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Research Paper

Rapid identification of bacterial agents in fecal samples of rodents by flow cytometry

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Abstract

Experiments were performed to determine whether flow cytometer could be used for rapid identification of bacteria based on their light scatter properties. Fresh fecal samples were collected from mice and were cultured on nutrient agar. Prominent colonies from the samples were sub-cultured again on differential media (MacConkey's and Eosin Methylene Blue) and further subjected to both biochemical and FACS (Fluorescent Activated Cell Sorter) analysis. In conventional method, samples were cultured and organisms were isolated using appropriate media and further identified by using specific biochemical methods. For flow cytometric analysis, 20,000 cells were acquired from each sample (n=5) and subjected them for analysis by using both forward scatter and side scatter properties. The dilution of microbial samples, cytometer used and the voltage of forward and side scatter on the cytometer were maintained constant throughout the study. These characters were observed together on two dimensional Dot plots, Density plots and Contour plots. Significant differences in individual characteristics were observed in forward and side scatter properties among *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Pseudomonas aerogenosa*. Further, bacterial populations were quantified and also mixed populations of microorganisms were prepared and they were further sorted as individual population using FACS Aria II sorter and subjected to usual microbial and biochemical analysis. In the present study, results obtained from conventional method and flow cytometric analysis were compared and found to be correlating each other very well. This rapid detection of microorganisms and their quantification by flow cytometry method in few hours would certainly benefit the animal facilities in adopting effective, preventive or curative measures in short span of time.

Keywords: Bacteria, Flow cytometry, Forward Scatter (FSC), Rodents, Side Scatter (SSC)

Introduction

The use of animal models is critical to the biological and biomedical research. Till recent years, almost every medical breakthrough in human and animal health can be directly related to the results of research programs using animals. Animals are biologically similar to humans and are susceptible to many of the same health problems as humans. Since animals have a shorter life span and a brief life cycle, this provides feasibility to study the animals throughout their entire life cycle. Animals used for biomedical research should be in a state of absolute good health for reliable and reproducible results. Sometimes apparent healthy animals also suffer from latent infections. Majority of these infections are subclinical and may go undetected in gross examination, but clinical symptoms may appear under conditions of stress during experimentation. It has been reported that infections[1-3], exogenous factors (environmental), genetic factors and interactions of all these may influence the suitability of an animal

for research. Also it has been reported that even subclinical infections in rodents modify or alter research outcome. Many infectious agents affect results in the field of immunology, physiology, reproductive physiology, oncology and many more research areas[4].

In India more than one million animals are being used every year in scientific research, covering various life science fields like cell biology, molecular biology, bio-chemical and bio-medical research, veterinary science, pharmaceutical science, food technology, drug development and analysis and cosmetic industries etc. To obtain authentic and reproducible results of the research experiments, it is important to have standardized animals with known health and genetic status. The main objective of laboratory animal management is to provide healthy standardized animals for research work[5-7].

Researchers are responsible for quality of animals used for the experiments, and they also must be aware to cause minimum distress and pain to the animals[4]. Health monitoring of experimental animals is of vital importance for reliable and reproducible research data and to reduce the risks of infections to personnel handling such animals or their products. Careful and systematic monitoring at regular intervals is a pre-requisite for a uniformly defined animal health status. This ensures the quality as well as the outcome of research[6]. Health monitoring data are part of the experimental work and have to be considered during interpretation of experimental results by the researcher[8].Traditional method of bacterial identification relies on phenotypic identification of the causative organism using gram staining, different culture media and biochemical methods. Also light microscopy is used to observe the shape and cellular arrangement of bacteria, and use this information for species identification.

Flow cytometry has evolved as an important tool for providing speedy identification of cell parameters along with great statistical accuracy. Cytometer can handle thousands of cells in few seconds and analyze them individually. Past decade has seen enormous development in the field of cytometry as well as its usage in biomedical research. Flow cytometry has vast application in microbiology too. This includes counting the bacterial cells, bacterial cell cycle analysis and assessment of antibiotic susceptibility of clinical samples, aquatic microbial studies etc. [9-14]

Flow cytometry is an analytical tool that allows the rapid measurement of light scatter and fluorescence emission from single cells, by which multiple cellular parameters can be analysed[15-17] .Flow cytometric measurements, can be made on several different characteristics of each cell. Such multi-parametric measurements are useful to correlate different characteristics and define sub-populations and distinguish between different cell types. Since measurements are made on single cells, heterogeneity within the population can be detected and quantified[15,16,18,19].

The data analysis system consists of software that allows the analysis of the huge amount of information produced by multi parameter data acquisition. The analytical software permits the study and independent analysis of any particular sub-populations. Besides all the statistical information, the data can be represented in several different formats such as mono-parametric histograms, bi-parametric plots and three dimensional plots. It is possible to perform qualitative and quantitative analyses of the samples at the same time. Thus, flow cytometry is a suitable technique for the study of heterogeneous populations in any cells[18,20,21].

Light scatter and flurochromes staining has been well used in the study of microbes from early 1980's onwards. Physiological effects of antimicrobial agents on bacterial cell size, membrane potential, quantity of DNA and cell cycle were some of the predominant studies[9,22]. Monitoring different parameters in clinical samples during the course of an illness allows an assessment of the effect of specific therapy on animals. This can also help in detecting emerging resistant strains. It has been reported by Walberg *et al*., 1996, Gant *et al.,* 1993, and Mason *et al.,* 1995[11,23,24] that light scattering profile of microbes is a useful parameter in the study of antimicrobial agents.

Encouraged by the reported results, the present study has been envisaged based on scatter parameters of microbes. In this study, we present a rapid method to detect few micro-organisms in rodents using Flow cytometry. Cytometry measurements of different bacterial colonies were carried out to evaluate whether cytometer can be used in scientifically reliable way to assess the bacterial load in laboratory animals and also to check whether scatter pattern can be used as a tool in identifying bacterial species.

Materials and Methods

Collection of samples
Fresh fecal samples from different strains of laboratory animals maintained at the Central Animal Facility, Indian Institute of Science, Bangalore, India were procured for this study. These animals were maintained under standard husbandry conditions with controlled temperature and humidity. Pelleted diet and water was provided *ad libidum.*

Preparation of samples
Fecal samples were collected from four different strains of laboratory mice such as BALB/c, C57BL/6, Swiss albino and C3HeJ. The age of these mice varied from 4 to 6 weeks. Half a gram of feces was weighed and suspended in five ml of 1XPBS. The suspension was thoroughly centrifuged at 1000 rpm for two minutes to remove debris. Different aliquots of the supernatant were stored at – 20^0Cuntil further analysis.

Isolation and identification of bacteria
A 0.1 ml of the stock solution was inoculated on nutrient agar by spread plate technique, the agar plates were incubated at 37^oC for 24 hours. Individual colonies from the medium were streaked on slides and further subjected to standard Gram staining using standard procedure. The heat fixed bacterial smear was subjected to four different reagents in the order listed: Crystal violet a primary stain for 1 min, Gram's iodine solution as a mordant for 1 min, Alcohol (70%) as a decolorizing agent for 30sec and Safranin to counter stain for 1min.The slides were analyzed under light microscope. Further, these colonies from the medium were grown individually in an appropriate selective and differential medium such as Mannitol Salt Agar (MSA), MacConkey's Agar (MCA), Eosin Methylene blue Agar (EMB). Subsequently standard biochemical tests such as Hydrogen sulfide production test (H_2S), Indole test, Methyl red (MR) and Voges-Proskauer (VP) test, Citrate utilization test, Catalase test, Gelatin liquefaction test, Oxidase test and Urease test were carried out to differentiate between the strains[25].

Preparation of single bacterial species stock suspensions
E. coli, Salmonella typhimurium, Pseudomonas aerogenosa and *Staphylococcus aureus* were grown aerobically at 37^oC in nutrient broth (Peptone-5g, Sodium chloride-5g and Beef extract-3g for 1000ml of distilled water) overnight. After incubation for 24hrs, the broth was centrifuged at 2000 rpm for 4 minutes and washed with Phosphate Buffered Saline (PBS) for three times, after the third wash, the bacteria were suspended in nutrient broth and stored at -20^oC.

Analysis of single species bacterial suspension
Single cell suspensions of *E. coli, Salmonella typhimurium, Pseudomonas aerogenosa* and *Staphylococcus aureus* were prepared in triplicates. Individual samples were diluted using PBS. Around 20,000 cells were acquired on Flow cytometer (Calibur BD).Viability maintenance was very important for microbial analysis. We have maintained the viability of microbial cells as well as their dilution constant during the flow cytometry studies. Sample flow rate was always maintained at "Low" allowing between 500 to 600 cells to pass the laser beam per second. The voltage of Forward Scatter (FSC) and Side Scatter (SSC) were also maintained constant throughout the study (Any change in these parameters can definitely express well in the spatial distribution pattern of microbes.

Preparation of mixed bacterial suspension
After the quantification of single species stock suspension by cytometer, a mixture of *E. coli, Salmonella typhimurium* and *Pseudomonas aerogenosa*were prepared. The single species stock suspension was diluted and equal volumes of these suspensions were mixed, so that the three species mixture roughly contained an equal amount of bacteria of each species and incubated for 20 min before sorting them on an Aria II sorter (BD).

Analysis of mixed bacterial suspension mixture
Further, an attempt was made to detect multiple infections by using mixture of all three bacterial samples such as *E. coli, Salmonella typhimurium*and *Pseudomonas aerogenosa*. These mixed samples were sorted by Cell Sorter (AriaII-BD) based on the forward and side scatter parameters after a brief incubation of 20 min. The individual sorted bacterial samples were again inoculated separately into nutrient broth and incubated. Overnight grown culture was again plated onto selective media and then subjected to the earlier biochemical tests.

Results and Discussion

Bacterial analysis by culture methods

Samples obtained from animals were subjected for bacterial screening. The samples were inoculated into nutrient broth, incubated overnight at 37^0C. Subsequently, overnight grown culture was plated onto nutrient agar by spread plate method. The agar plates were incubated at 37^0C for 24 hrs. Colony counts and characters such as color, elevation, margin and surface were noted (Table 1). Gram staining of these colonies revealed four different distinct colonies such as *E. coli, Salmonella typhimurium, Pseudomonas aerogenosa* and *Staphylococcus aureus* (Figure 1A-D).

Table 1: Colony characters of various rodent micro-organisms on Nutrient Agar

Sample No	Strain	No of colonies	Colour	Elevation	Margin	Surface	Grams reaction
1	C57BL/6	> 70	White	Flat	Round	Mucoid	Gram –ve rods
2	BALB/c	> 65	Cream	Raised	Round	Mucoid	Gram –ve rods
3	Swiss	> 48	Green	Flat	Round	Mucoid	Gram –ve rods
4	C3He/J	>93	Cream	Raised	Round	Mucoid	Gram –ve rods

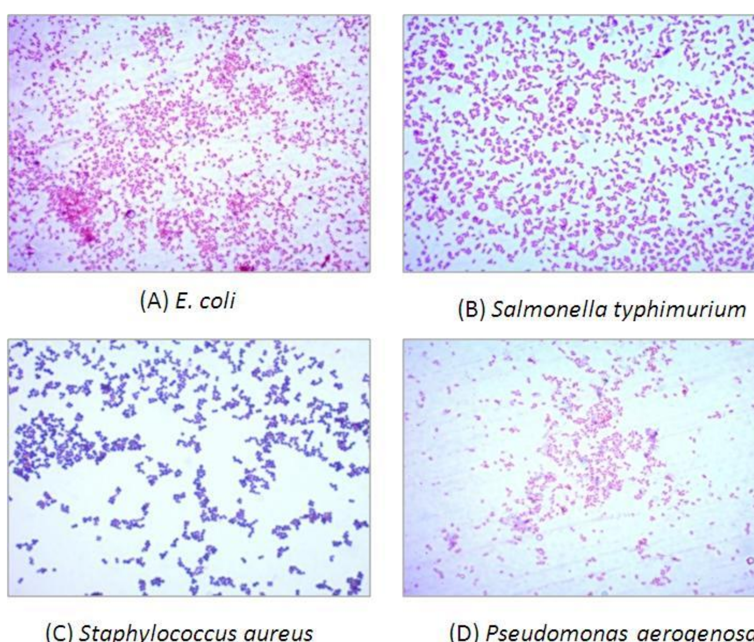

Figure 1: Cultural morphology of various rodent micro-organisms on Gram's staining

The specific white and cream colonies were picked up from nutrient agar plate and re-inoculated on specific agar such as MacConkey's agar (MCA) (Figure 2A) and MannitolSalt Agar (MSA) (Figure 2B). Pink colored colonies were observed on MSA and both pink and colorless colonies were seen on MCA. Further, pink color colony from MCA was streaked on Eosin Methylene Blue Agar (EMB) (Figure 2C), which gave green metallic sheen on overnight incubation. Green color colony (Figure 2D) was re-inoculated into nutrient agar and further used for biochemical tests. Biochemical analysis of samples for various tests such as Catalase, Oxidase, Indole, MR, VP, Citrate utilization, Gelatin liquefaction and H_2S production revealed four distinct microorganisms (Table 2).

Each sample was subjected to Flow cytometry by using FACS Calibur and analysis was done by using Cell quest Pro program. About 20,000 cells were acquired from each sample culture and both Forward (FSC) and Side Scatter (SSC) properties were determined.(Commercial beads 3µm were run separately and also along with samples, the data of which is not given here)

Table 2: Results of various biochemical tests

S. No.	Catalase	Oxidase	Indole	MR	VP	Citrate Utilization	Gelatin liquefactions	H_2S production	Inference
1	+ ve	-ve	+ ve	+ve	-ve	-ve	-ve	-ve	*E. coli*
2	+ ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	*Salmonella typhimurioum*
3	+ ve	+ ve	+ve	+ve	-ve	-ve	+ ve	-ve	*Pseudomonas aerogenosa*
4	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	*Staphylococcus aureus*

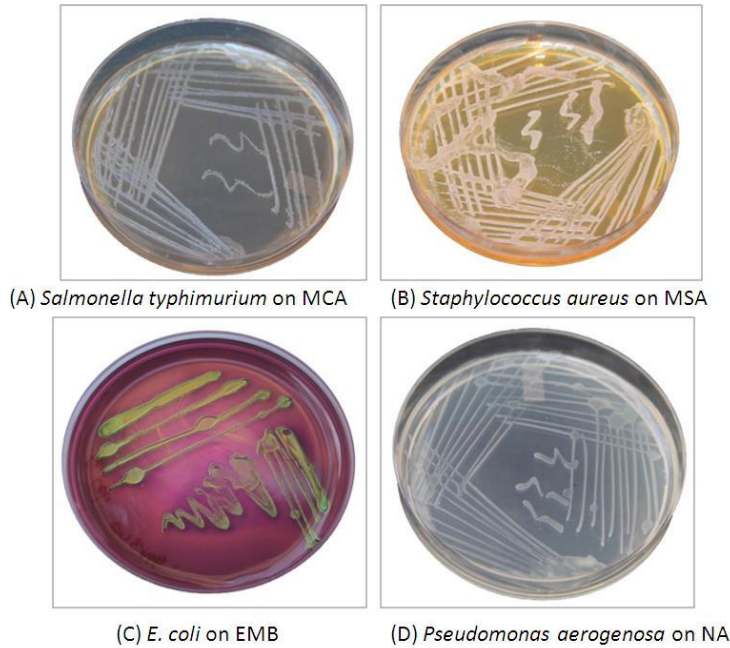

Figure 2: Cultural morphology of various rodent micro-organisms on different selective media

Each tube containing *E. coli*, *Salmonella typhimurium, Staphylococcus aureus* and *Pseudomonas aerogenosa* was diluted in such a way that flow rate of cells were around 600 – 800 per second in the cytometer. The scatter characters were individually taken as histogram plots. Individual characteristic differences in forward and side scatter were observed among *E. coli*, *Salmonella typhimurium, Staphylococcus aureus* and *Pseudomonas aerogenosa* (Figure3A-D).Both forward scatter and side scatter properties were observed together on Dot plots (Figure 3A-D),three dimensional density plots (Figure 4A-D) and Contour plots (Figure: 5A-D). An attempt was made to detect multiple micro-organisms by using FACS hence, single cell suspension of *E. coli*, *Salmonella typhimurium* and *Pseudomonas aerogenosa* were mixed. Cell characters were determined in the mixed sample (Figure 6A-D). The mixed sample was sorted on a Cell Sorter (FACS ARIA II was the cell sorter with “FACSDiva” program) and the number of cells that were sorted out individually is illustrated in Table 3. Sorted cells were confirmed for their identification by subjecting them again to culture and biochemical tests.

Table 3: Number of cells sorted (out of 20,000 cells)

Species	Number of cells Sorted
E. coli	15892
Salmonella typhimurium	24886
Pseudomonas aerogenosa	13320

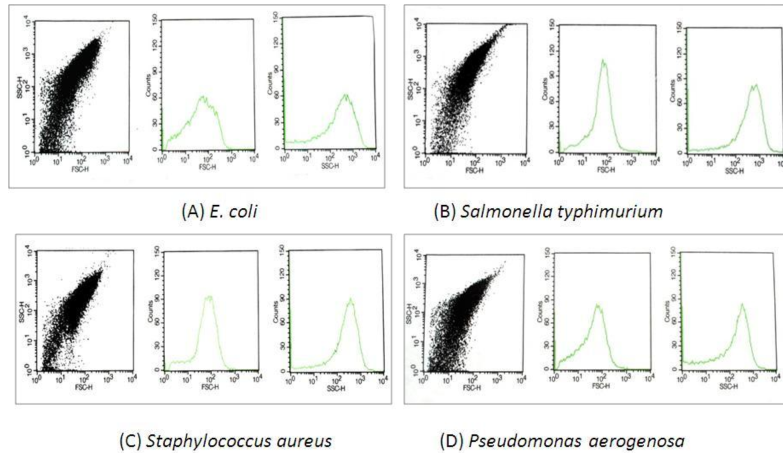

Figure 3: Dot plot and histogram of various rodent micro-organisms

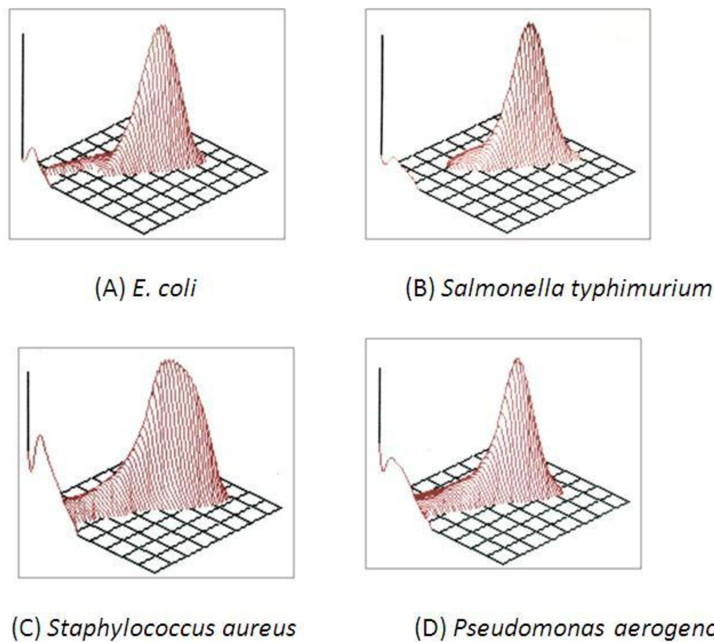

Figure 4: 3D density plot of various rodent micro-organisms

Conventional method of culture and isolation of micro-organisms requires microscopic and biochemical evaluation that necessitates considerable time for the accurate determination. However, flow cytometry is a rapid technique which allows evaluation of thousands of cells in fraction of a second. In this technique, individual cell can be analyzed quantitatively depending on their distribution properties. Flow cytometric method offers many advantages over conventional method in identification of bacteria. This rapid technique would certainly help in identifying the bacterial agent very quickly and thus aid in curtailing the spread of infection in the animal facility if appropriate actions are taken immediately. This novel method of identifying the microorganism by flow cytometry was employed in rapid diagnosis of bacterial infections in the present study.

In this study, we used flow cytometry for a speedy understanding of microbes. Microbial colonies were identified based on staining and biochemical tests. These preliminary studies helped in identifying of *E. coli*, *Salmonella typhimurium, Pseudomonas aerogenosa*, and *Staphylococcus aureus*. Further each microbe was grown separately and used for flow cytometry analysis.

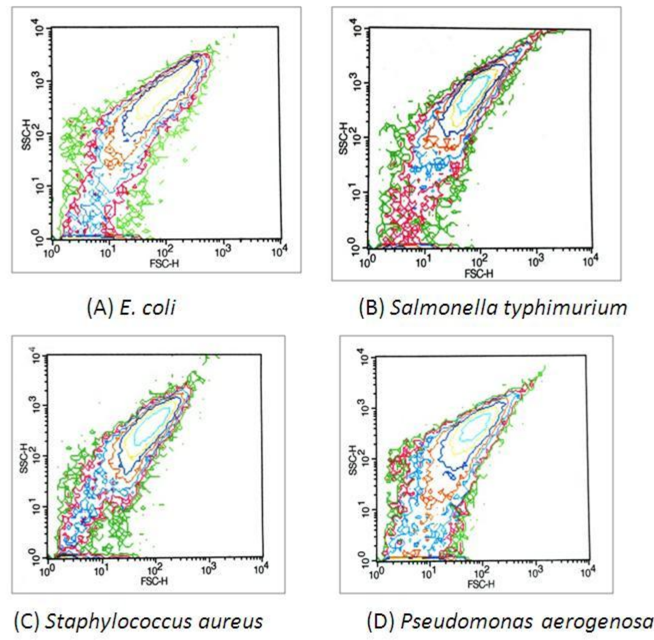

Figure 5: Contour plotof various rodent micro-organisms

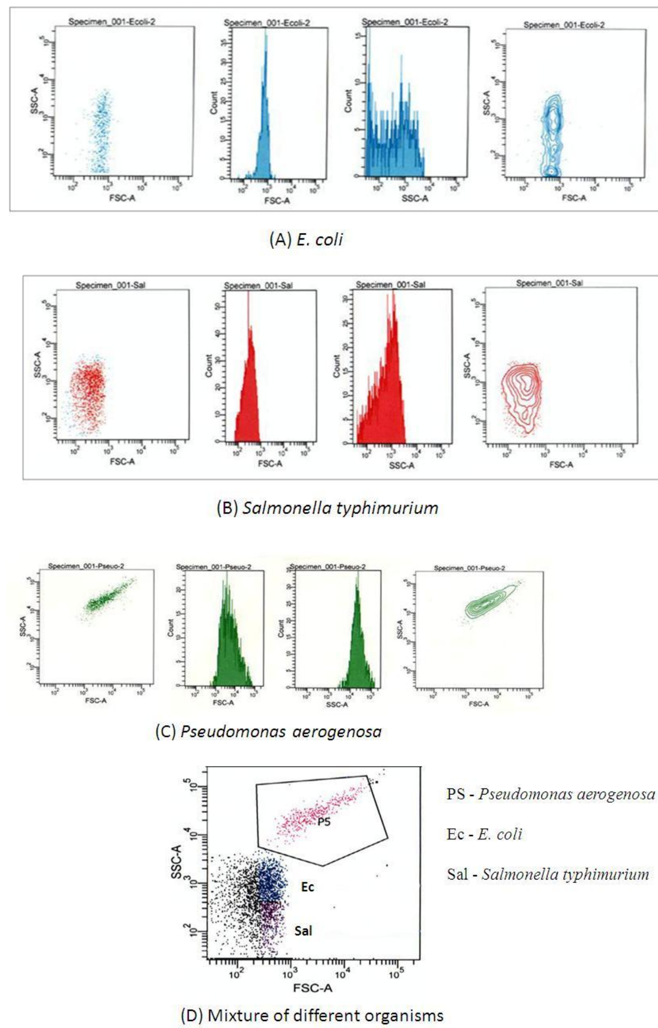

Figure 6: Contour and dot plot of *E. coli, Salmonella typhimurium* and *Pseudomonas aerogenosa*

Flow cytometry analysis was done to ascertain whether cytometers can be used in microbial species identification. Cytometers readily detect bacteria, and distinguish them from optical and electronic noise. Forward Scatter and Side Scatter dot plots of *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Pseudomonas aerogenosa* are represented in figure. Forward Scatter, Side Scattered histograms, contour plots and density plots were also prepared. Differences in pattern of each plot were clear from the figures provided for all four species.

Specific scatter patterns were observed for each identified bacteria (by culture and biochemical studies).In this experiment the dilution factor for bacterial samples remained similar for all the four species studied and also the voltage for forward scatter and side scatter on the Calibur cytometer was a constant factor. Hence the results obtained can be compared for identification.

Further, in our experiments three different species of bacteria such as *E. coli*, *Salmonella typhimurium* and *Pseudomonas aerogenosa* were mixed and subjected them for forward scatter and side scatters parameters. Further, each of these sorted bacteria was separately grown and analyzed by both culture and biochemical tests to verify bacterial species used for the mix and sort procedure. *Pseudomonas aerogenosa* was distinctively separate without any contamination which can be attributed to the cell granularity. However, *Salmonella typhimurium* and *E. coli* had shown a 6% to 8% contamination which can be attributed to the similarity in their cell size.

We have investigated the suitability of flow cytometry for the study of bacteria to determine whether this technique might be applicable to the study of the morphology and physiology of individual bacterial cells. Flow cytometer can readily detect bacteria and distinguish them from optical and electronic noise. Forward scatter and side scatter dot plot showed the distinct pattern for each micro-organism. From this, a forward scatter histogram and side scattered histogram was drawn. Both contour plot and density plot were also developed to identify the differences. Distinct difference in the pattern was observed very clearly in the present study.

Our work is supported by the earlier reports in microbial identification using flow cytometry by Gant *et al.*, (1993) and Hazel *et al.,*(2003) [11, 26]. Advantages of flow cytometer includes that large number of cells can be individually analyzed faster and also individual cell analysis helps in understanding heterogeneity among microbial cells. Further, sorted out cells from the mixture of *E. Coli*, *Pseudomonas aerogenosa* and *Salmonella typhimurium* based on forward and side scatter properties showed species specific characters on growth patterns and biochemical analysis. This substantiates our earlier result of bacterial scatter studies to identify different bacterial species. However, there are reasons yet to look into this pattern critically because, the patterns evolved can vary with the electrical voltage applied or with the density of microbes present in the culture sample. Hence, before one can use assertively this pattern for identification of microbes, lot more analytical thoughts have to be put in to standardize the same for each sample and specific cytometers.

Conclusion

The present study stresses that flow cytometry can be effectively used to determine the various micro organisms based on their size and granularity provided care should be taken to avoid possible variable factors.

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