncontrollable variables. Clinical specimens vary in viscosity and contain cellular and chemical constituents that may act as nutrients or toxins and that are often poly-microbial, that have the potential to affect the organism's viability. The survival of test organisms reported in this study may not faithfully reflect results with actual clinical material or recovery rates based on more common transport times; however, it allows comparisons of recovery rates to be made and most certainly permits insight into a swab's ability to sustain organism viability, a notion taken on faith if not investigated. An ideal swab is designed to collect many cells and allow for their release into media that needs to be verified by using scientifically competent and valid methods. Quantitative results obtained with swabs are similar to biopsy results, especially for wounds (Wheat *et al*., 1986). For example, a 75% concordance between the swab and biopsy specimens of peptic ulcers was observed (Sapico *et al*., 1986). Our method of collecting bacteria on the swab is one of the scientifically valid methods and offers a simple comparison of swabs' efficiencies.

The performance of various flocked and Macrofoam swabs of Puritan were compared to Nylon Flocked swab of Copan using scientifically competent and valid methods in a laboratory setting. A key finding of this study is the overall superior performance of Research Flocked swab based on high water and protein absorption capacities, and high recovery of bacteria. Further, the ability of the Macrofoam swab to recover the largest number of *H. influenzae*, *N. gonorrrhoeae*, and *P. anaerobius* deserves attention. The overall performance of Nylon Flocked (Copan) to recover viable bacteria did not surpass any one of the swabs manufactured by Puritan used in this study. The overall highest recovery efficiency of various bacteria coupled with its high water absorption capacity with the Research Flocked design is likely to have a greater effect on diagnostic sensitivity.

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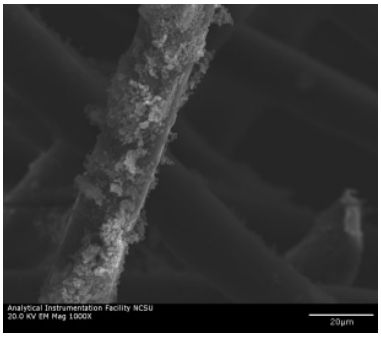
APPENDIX 1



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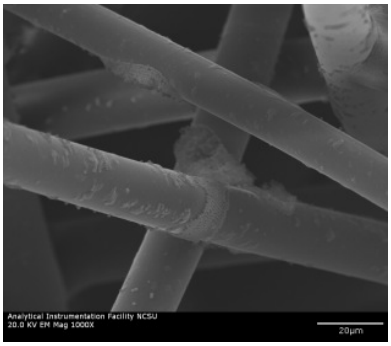

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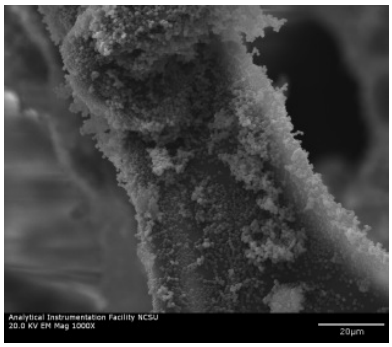
Nylon Flock (Copan)



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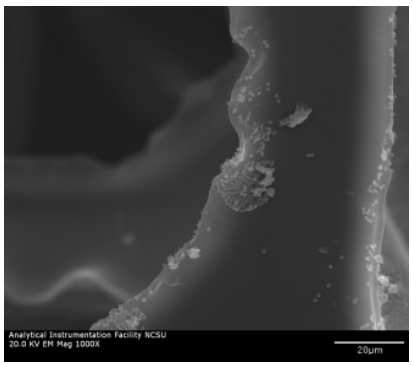

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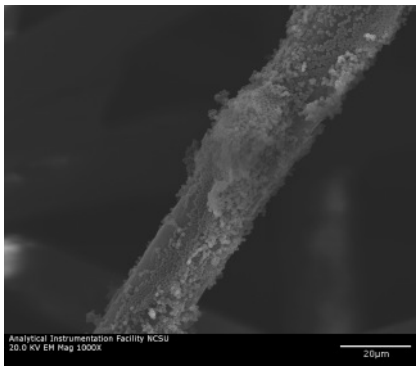
Macrofoam



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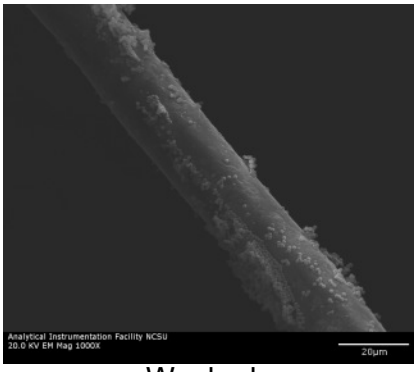
Nylon Flock



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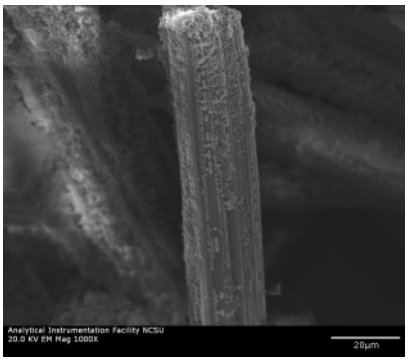

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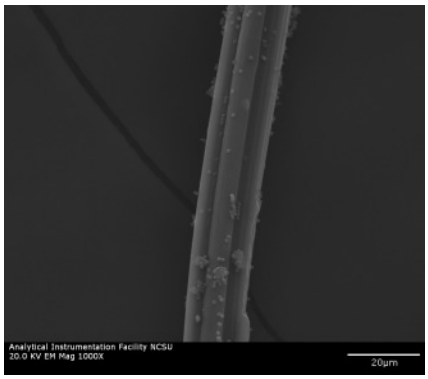
Rayon Flock



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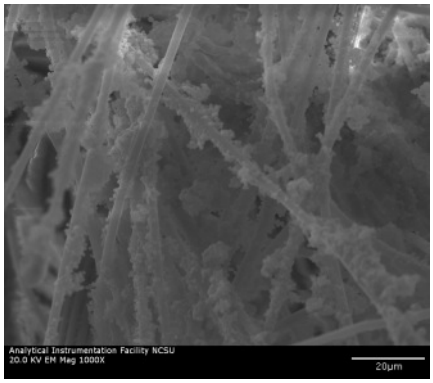

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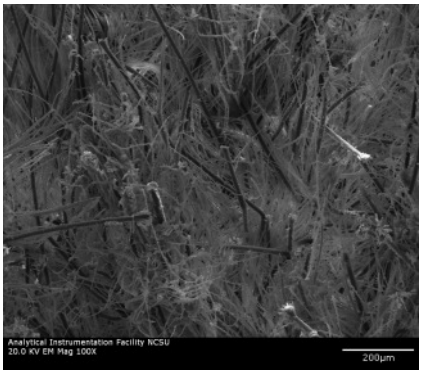

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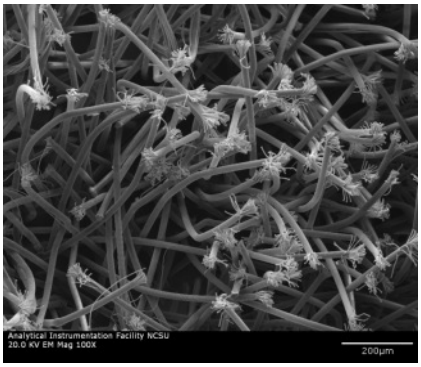

Research Flock