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The Screening of More Than 2,000 Schoolgirls for Bacteriuria Using an Automated Fluorescence Microscopy System

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Summary. After initial evaluation of a manual fluorescence microscopy system on a variety of urines the method was automated and subsequently tested in a population survey of urinary tract infection in schoolgirls. This automated Bactoscan system allowed a rapid analysis of urine samples and with the introduction of modifications to the staining protocol it correctly eliminated 91% of samples as being not significantly infected.

Key words: Bacteriuria, Rapid detection, Acridine Orange, Fluorescence, Bactoscan.

Introduction

Many of the automated systems presently being evaluated for their capability to detect bacteriuria require a minimum incubation period of 3 h. These accelerated growth detection systems are those which utilise impedance, nephelometry, respirometry and microcalorimetry to identy an increase in the number of bacteria in the sample under test.

Following the initial encouraging results obtained using a manual Direct Fluorescence Microscopy System (DFM) [1] to detect and enumerate microorganisms in urine, a prototype automated DFM instrument, "Bactoscan" (N. Foss Electric, Denmark), was employed using the similar staining method of alkaline acridine orange. More than 2,000 schoolgirls between the ages of four and twelve years were screened for bacteriuria using this new automated DFM instrument plus conventional plate count methods.

Methods

Specimen Collection

A trained children's nurse collected mid-stream specimens of urine from 2,234 choolgirls aged 4 to 12 years in the primary schools in Cumbernauld, Scotland [2]. The samples were held in cool boxes and transported to the Rapid Methods Microscopy Unit within 2 h of sampling.

Cultural Examination

The standard callibrated loop method using 1 μl of well mixed urine sample was plated over the surface of cystine lactose electrolyte deficient (CLED) agar medium and MacConkey agar medium. A minimum of 2 petri plates of each medium was used for each specimen. Dependent on the results of the Bactoscan count a maximum of 5 plates of each medium was used.

Normal Microscopic Examination

A wet film of each specimen was made and examined microscopically using phase contrast and a x40 phase contrast objective. When an average of more than 1 leucocyte per field was observed chamber counts were performed. Gram smears were also made of the urines.

Biochemical Examination

An N-Multistix (Ames Co.) was used to test each specimen of urine for protein, bilirubin, blood, glucose, nitrite, urobilinogen, ketones and pH.

Automated Direct Fluorescence Microscopy

The Bactoscan instrument is shown in Fig. 1. It is basically a flow through epifluorescence microscope equipped with a built-in detector system [3].

The rotating wheel carries 5 μl of prepared sample past the objective and all those particles exhibiting a fluorescence intensity above a controllable discriminator setting are detected and counted.

An upper discriminator level is used to reject the signals from large particles such as leucocytes.

The basic staining system is similar to that described in an earlier publication [1] i.e. adding to the sample 0.02 M disodium tetraborate containing 4 g of ethylene diamine tetra acetic acid, Triton X 100, formaldehyde and finally the acridine orange. A minimum of 10 ml of urine was necessary to carry out repeat Bactoscan runs. Five different modifications of the staining system were employed but only two methods, the standard staining system Protocol 1 and the fifth modification Protocol 6 were used on a large number of samples.

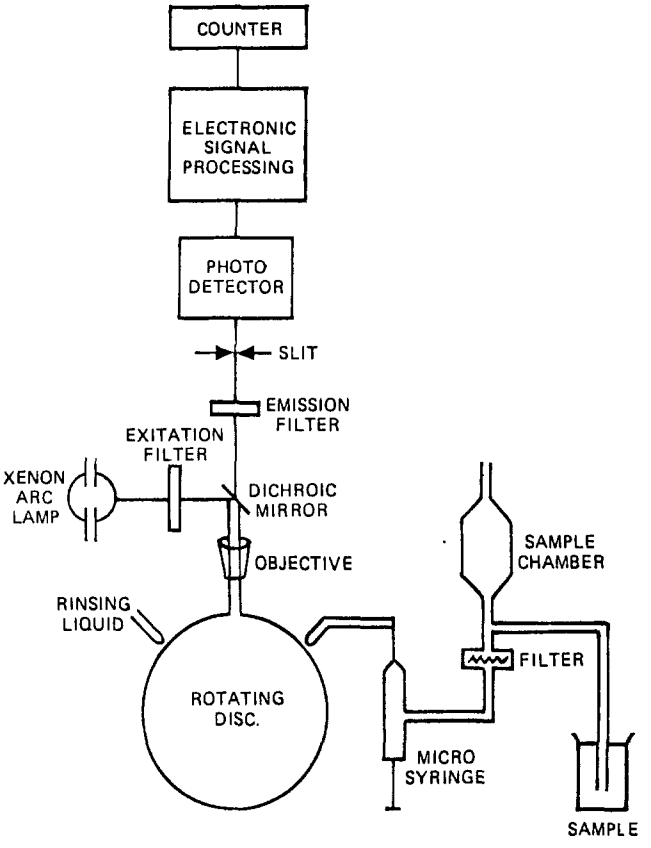

Fig. 1. Schematic of Bactoscan

Protocol 1 used one volume of urine plus two volumes of the disodium tetraborate buffer mixture and to this was added 16 µl per ml of 0.05% (w/v) aqueous acridine orange solution (Baker). The combined solution was mixed and held at room temperature (20 °C) for 3 min before being presented to the Bactoscan.

Protocol 6 involved an initial dilution of the urine sample, one volume to two volumes of sterile filtered distilled water before then adding two volumes of the borate buffer mixture and the acridine orange at 16 µl per ml of a 0.05% aqueous solution. This combined mixture was also held at room temperature for 3 min before testing by the Bactoscan.

Survey

Each schoolgirl attending primary school in Cumbernauld was given a questionnaire to be completed by the parents. More than 98% of the parents co-operated and the relevant data from these questionnaires have been presented [3]. The data from the questionnaire, with names coded to maintain confidentiality, along with that obtained from the DFM, cultural and other examinations were stored on a computer (ICL 2980) and analysed using the Statistical Package for the Social Sciences Program [4].

Results

A total of 2,234 schoolgirls in Cumbernauld agreed to take part in this survey. The urine specimens of all but four of these were tested for the numbers of bacteria present by

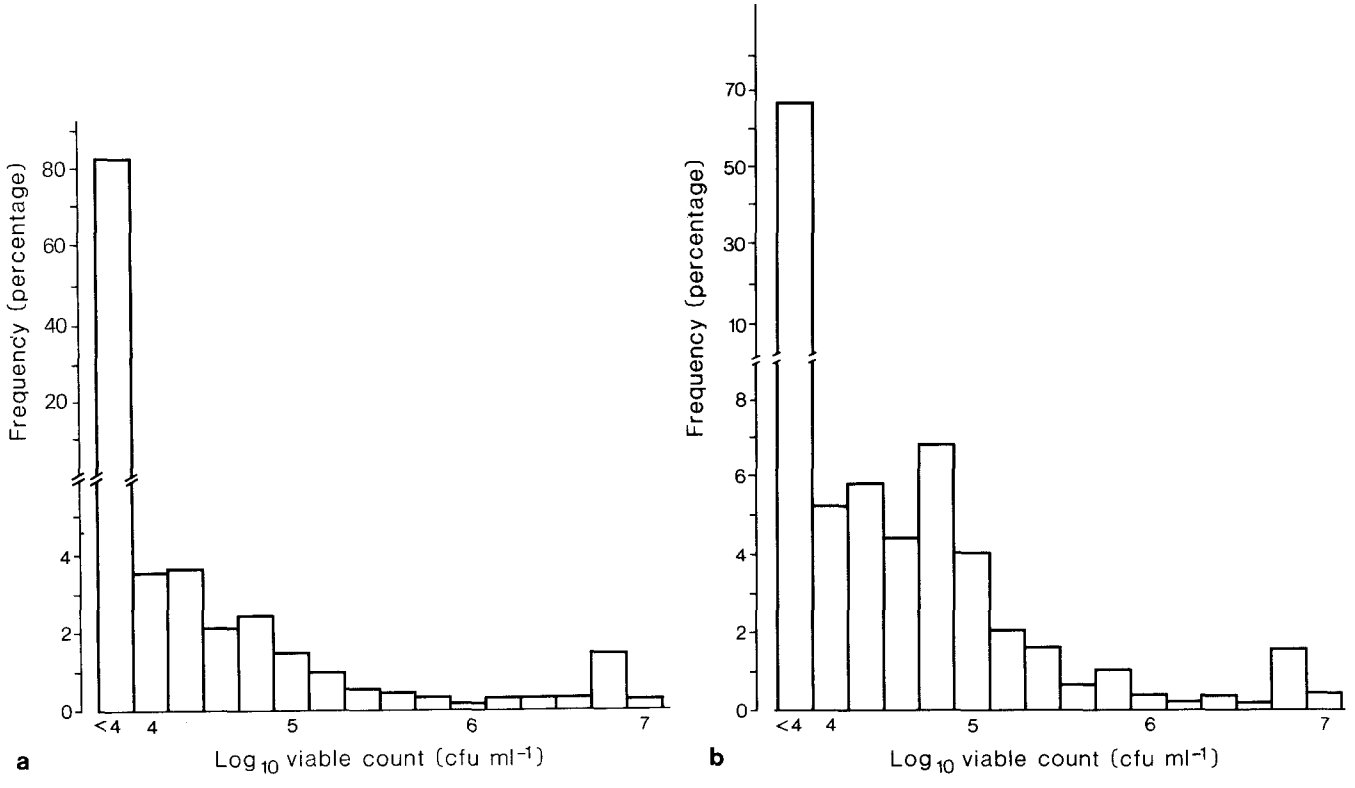

Fig. 2a, b. Frequency distribution of colony counts on MacConkey agar from 1,858 specimens of urine (a). b Frequency distribution of colony counts on CLED agar from 1,888 specimens of urine

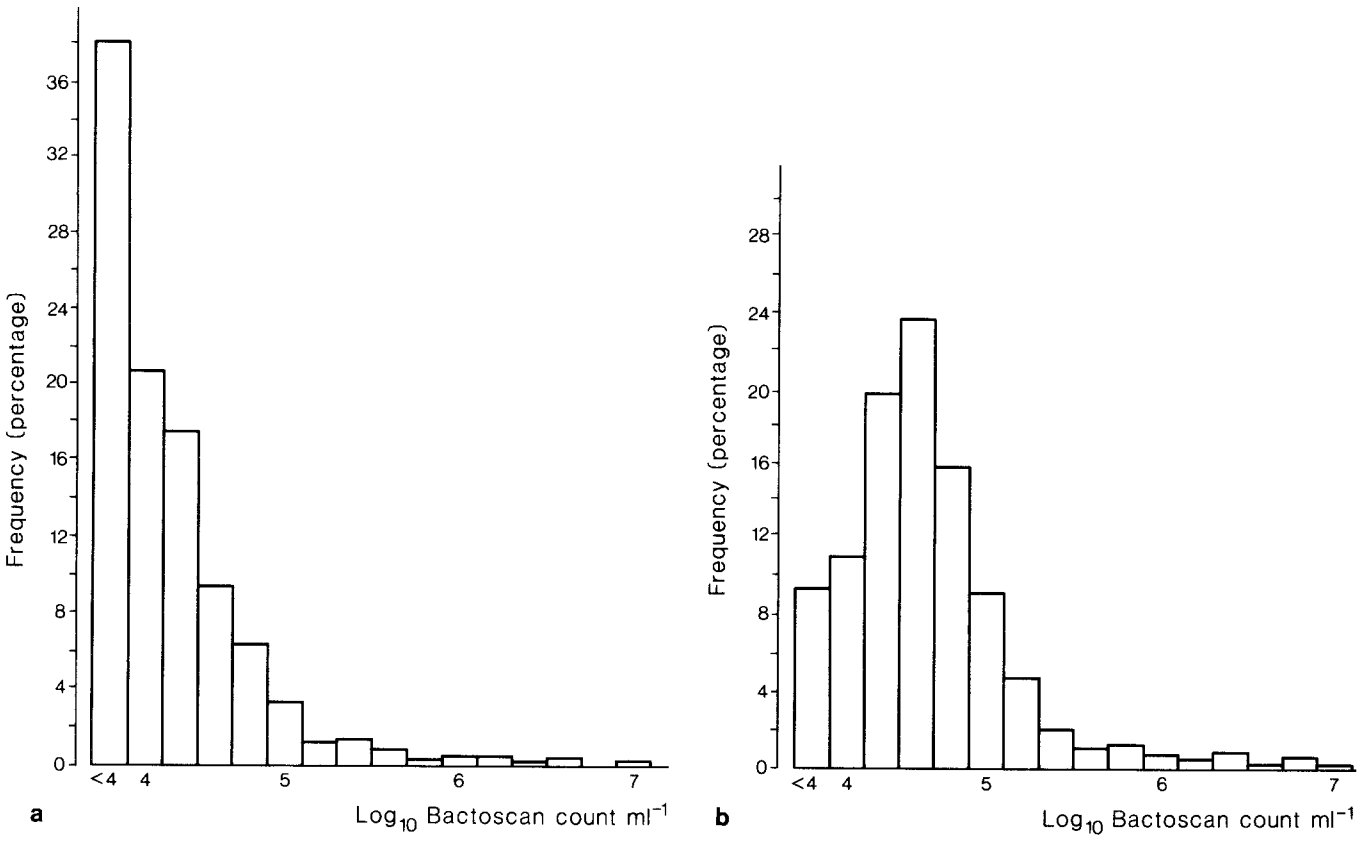

Fig. 3a, b. Frequency distribution of Bactoscan Protocol 1 counts from 1,893 specimens of urine (a). b Frequency distribution of Bactoscan Protocol 6 counts from 1,680 specimens of urine

conventional microbiological methods. These four were excluded because either they failed to produce a urine sample or they did not return the questionnaire.

Figures 2a and 2b show the distribution of colony forming units ml^{-1} (cfu ml^{-1}) on MacConkey and CLED media. Figure 3a shows the counts obtained on the Bactoscan using Protocol 1 preparation and staining technique. A total of 1,893 of the 2,230 urine specimens were examined using Protocol 1 with the other 337 excluded due to insufficient urine being available. Figure 3b shows the distribution of counts ml^{-1} for those urine samples tested on the Bactoscan using Protocol 6 staining technique, a total of 1,680 specimens. An examination of these two sets of figures show that the Bactoscan counts of both Protocol 1 and Protocol 6 are distributed over a wider range of the lower counts than are the plate counts, particularly those on MacConkey agar. Many of the Bactoscan results especially when using Protocol 6 have an inherent count, so although 65% and 82% of the plate counts on CLED and MacConkey agars respectively have counts of less than 10×10^3 cfu ml^{-1} only 9% of the Bactoscan Protocol 6 results have counts below 10×10^3 ml^{-1} (Figs. 2a, b and 3b).

The direct relationship between the two methods on Bactoscan and the colony counts on MacConkey and CLED media is shown in Figs. 4a and 4b for Protocol 1 and in Figs. 5a and 5b for Protocol 6.

Table 1a. Comparison of Bactoscan Protocol 1 counts with plate counts on MacConkey agar – 1,858 urine specimens

	Bactoscan Protocol 1 counts ml^{-1}	
MacConkey agar cfu ml^{-1}	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
$< 100 \times 10^3$	1,725	50
$\geqslant 100 \times 10^3$	42	41

Table 1b. Comparison of Bactoscan Protocol 1 counts with plate counts on CLED agar – 1,888 urine specimens

	Bactoscan Protocol 1 counts ml^{-1}	
CLED agar cfu ml^{-1}	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
$< 100 \times 10^3$	1,696	44
$\geqslant 100 \times 10^3$	101	47

This direct comparison graph, containing such a high proportion of low count urine specimens does not show the potential of the Bactoscan. Tables 1a, 1b and 2a, 2b with the counts in bands show the comparison of the

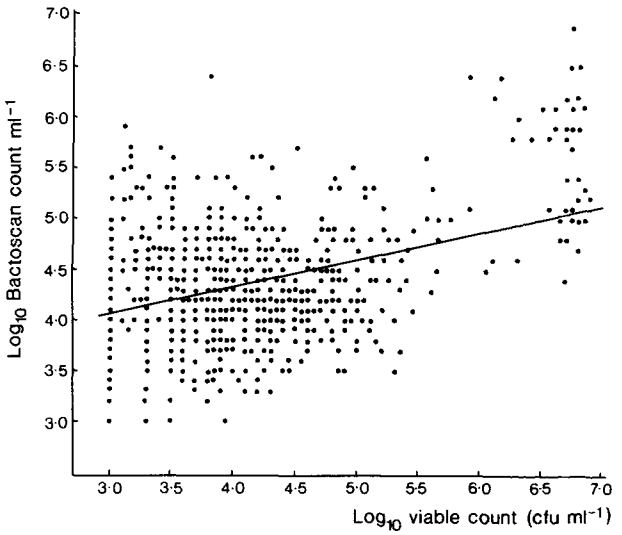

Fig. 4a. Relationship between plate counts on MacConkey agar and Bactoscan Protocol 1 counts from 1858 specimens of urine. Regression line y = 0.28x + 3.21

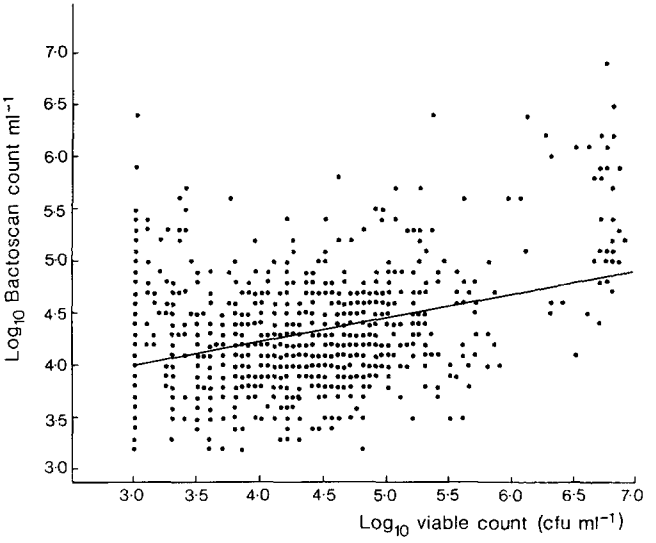

Fig. 4b. Relationship between plate counts on CLED agar and Bactoscan Protocol 1 counts from 1888 specimens of urine. Regression line y = 0.22x + 3.34

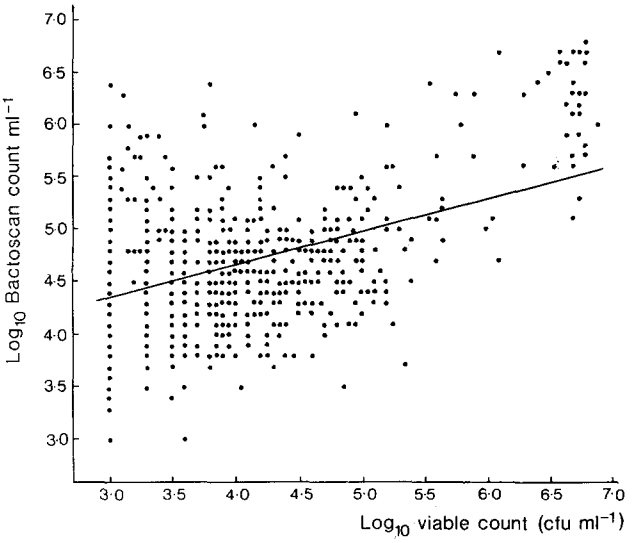

Fig. 5a. Relationship between plate counts on MacConkey agar and Bactoscan Protocol 6 counts from 1645 specimens of urine. Regression line y = 0.31x + 3.47

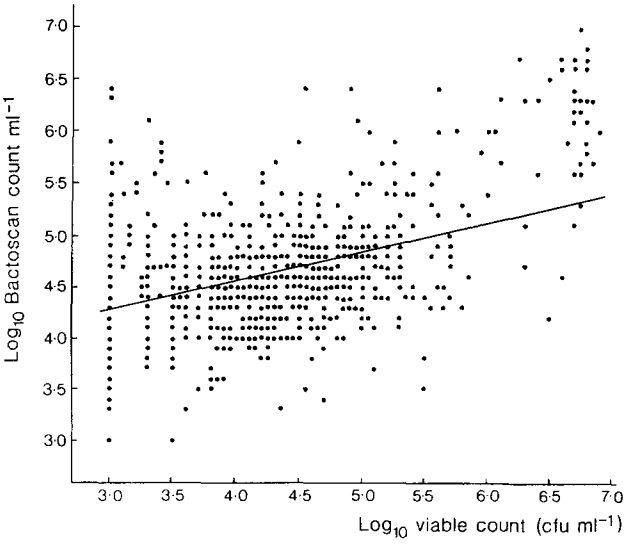

Fig. 5b. Relationship between plate counts on CLED agar and Bactoscan Protocol 6 counts from 1677 specimens of urine. Regression line y = 0.26x + 3.55

Table 2a. Comparison of Bactoscan Protocol 6 counts with plate counts on MacConkey agar – 1,645 urine specimens

Bactoscan Protocol 6 counts ml^{-1}		
MacConkey agar cfu ml^{-1}	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
$< 100 \times 10^3$	1,439	133
$\geqslant 100 \times 10^3$	20	53

Table 2b. Comparison of Bactoscan Protocol 6 counts with plate counts on CLED agar – 1,677 urine specimens

Bactoscan Protocol 6 counts ml^{-1}		
CLED agar cfu ml^{-1}	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
$< 100 \times 10^3$	1,416	122
$\geqslant 100 \times 10^3$	67	72

instrument and cultural results. This strictly quantitative assessment shows that, taking a cut off point at 100×10^3 counts ml^{-1} with both the Bactoscan and cultural results, Protocol 1 correctly screened out over 97% of the negative cultural results on MacConkey agar i.e. 93% of total specimens and 97% of the negative cultural results on CLED agar i.e. 90% of total specimens.

The false positive rate is given by the number of samples found to be positive by the test method but negative by the

Table 3. Comparison of Bactoscan Protocol 1 results with cultural status of 1,893 urine specimens

	Bactoscan Protocol 1 counts ml^{-1}	
Culture status	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
No growth	532	2
No significant growth	926	35
Contaminated	328	17
Mixed significant	1	4
Pure significant	15	33

Table 4. Comparison of Bactoscan Protocol 6 results with cultural status of 1,680 urine specimens

	Bactoscan Protocol 6 counts ml^{-1}	
Culture status	$< 100 \times 10^3$	$\geqslant 100 \times 10^3$
No growth	457	15
No significant growth	767	68
Contaminated	258	69
Mixed significant	1	3
Pure significant	2	40

reference method, as a percentage of the total number of samples negative by the reference method.

The false negative rate is given by the number of samples found to be negative by the test method but positive by the reference method, as a percentage of the total number of samples positive by the reference method.

The false positive rate for Protocol 1 was 2.8% and 2.5% on MacConkey and CLED media respectively and the false negative rates 51% and 68% using the same quantitative cut off point of 100×10^3 counts ml^{-1}.

Using Protocol 6 the Bactoscan correctly screened out 91.5% of the negative cultural results on MacConkey agar i.e. 87% of total specimens and 92% of the negative cultural results on CLED agar i.e. 84% of total specimens. The false positive rates using Protocol 6 were 8.5% and 7.9% on MacConkey and CLED media respectively and the false negative rates were 27% and 48%.

However, urine cultural results are deemed to be positive not on a quantitative assessment alone but also on the type of organism and the number of different organism types present. Using quantitative and qualitative assessments the samples were graded on a cultural basis as follows:

1) No growth i.e. no colony forming units in 1 μl of urine.

2) No significant growth i.e. less than 50×10^3 cfu ml^{-1} on MacConkey or CLED agars.

3) Contaminated i.e. (i) greater than 50×10^3 but less than 100×10^3 cfu ml^{-1} on MacConkey or CLED agars (ii) greater than 100×10^3 ml^{-1} on MacConkey or CLED agars but composed of two or more types of organisms with at least one organism being recognised as a possible contaminant.

4) Mixed but significant i.e. greater than 100×10^3 cfu ml^{-1} on MacConkey or CLED agars with no more than two types of organisms present both recognised pathogens and one being present at a minimum level of 100×10^3 cfu ml^{-1}.

5) Pure and significant i.e. greater than 100×10^3 cfu ml^{-1} on MacConkey or CLED agars of a single organism type recognised as a urinary tract pathogen.

From the 1,893 specimens examined with Protocol 1 (Table 3) 53 were regarded culturally as being positive and the remainder 1,840 as negative. The Bactoscan, using Protocol 1, correctly identified 1,786 of the negative specimens i.e. 97%, using a cut-off point of 100×10^3 counts ml^{-1}. A count of more than 100×10^3 ml^{-1} was produced in 54 of the culture negative samples, a false positive rate of 3%. Of the 53 culture positive urines the Bactoscan with Protocol 1 produced counts greater than 100×10^3 ml^{-1} for 37 of them but failed to give counts greater than 100×10^3 ml^{-1} for 16 of the specimens, a false negative rate of 30%.

A total of 1,680 of the urine specimens were examined using Bactoscan with Protocol 6 (Table 4) and of the 1,634 culture negative specimens the Bactoscan with a cut off point of 100×10^3 counts ml^{-1} agreed in 1,482 of cases, a 91% agreement rate. A total of 152 urines were falsely judged positive using Protocol 6, a false positive rate of 9%. Forty-six of these urine specimens were classed as culture positive specimens and Bactoscan Protocol 6 identified 43 of these, but failed to register counts of 100×10^3 ml for three of the urines giving a false negative rate of 6.5%.

Of the three false negative urine specimens two produced growths of *E. coli* greater than 100×10^3 cfu ml^{-1} on MacConkey agar only and the third specimen showed growth of a non-lactose fermenting *E. coli* with greater than 1×10^6 cfu ml^{-1} on MacConkey and CLED media.

Discussion

To screen a normal population for the presence of bacteriuria can be a time consuming and costly exercise, particularly when using conventional microbiological methods. A system is required to rapidly identify all the suspect specimens and eliminate all the negatives. Allowing for true infections plus contamination rates, this would reduce the specimens to around 10–2-% of the original number. It would also be useful if the system produced results sufficiently quickly to allow further investigation of the suspect patients while they were present at the clinic or survey centre. The system should be capable of producing a low rate of false positives and close to zero rate of false negatives.

The Bactoscan prototype machine goes some way towards achieving these objectives with a throughput of 60 to 70 samples/h and a lead time of under 5 min. However, using Protocol 1, although a low rate of false positives was obtained, the very high rate of false negatives was unacceptable.

The use of the Bactoscan with Protocol 6 did, however, go further towards a suitable system and with a total of over 1,600 specimens examined, it correctly rejected 91% as being negative. It did fail to recognise three positive specimens out of the total of 46, a false negative rate of 6.5%; however, this false negative rate is comparable to other automated systems [5]. The main criticism of the instrument culture and staining methods is the high level of counts in definite culture negative specimens. Although these specimens are still classed as negative by the Bactoscan using Protocol 6 the decision level on Bactoscan cannot be lowered from 100×10^3 counts ml^{-1}.

This prototype system does, however, show that automated Direct Fluorescence Microscopy is a viable concept for the truly rapid detection of bacteriuria.

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