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Rapid Detection of Bacteria from Blood Culture by an Electronic Nose

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The treatment of patients with bacteraemia and septicemia requires accurate and rapid identification of the pathogen so that the physician can be guided regarding the selection of the proper antimicrobial therapy. The usual procedure is to withdraw an aliquot of the positive blood culture sample for gram staining and subculturing on the media for the growth and subsequent identification, and susceptibility determinations. It was noticed that during the process some microbiologists would sniff the effluent gases that are products of metabolism and in some cases guess the identity of the bacterium. That prompted us to engage in systematic investigation of two gram positive and two gram negative bacteria using an electronic nose that had been proven successful in distinguishing the aroma of coffee beans from different sources. The investigation was successful in illustrating the efficacy of such a device in this clinical setting to distinguish *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis*. A representative set of patterns obtained with this apparatus is displayed as well. No effort was made to determine an optimal set of sensors for some specific set of bacterial metabolism gaseous products.

Key words: electronic nose, bacteria identification, blood culture

Blood is one of the most important specimens received by the clinical laboratory. It is submitted as an aid to identification of bacteria and fungi responsible for many conditions including bacteremia, sepsis, infections of native and prosthetic valves, suppurative thrombophlebits, and infections of vascular grafts. Because a wide variety of microorganisms can be involved in blood stream-related infections, the methods used to recover organisms from the blood should be capable of supporting detection of a range of microorganisms. Accurate and rapid identification of microorganisms present in the blood culture of a specimen is an essential practice in the clinical microbiology laboratory. Fortunately, there have been many improvements in the media and technology for culturing blood during the past decade, resulting in highly reliable manual and automated systems (7). Fully automated continuously monitoring blood culture systems are the newest type of system developed for the detection of bacteria and fungi in blood. The systems available in the United States include BacT/Alert (Organon Teknika Corp; Durham, NC), BACTEC 9000 (Becton Dickinson Microbiology System, Sparks, MD) and ESP (Trek Diagnostic Systems Inc; Westlake, Ohio). The systems are alike in that the culture bottles are incubated in an instrument where they are continuously monitored (typically at 10 minute intervals) for the production and/or consumption of gas. The data collected are transmitted to a computer and analyzed to allow rapid detection of growth. Each system utilizes a noninvasive method (e.g. colorimetric, fluorescent or manometric methods for detecting CO_2 or other gases) to monitor growth (7). Once the instrument indicates the presence of a microorganism, the laboratory technologists use traditional staining procedures for determination of morphological types and also to subculture the sample on pertinent culture plates for further identification procedures. Another 12-36 hours is required before an accurate identification can be determined. Time constraints associated with these diagnostic procedures severely limit the amount and quality of the available information that one might extract. Microbiology laboratory personnel have long known that microorganisms usually have distinctive odors likely caused by the metabolizing of nutrients. Thus one might expect that such odors could be used to identify the microorganisms. One might also expect that at least the morphological groups of the microorganism could be determined via an Electronic Nose (EN) using biosensor technology.

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Electronic Nose

The human olfactory system has many limitations such as olfactory fatigue, a limited range of classes of molecules that may be detected, and its nonapplicability in toxic environments. On the other hand the EN has fewer such constraints in its applicability. The human olfactory system involves an elaborate arrangement of interconnected neurons and receptors that conduct signals to the brain for interpretation. When the molecules from a gas interact with numerous receptors in the nose, that starts a sequence of events that may result in recognition.

The particular EN used in this work was designed and built by several undergraduates (academic year 1998-99) of the Illinois Institute of Technology as part of an Interprofessional PROject (IPRO's) (5). The EN works in a fashion similar to a nose. An array of chemical sensors is used to mimic the range of protein receptors in the human nose. Each of these sensors is designed to respond differently to different classes of molecules. The signals from the sensors are appropriately transmitted to the pattern recognition program in the computer, which acts as the brain.

In recent years intensive research has been undertaken towards the development of portable, rapid, and sensitive biosensor technology with immediate results interpretation. The results are well-suited for the purpose of the identification of the microorganisms from various sources such as blood, serum, urine, and other body fluids and secretions, with high sensitivity and specificity.

There are many applications for the EN including use in the military, environmental monitoring, medical diagnosis, and quality testing of food and drugs. Because each human's nose and perception of smell varies, and indeed cannot detect certain classes of molecules, the EN may actually be a better tool. In addition, the EN results are consistent and reproducible (4).

Biosensors for bacterial detection generally involve biological recognition components such as receptors, nucleic acids, or antibodies in intimate contact with an appropriate transducer. Depending on the method of signal transduction, biosensors can be divided into four basic groups: optical, mass, electrochemical, and thermal sensors (2, 3, 8). In addition, biosensors can be classified into two broad categories: sensors for direct detection of the target analyte and sensors with indirect, labeled, detection. Direct detection biosensors are designed in such a way that the biospecific reaction is directly determined in real time by measuring the physical changes induced by the complex formation (1). Indirect detection biosensors are those in which a preliminary biochemical reaction takes place and the products of that reaction are then detected by a sensor (1).

The operation of the EN, used in this study, can be resolved into four simple steps. First, the gas flows to each of the sensors in the manifold. Second, an electronic circuit takes the voltage signals from the electrochemical sensors and amplifies or conditions the signals as necessary. Third, the output from the circuits is taken through a data acquisition board that has been added to a personal computer. A pattern recognition program then analyzes the data thus collected. In this study, our approach was to determine the byproducts produced by various bacteria while they are growing in a nutrient medium.

All of these events are controlled and orchestrated within a widely used laboratory automation software environment called LabVIEW (National Instruments, Austin, Texas). Using principal component analysis (PCA), the corresponding "signature" of each gas sample is displayed as a distinctive cluster of points, usually around the first two mutually perpendicular principal components in a 2-D array. Information about LabVIEW may be found at www.iit.edu/~labview, a website created and maintained by one of the authors, Christopher Morong.

Materials and Methods

Test organisms

The organisms used in this study were: *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. The stock cultures were kept at -50°C in skim milk. Each culture was thawed, aseptically inoculated onto the Trypticase Soy Agar (Table 1) with 5% Sheep Blood Plate (BAP), and incubated overnight at 35°C in air. The culture was transferred again onto the BAP and incubated overnight at 35°C in air. A suspension of each culture, equivalent to 0.5 McFarland Standard, was made using sterile 0.15M NaCl. One ml of the suspension was aseptically transferred into a 125 ml Erlenmeyer Flask that contained 25 ml of Trypticase Soy Broth (TSB), or Brain Heart Infusion Broth (BHIB). The inoculated flasks, along with the non-inoculated (control) flask, were incubated at 35°C in air for 18 hours. For the second part of the experiment, BACTEC Blood Culture Bottles (BACTEC plus Aerobic/F B.D. 442192, BCB) were used with, and without, supplement of human blood with the same broth-organism ratio. The components of the TSB, BHIB and BCB are indicated in Tables 2-4.

Table 1. Approximate formula per liter purified water of trypticase soy agar with 5% sheep blood plate

Pancreatic digest of casein	14.5 g
Papaic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	1.0 g
Growth factors	1.5 g
Sheep blood, defibrinated	50.0 ml

Table 2. Classical formula of trypticase soy broth per liter purified water

Pancreatic digest of casein	17.0 g
Papaic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Dextrose	2.5 g
Disodium phosphate	2.5 g

Table 3. Classical formula of brain heart infusion broth per liter purified water

Brain heart, infusion from (solids)	6.0 g
Peptic digest of animal tissue	6.0 g
Dextrose	3.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Pancreat digest of gelatin	14.5 g

Table 4. Reactive ingredients in the BACTEC plus aerobic/F blood culture bottles

Processed water	25 ml
Soybean casein digest broth	2.75% w/v
Yeast extract	0.25% w/v
Dextrose	0.06% w/v
Sucrose	0.084% w/v
Hemin	0.0005% w/v
Menadione	0.00005% w/v
Pyridoxal HCl	0.001% w/v
Sodium polyanetholesulfanate	0.05% w/v
Nonionic adsorbing resin	16.0% w/v
Cationic exchange resin	1.0% w/v

Gas identification

At the end of the incubation, 5 ml of medium was transferred into 20.0 ml of sterile 0.15M NaCl. A Teflon coated 1 inch magnetic stirrer was placed in the diluted culture. Then a rubber stopper was secured into the top of the flask with two glass tubes. The glass tube assembly (one input, one output) was connected to the EN system through Bev-A-Line tubes. The flask was placed on top of the magnetic stirrer and set to a level where the magnetic stirrer stirred the solution vigorously without creating excessive gas bubbles. Artificial air (20% dioxygen and 80% dinitrogen) from a pressurized tank was the carrier gas that passed through the head space over the samples and into the bioanalytical system. The average elapsed time per sample was approximately six minutes. The entire process by which the gas flows through the EN is displayed in Fig. 1. All the operations are under programmed computer control. First, the system is flushed with artificial air until the response from the sensors becomes stable (determined by observing the graphical dynamic display provided by LabVIEW). Then a computer controlled valve is actuated such that the artificial air stream now passes through the head space in the Erlenmeyer flask for ten seconds diluting and

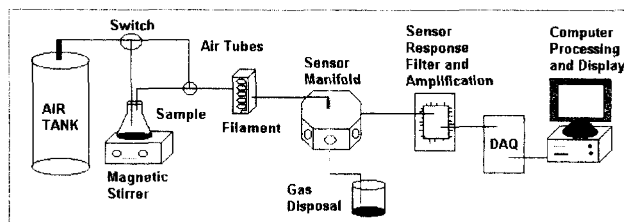

Fig. 1. Basic electronic nose diagram.

carrying the sample. The sample-air mixture next passes over a platinum filament in series with the gas flow in one of two possible modes. Either the filament is cold and passive or, via an electric current, the platinum helix is heated, again under computer control, to preselected temperatures and preselected durations.

Thus the sensors alternate between (a) exposure to sample gas-air mixture, possibly modified by exposure to the filament at each of several temperatures, and (b) purging by air. The composition of the products is dependent upon the filament temperature, duration of contact and the gas composition. The heated filament, together with the dinitrogen and dioxygen in the carrier gas, converts some of the gas constituents to molecular species that are more likely to change the characteristics of the electrochemical sensors.

On leaving the filament the emergent gas stream is divided into eight equal parts within the manifold for distribution to each of the sensors. This manifold has a unique parallel construction (unlike conventional ENs with sensors aligned serially) such that the gas stream is divided into eight identical streams, one for each sensor. After 10 seconds (the elapsed time is variable and under computer control), the system is flushed by air. Thus the system operation is such that the sensors are alternately bathed in sample-air mixture and just air. The signal from the sensors, electronically conditioned by sensitive components, is now sent to the Data Acquisition board (DAQ) where it is digitized and stored in the computer at a one hertz sampling rate. A LabVIEW program displays the data on the screen (see Fig. 2) as an easily interpretable graph. Further development of the data was done using Microsoft Excel. Through the technique of PCA the similarities and differences between the graphs of the signals from different bacterial odors can be determined (see Fig. 3). Using this as a basis, the signature of the unknown bacterial odors can be identified by comparing with a library of signatures of known substances.

The PCA transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. Using eigenanalysis, the eigenvalues and eigenvectors determine the direction of the principal components (6).

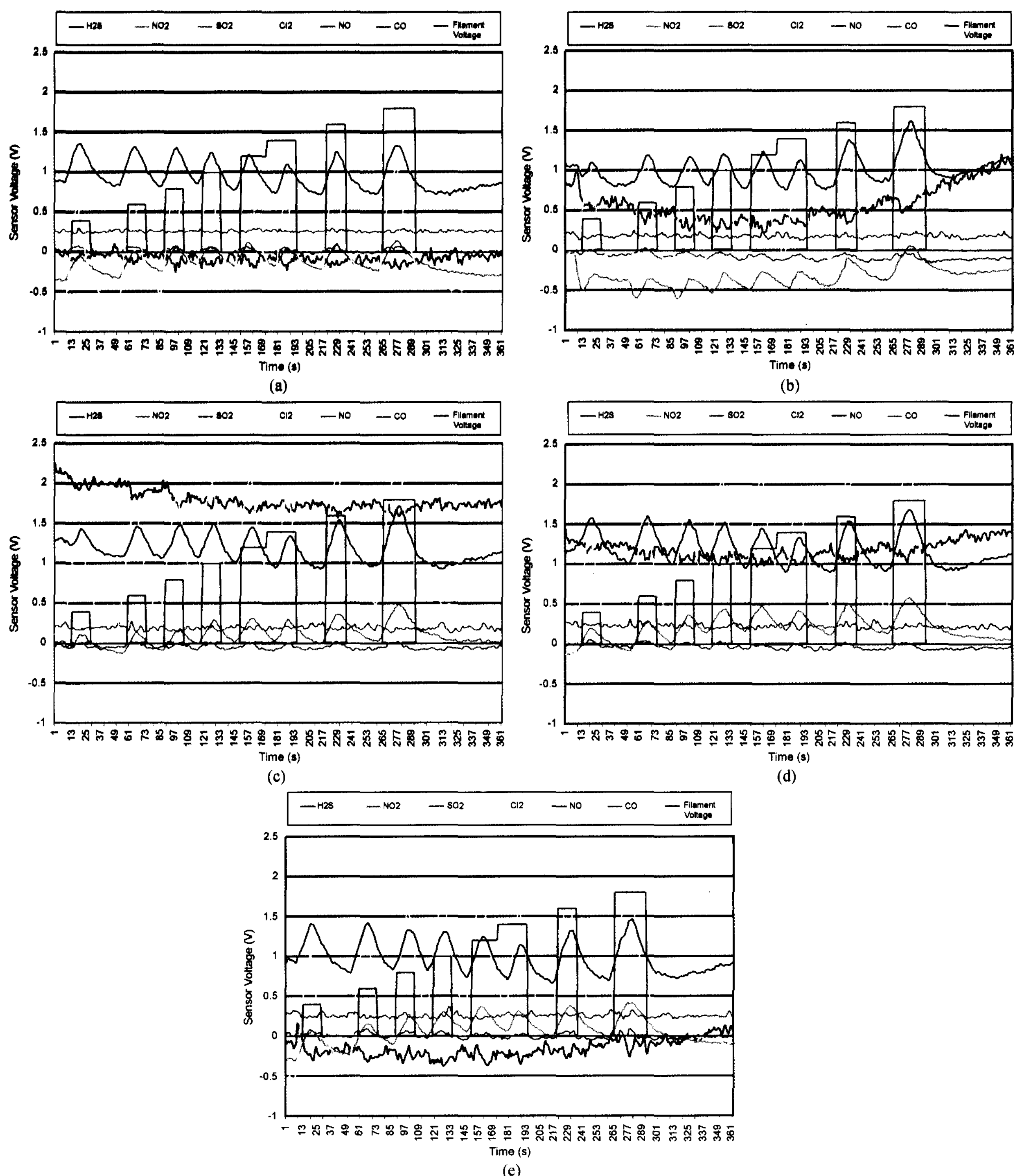

Fig. 2. Sensor voltage chart. 2a. Control, 2b *Escherichia coli* ATCC 35218, 2c *Pseudomonas aeruginosa* ATCC27853, 2d *Staphylococcus aureus* ATCC 29213 and 2e *Enterococcus faecalis* ATCC 29212 were grown from stock culture as described in the text. Erlenmeyer Flask with Brain Heart Infusion Broth were inoculated with one ml organism suspension. The inoculate flask and uninoculated flask (control) were incubated at 35°C in air for 18 hours. At the end of the incubation, 5 ml of medium was transferred into 20.0 ml of sterile 0.15 M NaCl. The flask containing diluted medium was placed on top of a magnetic stirrer. Artificial air (20% dioxygen and 80% dinitrogen) was the carrier gas that passed through the head space, the samples, and into the bioanalytical system for the elapsed time for 6 minutes. The control set does not contain test organisms. The experiments were run in duplicate.

In this application PCA projects a point in N-dimensional space (number of sensors times the number of filament temperatures) into a 2-D plane that can be displayed on a graph while maintaining the relative distances between the points.

Seven electrochemical sensors were used within the EN that were sensitive primarily to the following substances: Carbon Monoxide, Dihydrogen Sulfide, Nitrogen Dioxide, Sulfur Dioxide, Dichlorine, Nitric Oxide and Ethylene Oxide. Electrochemical sensors were chosen because they operate at room temperature and are insensitive to water vapor that is ubiquitous in biological systems.

Results

TSB, BHIB, BCB (without human blood supplement) and BCB (with 2.0 ml of human blood supplement) were used for the nutrient media. A total of five runs was made consisting of all four organisms plus a non-inoculated flask or bottle acting as a blank. For each flask at least four air samples were exposed to the EN and a PCA was constructed. Representative sensor voltage changes start with BHIB as is shown in Fig. 2. Fig. 2a represents sensor voltage changes in the case of BHIB without an inoculation of the organisms (blank). Figs 2b, 2c, 2d and 2e, represent sensor voltage changes in the case of BHIB with the *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 respectively. Each organism produces a specific pattern of gas composition. Each pattern depends upon the composition of the medium used to support the growth since different patterns of the sensor voltage changes in the case of BHIB, TSB, CB with and without human blood supplements were found. PCA for the same sensor voltage change data is shown in Fig. 3. The chart depicted in Fig. 2 displays the color coded conditioned voltage for each of the seven electrochemical sensors used in this particular run as well as the applied filament voltage over the sampling period of six minutes. The filament voltage is the histogram-like function that increases in 0.2 volt increments approximately every ten seconds. Each sensor has a different response rate and peak value for the gas components. The baseline is the sensor response to the artificial air pulse that alternates with the passage of the gas sample. The response and recovery rates for the sensors are not equal and need to be taken separately.

Each of the peak to peak values for the start to end times for a given sample and pure air, as displayed in Fig. 2, are stored as a vector. The vectors from the different sample runs are arranged as a matrix. The eigenvalues and eigenvectors of that matrix are the basis for the Principal Component Analysis (PCA). The final analysis of all four samples, and the blank, appear as distinct clusters in Fig. 3. The super positioning of all the points is not complete in each case. That is likely due, somewhat, to inexact reproducibility of each sample but, more likely, given the sequencing of the points, to incomplete purging with air between each sample run.

When the BCB (with or without human blood supplement) were inoculated with test organisms, unique sensor voltage changes clusters were found. Changes in the sensor voltage indicate the presence of blood. Representative PCA are shown in Figs 4 and 5.

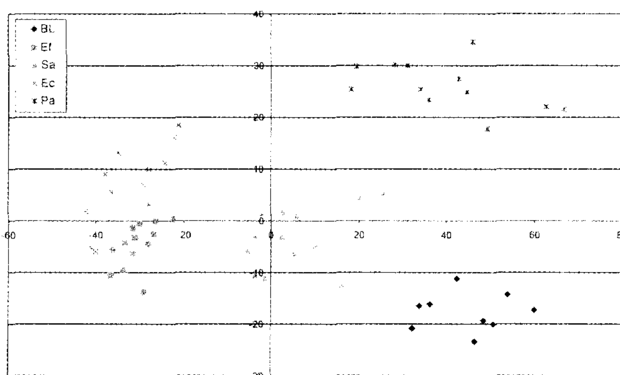

Fig. 3. Principal Component Analysis in 2-D. Control (BL), *Escherichia coli* ATCC 35218 (Ec), *Pseudomonas aeruginosa* ATCC 27853 (Pa), *Staphylococcus aureus* ATCC 29213 (Sa) and *Enterococcus faecalis* ATCC 29212 (Ef). The experiment was performed as described in Fig. 2. Sensor voltage changes as appear in Fig. 2 were used to construct this chart.

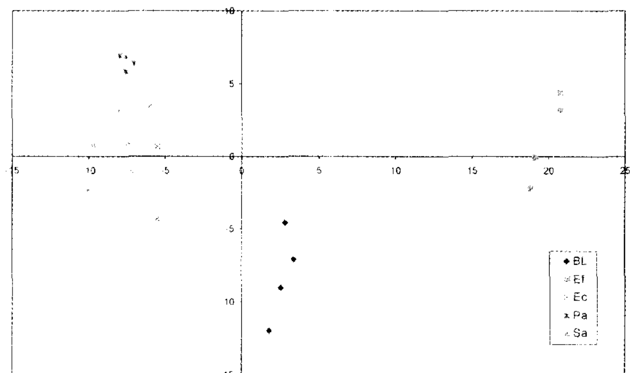

Fig. 4. Principal Component Analysis in 2-D. Control (BL), *Escherichia coli* ATCC 35218 (Ec), *Pseudomonas aeruginosa* ATCC 27853 (Pa), *Staphylococcus aureus* ATCC 29213 (Sa) and *Enterococcus faecalis* ATCC 29212 (Ef) were grown from stock culture as described in the text. The BACTEC bottles (BACTEC plus aerobic/F B.D. 442192) were inoculated with one ml of each organism suspension. The inoculate bottles were incubated into the BACTEC 9240 until indicated positive by the instrument. At the end of the incubation, 5 ml of medium was transferred into 20.0 ml of sterile 0.15 M NaCl. The flask containing diluted medium was placed on top of the magnetic stirrer. Artificial air (20% oxygen and 80% nitrogen) from a pressurized tank was the carrier gas that passed through the head space the samples and into the bioanalytical system for the elapsed time of 6 minutes. The control set does not contain test organisms. The experiments were run in duplicate.

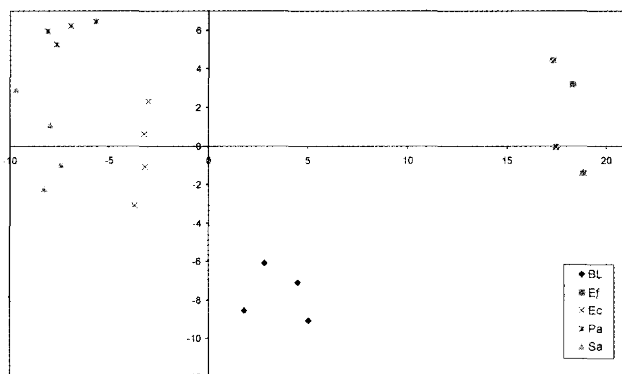

Fig. 5. Principal Component Analysis in 2-D. Experiment was performed as described in Fig. 4 except the BACTEC bottles were aseptically supplemented with 2.0 ml of human blood. The experiments were run in duplicate.

Discussion

The instrument systems available for the continuous monitoring of the blood cultures are limited. When a culture becomes positive, the instrument system indicates such an event electronically which alerts the microbiology personnel. At this time the nature of the organism in the blood culture bottle is unknown. The procedure and the instruments described in the article on Electronic Noses (4), can be easily adapted and applied with the currently available blood culture instrument systems. It is possible to determine the nature of the organism (e.g. Gram positive or Gram negative) with the help of an Electronic Nose. An overview of the biosensors for detection of pathogenic bacteria has been well developed by Abdel-Hanid *et al.* (1).

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