

Comparison of five tests used in diagnosis of neonatal bacteraemia

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SUMMARY The neutrophil count, immature:total neutrophil ratio, C reactive protein assay, nitroblue tetrazolium test and an acridine orange leucocyte cytospin test were evaluated for the diagnosis of neonatal bacteraemia. The acridine orange leucocyte cytospin test gave the highest specificity and positive predictive accuracy, but was less sensitive than the neutrophil count, C reactive protein assay or nitroblue tetrazolium test, particularly for the diagnosis of bacteraemia caused by coagulase negative staphylococci. No single test gave the sensitivity, specificity, and positive predictive accuracy of the combined results of the acridine orange leucocyte cytospin, C reactive protein, and nitroblue tetrazolium tests.

Bacteraemia is a common cause of morbidity and mortality among neonates in intensive care units,¹ but the clinical signs are often non-specific.² It has been suggested that a combination of haematological and biochemical tests may provide a more rapid and accurate diagnosis of bacteraemia than conventional microbiological methods^{3,4} and the use of a 'sepsis screen' may reduce the amount of antibiotics used.⁵

We selected five tests including the acridine orange leucocyte cytospin test that could be performed rapidly and economically, and adapted them for use with small amounts of blood. We report an evaluation of these tests in neonates in a regional referral neonatal unit with suspected bacteraemia.

Patients and methods

The regional neonatal unit admits about 500 babies each year with both medical and surgical illnesses from this and other units in West Yorkshire. More than 60% of the admissions to the regional neonatal unit are premature neonates with the respiratory distress syndrome. Blood, cerebrospinal fluid, and urine were collected from all neonates with suspected bacteraemia. Clinical signs of bacteraemia included unstable temperature, apnoea, bradycardia, poor peripheral perfusion, or hypotension.

Blood was collected by arterial or venous puncture after skin disinfection with 70% isopropyl alcohol or povidone iodine. Up to 2 ml was divided into three containers as follows: one 0.5 ml sample was anticoagulated with edetic acid (final concentra-

tion 1.5-2 mg/l), 0.5 ml was placed in a tube without anticoagulant and 0.5-1 ml was inoculated aseptically into 80 ml of broth (Signal medium BC 102, Oxoid Ltd, Basingstoke). An indicator was inserted through the rubber seal⁶ and the broth culture incubated at 37°C on a Denley (prototype) continuous shaker at 160 rpm for 4-18 hours, it was then left in the incubator for up to 10 days. Bottles with signs of bacterial growth and all bottles after 48 hours of incubation were subcultured by inoculation of 0.1 ml aliquots on to heated blood agar and 5% horse blood agar plates, which were incubated at 37°C in 5% carbon dioxide and at 37°C anaerobically, respectively, for up to 48 hours. Isolates from the blood cultures were identified using conventional microbiological techniques.⁷ Coagulase negative staphylococci were defined as catalase positive, coagulase negative, deoxyribonuclease negative Gram positive cocci.

The sample containing edetic acid was used for the neutrophil count, immature:total neutrophil ratio, nitroblue tetrazolium test, and the acridine orange leucocyte cytospin test. The total nucleated cell count was made on a Coulter counter and a differential cell count from a Giemsa stained blood film gave the immature:total neutrophil ratio.⁸ An age adjusted normal range⁹ was used to assess the neutrophil counts, and neutropenia or neutrophilia were considered abnormal. A normal range of up to 0.2 was used for the immature:total neutrophil ratio.⁴

The nitroblue tetrazolium test was done by a modified cytocentrifuge technique.¹⁰ 50 µl of blood

containing edetic acid was added to 100 µl of 0.15M phosphate buffered saline (pH 7.2) containing 0.075% (w/v) nitroblue tetrazolium in polystyrene tubes. The mixture was incubated at 37°C for 20 minutes, and then for a further five minutes at room temperature. Red cells were lysed by the addition of 1.2 ml of hypotonic formal saline (10% formalin containing 0.025M sodium chloride). When red cell lysis was complete (usually after one minute), 2.8 ml of 0.2M sodium chloride was added, and after mixing the tubes were centrifuged at 258 g for five minutes. The supernatant was discarded by inversion and 50 µl of 22% (w/v) bovine albumin (Ortho Diagnostics, New Jersey, USA) was added to resuspend the deposit that was then prepared as a cellular monolayer with a Shandon 2 cytospin at 1000 rpm for five minutes. The cytospin preparation was heat fixed at 70°C for two minutes and stained with 0.15% (w/v) aqueous rhodanile blue dye that had been passed through an 0.2 µm cellulose acetate filter to remove any deposit. Stained slides were examined by light microscopy at a magnification of 400. The percentage of 100 neutrophils containing nitroblue tetrazolium formazan gave the nitroblue tetrazolium score, with a normal range of up to 13%.¹¹ The metabolic integrity of the neutrophils was assessed using phorbol myristic acid.¹²

The acridine orange leukocyte cytospin test was used to detect bacteria in neutrophils from whole blood. The test was performed by adding 50 µl of acridine orange (100 mg/l in 0.15M sodium chloride) to 50 µl of whole blood containing edetic acid in polystyrene tubes. Red cells were lysed and a cellular monolayer prepared as for the nitroblue tetrazolium test. The cytospin slide preparations were heat fixed and then examined by ultraviolet microscopy using a rhodamine filter set at a magnification of 1000. Intracellular bacteria were coloured bright red against a dull red background. The presence of bacteria was confirmed with a duplicate cytospin preparation or by overstaining the acridine orange leukocyte cytospin preparation with Gram's stain.

A latex agglutination method¹³ was used to screen serum samples for C reactive protein concentrations of more than 10 mg/l after dilution of serum 1/10 in 0.15M sodium chloride. C reactive protein concentrations of more than 10 mg/l were quantified using an enzyme multiplied immunoassay.¹⁴

Results

Two hundred samples of blood were collected from 100 neonates including 68 who had been admitted with prematurity and the respiratory distress syndrome. The mean gestational age of the premature

neonates was 30.2 weeks (range 24–35) and the mean birth weight was 1410 g (range 700–2470). The remaining 32 babies were admitted with a variety of other surgical and medical diagnoses. One sample of blood only was collected from each of 65 of the neonates; the number of samples taken from the other 35 varied from two to 10.

After making certain that only one sample was considered from each episode of suspected bacteraemia,⁵ and that contaminated samples had been excluded,⁷ 188 episodes of suspected bacteraemia were considered for further study, of which 34 were associated with positive and 154 with negative blood cultures. The bacterial species isolated during episodes of suspected bacteraemia are shown in table 1. Twenty one (62%) of the 34 blood culture isolates were coagulase negative staphylococci. The proportion of these that were caused by contamination was not known. There were no episodes of bacterial meningitis during the study. There was one urinary tract infection that was associated with *Escherichia coli* septicaemia.

The sensitivity, specificity, and positive predictive accuracy of each test are shown in table 2. The neutrophil count was abnormal in 24 (71%) of the 34 episodes associated with positive blood cultures, but was also abnormal in 65 (42%) of 154 episodes with negative blood cultures. Though the immature:total neutrophil ratio was less often abnormal when the blood culture was negative, only 10 (29%) of 34 episodes associated with positive blood cultures gave an abnormal immature:total neutrophil ratio. Even after exclusion of coagulase negative staphylococci, eight of 13 episodes were associated with immature:total neutrophil ratios of less than 0.12.

The C reactive protein concentration was less than 10 mg/l in 10 (29%) of the 34 episodes associated with positive blood cultures, but in only two (15%) of 13 episodes after the exclusion of

Table 1 Bacterial species isolated from neonates with episodes of suspected bacteraemia

Bacterial species	No of isolates
Coagulase negative staphylococci	21
<i>Pseudomonas aeruginosa</i>	3
<i>Staphylococcus aureus</i>	3
<i>Enterococcus faecalis</i>	2
<i>Enterococcus faecium</i>	1
Group B streptococcus	1
<i>Listeria monocytogenes</i>	1
<i>Escherichia coli</i>	1
<i>Serratia liquefaciens</i>	1
Total	34

Table 2 Sensitivity, specificity, and positive predictive accuracy of tests for the diagnosis of bacteraemia

	Sensitivity (%)	Sensitivity excluding coagulase negative staphylococci (%)	Specificity (%)	Positive predictive accuracy (%)
Neutrophil count	70.6	84.6	57.8	27.0
Immature:total neutrophil ratio	29.4	30.8	82.5	27.0
C reactive protein assay	61.8	84.6	81.2	42.0
Nitroblue tetrazolium test	76.5	100.0	70.8	36.6
Acridine orange leucocyte cytospin test	38.2	76.7	94.2	59.1
Two or three of C reactive protein assay, nitroblue tetrazolium, acridine orange leucocyte cytospin test positive	61.8	92.3	90.3	56.8

Sensitivity—positive blood cultures with positive test; specificity—negative blood cultures with negative test; positive predictive accuracy—positive test with positive blood culture.

coagulase negative staphylococci. Though the nitroblue tetrazolium test was positive in all 13 episodes, 45 (29%) of 154 episodes with negative blood cultures were also associated with positive nitroblue tetrazolium tests. The acridine orange leucocyte cytospin test identified 10 (77%) of 13 episodes of bacteraemia after exclusion of coagulase negative staphylococci and only nine (6%) of 154 episodes associated with negative blood cultures gave positive acridine orange leucocyte cytospin tests. The acridine orange leucocyte cytospin test, however, only detected 13 (38%) of 34 episodes associated with positive blood cultures when coagulase negative staphylococci were included.

The C reactive protein assay, the nitroblue tetrazolium and the acridine orange leucocyte cytospin test gave the highest positive predictive accuracy. Two or three tests were positive in 12 (92%) of the 13 episodes of bacteraemia after exclusion of coagulase negative staphylococci and in 21 (62%) of the 34 episodes associated with positive blood cultures. Coagulase negative staphylococci were isolated from 12 (8%) of the 151 episodes when none or only one of the three tests was positive, and from nine (24%) of the 37 episodes when two or more of the tests were positive. The results of total neutrophil counts and immature:total neutrophil ratios did not improve sensitivity, specificity, or positive predictive accuracy when included in three, four, or five test combinations.

In 15 (10%) of the 154 episodes associated with negative blood cultures two or three of the C reactive protein, nitroblue tetrazolium, and acridine orange leucocyte cytospin tests were positive. These 15 episodes occurred in neonates who had undergone major operations (n=4), had a tracheo-oesophageal fistula and pneumonia (n=1), and had necrotising enterocolitis (n=1); three babies had

received antimicrobial drugs within 24 hours of blood sampling. The remaining six episodes were all associated with positive acridine orange leucocyte cytospin tests, but cultures of the urine and cerebrospinal fluid grew no pathogens.

Discussion

The neutrophil count, immature:total neutrophil ratio, C reactive protein assay, nitroblue tetrazolium test, and acridine orange leucocyte cytospin test were evaluated for the diagnosis of bacteraemia in neonates in a regional unit. All these tests could be done within one hour of blood sampling and used a total blood volume of less than one ml.

Many studies have reported the use of total neutrophil count, immature:total neutrophil ratio or C reactive protein assay for the diagnosis of bacteraemia in neonates. The white cell count is of poor predictive value in neonatal bacteraemia, but the total neutrophil count may be high or low in 80% of such babies.¹⁵ Immature:total neutrophil ratios may also be raised in more than 80%.¹⁵⁻¹⁷ The poor sensitivity of the immature:total neutrophil ratio in this study may have been the result of sampling at the wrong time, because this unit admits a large proportion of very low birthweight 'high-risk' neonates, and also because the immature:total neutrophil ratio may be less sensitive after the first week of life.¹⁸ Though a positive blood culture was never associated with a negative nitroblue tetrazolium test, either C reactive protein assays or nitroblue tetrazolium tests were often positive in association with negative blood cultures.

High specificity and sensitivity have been reported for buffy coat smears prepared from capillary blood and stained with acridine orange.¹⁹ We

modified the method to use 50 µl of whole blood mixed with edetic acid and were able to identify 10 (77%) of 13 episodes of bacteraemia after the exclusion of coagulase negative staphylococci. Positive acridine orange leucocyte cytospin tests with negative blood cultures may have been false positive results or may have resulted from transient bacteraemia.

Infections with coagulase negative staphylococci are rarely associated with neonatal mortality²⁰ but coagulase negative staphylococci have replaced Gram negative bacterial species as the commonest isolates from neonatal blood cultures after the first 48 hours of life.^{21 22} The high incidence of bacteraemia with coagulase negative staphylococci in premature neonates has been attributed to colonisation of plastic intravascular devices by antibiotic resistant, slime producing strains of coagulase negative staphylococci.²³ Bacteraemia with coagulase negative staphylococci may be associated with the clinical features of septicaemia,^{24 25} so coagulase negative staphylococci isolates were included in this study even though the proportion of isolates of coagulase negative staphylococci in blood cultures that could be attributable to contamination was not known.

Only three (14%) of 21 isolates of coagulase negative staphylococci in blood cultures were associated with positive acridine orange leucocyte cytospin tests. The minimum concentration of organisms detectable by the acridine orange leucocyte cytospin technique using 50 µl of whole blood was 20 organisms per ml, suggesting lower concentrations for most of the neonates from whose blood cultures coagulase negative staphylococci had been isolated. This may reflect both the low virulence of coagulase negative staphylococci and the proportion of coagulase negative staphylococci contaminants.

The acridine orange leucocyte cytospin test gave the highest specificity and positive predictive accuracy but was less sensitive than neutrophil count, C reactive protein assay, or the nitroblue tetrazolium test, particularly for the diagnosis of bacteraemia caused by coagulase negative staphylococci. No single test gave the sensitivity, specificity, and positive predictive accuracy of the combined results from the acridine orange leucocyte cytospin test, C reactive protein assay, and nitroblue tetrazolium test.

Philip⁵ reported a reduction in the use of antibiotics after he started using a combination including estimations of the white cell count, the band:neutrophil ratio, the erythrocyte sedimentation rate, the latex C reactive protein assay and serum haptoglobin concentration for the diagnosis of neonatal sepsis. Many neonatal units use unnecessary

antibiotics.²⁶ This study confirms that a combination of tests may improve the accuracy of diagnosis of neonatal bacteraemia.⁴ We intend to follow this study with an evaluation of the influence of this combination of tests on neonatal morbidity and mortality, use of other diagnostic tests, and use of antibiotics in this regional neonatal unit.

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