Diagnosis of Neonatal Sepsis: A Clinical and Laboratory Challenge

The evaluation of tests for neonatal sepsis is important because the infection may present a very serious threat to the baby. There is an urgent need to know whether the baby has sepsis to institute treatment as quickly as possible. Confirmation of the diagnosis may take time, and diagnostic tests are used to obtain a rapid indication of the infection status. These tests are not perfect. Some real cases of infection will produce negative test results, whereas some babies without infection will test positive. The potential usefulness of the test will depend, above all, on the clinical condition of the baby. If the baby is really very sick, the test will not give very much additional information. Similarly, if the baby is evidently well, a clinical examination will be sufficient and a positive test result would not dramatically increase the probability that the baby is infected. It is in situations in which the clinical picture leaves the physician in doubt about the infection status that a diagnostic test is likely to be most useful. Thus, the result of a diagnostic test must be evaluated in the light of the clinical condition of the baby.

Extensive literature exists on single laboratory tests or combinations of tests, as well as tests used together with risk factors and/or clinical signs, to diagnose neonatal sepsis. In many instances, the results of the evaluations have been conflicting. There are several possible explanations for the divergent results, and the purpose of this review is to update readers on the topic and raise issues that should be addressed in the future.

Basic Physiology of Neonatal Infection

Throughout pregnancy and until the membranes rupture, the fetus is relatively protected from the microbial flora of the mother by the chorioamniotic membranes, the placenta, and poorly understood antibacterial factors in amniotic fluid *(1)*. However, there are many ways that infectious agents can reach the fetus or newborn to cause infection. Procedures disturbing the integrity of the uterine contents, such as amniocentesis *(2)*, cervical cerclage *(3)*, transcervical chorionic villus sampling *(4)*, or percutaneous blood sampling *(2, 5)*, can permit entry of skin or vaginal organisms, causing amnionitis and secondary fetal infection. Certain bacteria, particularly *Treponema pallidum* and *Listeria monocytogenes*, can reach the fetus through the maternal bloodstream despite placental protective mechanisms, causing transplacental infection. This process is uncommon, but it leads to either congenital infection not unlike infections caused by certain viruses or *Toxoplasma* or to stillbirth resulting from overwhelming infection.

Initial colonization of the neonate usually takes place after rupture of the maternal membranes *(1)*. In most cases, the infant is colonized with the microflora of the birth canal during delivery. However, particularly if the rupture of membranes lasts longer than 24 h, vaginal bacteria may ascend and in some cases produce inflammation of the fetal membranes, umbilical cord, and placenta *(6, 7)*. Fetal infection can result from aspiration of infected amniotic fluid *(8)*, leading to stillbirth, premature delivery, or neonatal sepsis *(2, 7, 9, 10)*. The organisms most commonly isolated from infected amniotic fluid are anaerobic bacteria, group B streptococci, *Escherichia coli*, and genital mycoplasmas *(2, 9)*. Infection of the mother at the time of birth, particularly genital infection, is the principal pathway of maternal transmission *(1, 11)* and can play an important role in the development of infection in the neonate. Transplacental hematogenous infection during or shortly before delivery (including the period of separation of the placenta) is possible, although it seems more likely that the infant is infected during passage through the birth canal. Finally, bacteria can be introduced after birth from the environment surrounding the baby, either in the nursery or at home.

Many pre- and intrapartum obstetric complications are associated with an increased risk of infection in newborn infants. Among these are premature onset of labor, prolonged rupture of the fetal membranes, uterine inertia with high forceps extraction, and maternal pyrexia *(12)*.

Sophisticated equipment for respiratory and nutritional support combined with invasive techniques provide life support to the ill infant. Arterial and venous umbilical catheters, central venous catheters, peripheral arterial and venous cannulas, urinary indwelling catheters, hyperalimentation infusions, and assisted ventilation provide enormous opportunities for relatively nonvirulent pathogens to establish infection and to invade the host *(12)*.

Transient bacteremia may accompany procedures that traumatize the skin and mucosal membranes. Bacteremia was identified in infants who received endotracheal suctioning; the bacteremia was present immediately after the procedure, but culture results were negative at 10 min *(13)*. Invasion of the bloodstream may follow multiplication of the organisms in the upper respiratory tract or other foci. Once bacteria gain access to the blood stream, mechanisms are activated by the host to eliminate the microbial intruder. Usually the organism is efficiently cleared by the monocyte-macrophage system after opsonization by antibody and complement. Thus, the bacteremia produces only short-lived illness. Sometimes, however, depending on the age of the patient, the virulence and number of bacteria in the blood, the nutritional and immunologic status of the host, and the timing and nature of therapeutic intervention, a systemic inflammatory response is established that can progress independently of the original infection *(14, 15)*.

Perinatally vs Postnatally Acquired Infections

Infections that are manifest early in the first week of life are usually attributable to microorganisms transmitted from mother to infant and have an epidemiology different from those of infections acquired later in the neonatal period *(16)*. There is no time point that clearly distinguishes maternally transmitted infections from environ-

mentally transmitted infections *(17)*. However, epidemiologic studies of neonatal infections usually divide so-called early-onset infections from late-onset infections at somewhere between days 3 and 7 of life with the assumption that early-onset infections are presumably transmitted perinatally from the mother and late-onset infections are acquired postnatally from an environmental source *(16–26)*. Therefore, a report of a new test for the diagnosis of neonatal sepsis must be critically evaluated taking into account the arbitrary time points used for separating maternally derived infections from postnatal infections. The postnatal age provided as a basis for diagnosing either early- or late-onset disease may have a striking effect on the concentrations of analytes in cases. This is equally important for controls. Only when the time points for separating the two patterns of disease are uniform can consistency in all studies be expected. We are far from this. In the last decade, many studies have not even differentiated between early- and late-onset infection, and still more disappointing, they have examined the accuracy of modern laboratory tests in groups of newborn infants with wide-ranging postnatal ages *(27–29)*. These differences may alter the diagnostic characteristics of a particular test and confound the comparison of published reports *(30)*.

Systemic Response to Infection in Newborns

Neonatal sepsis, sepsis neonatorum, and neonatal septicemia are terms that have been used to describe the systemic response to infection in newborn infants. There is little agreement on the proper use of the terms, i.e., whether their use should be restricted to bacterial infections, positive blood cultures, or severity of illness *(31)*.

In 1991, the American College of Chest Physicians and the Society of Critical Care Medicine convened a Consensus Conference in an attempt to provide a conceptual and practical framework to define the systemic inflammatory response to infection, which is a progressive injurious process that falls under the generalized term "sepsis" and includes sepsis-associated organ dysfunction as well *(32)*. The term systemic inflammatory response syndrome (SIRS) is used to describe a clinical syndrome characterized by two or more of the following: (*a*) fever or hypothermia, (*b*) tachycardia, (*c*) tachypnea or hyperventilation, and (*d*) abnormal white blood cells or increase in immature forms. SIRS may be a result of a variety of immunologic, endocrinologic, traumatic, surgical, chemotherapeutic, and infectious insults *(32, 33)*. Sepsis is considered when there is a systemic response to a possible infection. Evidence of bacteremia or an infectious focus is not required *(32, 33)*. When sepsis is accompanied by organ dysfunction, hypoperfusion, or hypotension, the sepsis is considered severe. Septic shock ensues when hypotension develops despite adequate fluid replacement. Finally, in the presence of altered organ function in an acutely ill patient, so severe that homeostasis cannot be maintained without intervention, multiple-organ dysfunction syndrome is diagnosed. Very recently, a group of experts and opinion leaders revised the 1991 sepsis guidelines and found that apart from expanding the list of signs and symptoms of sepsis to reflect clinical bedside experience, no evidence exists to support a change to the definitions *(34)*.

The application of the above terminology guidelines to septic newborns, however, needs careful assessment (i.e., age-related reference values for blood pressure, heart rate, respiratory rate, and leukocyte count). Furthermore, the application of a staging system (including sepsis, severe sepsis, septic shock, and multiple-organ dysfunction syndrome) may not be the best approach to disease or risk stratification in the newborn. Early organ abnormalities may not be manifest, so that earlier stages in the evolution of the syndrome may not be identified. Furthermore, the often fulminant or rapid course of the disease in the newborn may limit the staging system outlined above to just a snapshot in time of this dynamic process.

Currently, criteria for neonatal sepsis usually include documentation of infection in a newborn infant with a serious systemic illness in which noninfectious explanations for the abnormal pathophysiologic state are excluded or unlikely. However, even culture is not free from error because it can be falsely sterile, as suggested by postmortem cultures *(35)*, or because of the low yield caused by insufficient sample volumes, intermittent or low-density bacteremia, or suppression of bacterial growth by earlier (i.e., intrapartum) antibiotic administration. Theoretically, this would lead to an underrepresentation of truly infected newborn infants. On the other hand, in only a few studies were the definitions of sepsis stringent enough to distinguish culture contaminants from true systemic infections. A decade ago, Pourcyrous et al. *(36)* recognized that 83% of blood cultures yielding organisms with low-grade or questionable virulence were the result of contamination during collection. In all of these cases, antibiotic therapy was not administered or was inadequate by generally accepted standards, but clinical courses were uneventful. If the term "culture-proven" sepsis is used to mean infants whose clinical signs of infection and/or abnormal laboratory results are fully explained by the yield of typical skin or upper respiratory flora from a single blood culture, it is necessary to specify whether, in this situation, clinical signs and abnormal laboratory results have been resolved with specific antimicrobial therapy or worsened without it *(37)*. This approach, taking full account of the clinical course, should be more fruitful, leading to an unequivocal standard criterion.

Faced with these questions, and as found in studies on sepsis in adult patients, part of the neonatology literature has abandoned positive cultures as a critical point of the "gold standard". However, it must be emphasized that there is no universal agreement on the definition of neonatal clinical septicemia. In recent years, some authors have suggested that presence of just one clinical sign (compatible with infection) along with a C-reactive protein (CRP) value >10 mg/L, is sufficient to make the diagnosis of early- and late-onset neonatal clinical septicemia *(38, 39)*. Others have assigned infants with only

maternal risk factor(s) for infection to the sepsis group "to develop criteria that are relevant and simple enough for clinical practice" *(40)*. In other studies, the presence of two or three categories (by organ system) of clinical signs of infection in the infant has been taken to strongly support a diagnosis of septicemia *(41–43)*. Absent from neonatology publications, however, are data on just how common a given clinical sign is in all infants ever evaluated (rather than in infants with positive cultures or infants with a specific type of infection). Thus, with the exception of the clinical scenario of a newborn with clear-cut signs of infection such as septic shock, the possibility that infants with only clinical evidence of infection may have been assigned an incorrect diagnosis is intrinsic to all studies of this nature *(37)*. In contrast to our insistence on the need for an unequivocal and uniform standard criterion for establishing culture-positive as well as culture-negative sepsis, some authors have considered a CRP value >10 mg/L combined with an immature:total neutrophil ratio >0.25 as a criterion to start antibiotic therapy even in babies with no symptoms of infection *(44)*.

In conclusion, it would seem that the absence of a universally accepted standard definition has led to authors creating their own definitions to suit the purposes of their own particular studies. However, these definitions should be explicit with regard to type of baby (defined by gestational age and birth weight), baby's age, culture status, symptom status, and illness severity.

Illness Severity in Newborns

Although illness severity is a familiar medical concept, it is sometimes difficult to assess. An important feature of morbidity in newborn infants is its integrative and global nature. Individual attributes (e.g., hypoglycemia, oral feeding difficulties, and seizures) fail to capture the overall neonatal health and morbidity status. Until recently, birth weight, gestational age, and Apgar score were often considered sufficient proxy measures of morbidity at birth. Although birth weight and gestational age stratification may be adequate for some purposes, they do not account for variations in illness severity completely. The Apgar score was originally designed to identify neonates in need of immediate cardiopulmonary intervention *(45–48)* and is determined predominantly by acute intrapartum events. Low Apgar scores may actually reflect gestational age, birth weight, sedation, or congenital malformations *(47)*.

In recent years, two major neonatal severity measures, the Score for Neonatal Acute Physiology (SNAP) and the Clinical Risk Index for Babies (CRIB), have been developed. Both SNAP and CRIB focus on measuring and scoring physiologic derangements, following the example of the Acute Physiology and Chronic Health Evaluation in adult intensive care units (ICUs) *(49)* and the Pediatric Risk of Mortality in pediatric ICUs *(50)*. The rationale is that, regardless of disease or diagnosis, derangements from the physiologic norm increase the likelihood of adverse outcome and that the greater the derangements, the greater the risk. The composite severity can be represented by the weighted sum of derangements across all organ systems *(51)*.

CRIB was designed for ease of data collection. It uses a 12-h baseline period from birth *(52)*. In the population ≤31 weeks of gestational age, most illness is adequately captured by sampling only three items in the respiratory/metabolic systems (worst base deficit and highest and lowest appropriate oxygen requirements). SNAP, first described in 1993 *(53)*, uses the worst recorded values of 26 routinely measured physiologic variables (including 8 that can be scored for high or low values) during the first 24 h of stay in the neonatal ICU (NICU). It has been prospectively validated in multiple NICUs covering very different population groups and has been found to be highly correlated with other indicators of illness severity *(53–56)*. From this, the SNAP-Perinatal Extension (SNAP-PE) captures SNAP physiology scores, combining them with additional scoring for three potent perinatal mortality risks (i.e., birth weight, low Apgar score, and small for gestational age status), all of which are independent of physiologic derangement *(57)*. Thus the SNAP-PE score is a combined physiologic and perinatal measure of mortality risk.

As a first-generation newborn illness severity score, SNAP is suitable for outcomes research, but it is cumbersome for routine clinical use because of the number and complexity of its items. On the other hand, CRIB is difficult to apply to infants born outside the hospital. The recently revised SNAP-II scores six items, covering six systems (pH, temperature, blood pressure, oxygenation, urine output, presence of multiple seizures), but has performance equivalent to that of SNAP and CRIB for both very low birth-weight infants and larger infants *(58)*.

Laboratory Tests

NORMAL STANDARD: GENERAL CONSIDERATIONS

A normal standard, in the context of a laboratory test, refers to what is commonly called a normal range by statisticians. It should not, however, be confused with the normal (gaussian) distribution. The normal distribution is a symmetric distribution in which the interval defined by the mean ± 1.96 SD includes the central 95% of the values. The distributions of most clinical indices do not approximate the normal distribution, and this simple interval cannot be used to define an interval that includes the central 95% of the values of a clinical index. A normal range, or normal standard, is an interval that refers to the distribution of the index in healthy individuals; the normal range includes the central 95% of these values. In statistical terms, the normal range can be defined as the interval between the 2.5th percentile and the 97.5th percentile of the distribution of the index in healthy individuals. If this interval is used in the context of a diagnostic test, it has, by definition, a specificity of 95% (the specificity is 97.5% only if one tail of the distribution is considered a positive test result). The normal range provides no information about the sensitivity of the test.

normal (and abnormal) ranges in the newborn infant

At birth, the fetus makes an abrupt transition from the protective environment of the uterus to the outside world; the newly born baby must undergo extreme physiologic changes to survive this transition *(59)*. It is therefore not surprising that many physiologic and metabolic processes change constantly during the first few days of life. These changes profoundly affect various kinds of laboratory values (e.g., hormones, biochemical indices, immunologic products, and cytokines), and the mean reference values in the early neonatal period differ from those measured in later periods *(60, 61)*. Analysis of the reliability of laboratory tests in the diagnosis of neonatal sepsis must therefore take account of the fact that postnatal age may dramatically affect the interpretation of what constitutes the normal (and equally important the abnormal) value of a laboratory test *(60)*.

Virtually all published guidelines stress the need for reference values. Unfortunately, the agreement ends here. In this context, no discussion of the issue would be complete without consideration of the interpretation of two laboratory aids whose performance has acquired an almost ritual quality: CRP and complete blood cell count (CBC).

In the majority of published reports, upper limits for CRP during the neonatal period have been obtained from symptomatic uninfected patients *(62–72)*. Thus there are few studies of upper limits for CRP in the healthy newborn *(73–76)*. Furthermore, most of these studies of healthy neonates were cross-sectional and based on small samples with wide-ranging postnatal ages. Gutteberg et al. *(73)*, who determined (by radial immunodiffusion) CRP concentrations in 16 apparently healthy newborns at unspecified sampling times during the first month of life, obtained normal 97.5th percentile upper limits for CRP of ~5 mg/L. In the study by Forest et al. *(74)*, 69 newborns labeled as "apparently healthy newborns" for their normal postnatal course, despite their initial admission to the NICU, were sampled at unspecified times from birth up to the 6th week of life. Of these, 68 had a CRP concentration (as measured by enzyme immunoassay) <10 mg/L. In the study by Schouten-Van Meeteren et al. *(75)*, 95% of 38 apparently healthy newborns who were sampled between 12 and 24 h after birth had CRP concentrations ≤10 mg/L (as determined by a fluorescence polarization immunoassay). Nonetheless, it is less well known that the false-positive rate for these methods in the apparently healthy neonate throughout the first 30 days of life is 8% for CRP *(30)*. In addition, a lack of follow-up (or outcome) data has been a notable weakness in the literature on the "squeaky clean well infants".

We have followed these precepts in a recent longitudinal study investigating the pattern of CRP response in the healthy neonate at three fixed neonatal ages: 0, 24, and 48 h *(76)*. We were not interested in establishing CRP ranges for an ideal population of healthy infants (i.e., term infants with no risk factors), but in setting up ranges that may be important for clinicians' everyday experience. The CRP reference intervals that we established at birth (95th percentile, 5.0 mg/L), at 24 h (95th percentile, 14.0 mg/L), and at 48 h of life (95th percentile, 9.7 mg/L) included neonates who were not necessarily free of history of maternal and intrapartum complications but whose postnatal clinical course from birth to the 4-week follow-up visit was unremarkable, implying therefore, no need of management (including antimicrobial treatment) throughout this study period.

The most commonly cited study of neonatal CBC is that of Manroe et al. *(77)*. In the period from 0 to 24 h of age, the critical decision time for most neonatal "sepsis workups" *(78)*, Manroe et al. based their graphs on 108 infants sampled in a cross-sectional way. The 10th and 90th percentile envelopes were defined by visual inspection. It is not comforting to think that so many of us have long accepted these norms without question. From the study by Schelonka et al. *(78)*, the normal ranges for leukocyte indexes in 193 healthy term infants with no identifiable perinatal risk factors for infection were at 4 h of life considerably broader than those described previously by Manroe et al. *(77)* for the first 24 h of life. Thus, if one applies the reference intervals of Manroe et al. *(77)* to healthy term infants, one would label huge numbers of them as being at extremely high risk for sepsis. Leaving aside the methodologic aspects, it is important to remember that the actual physical sampling can lead to dramatic changes in the CBC results. It has been well documented that the CBC depends on the infant's age *(77–80)*, on whether the sample is arterial or venous *(81)*, and on whether the infant is crying vigorously *(81)*. This means that for a given infant, a test value is not a static value; there is considerable intraindividual variability. In this context, it is worrying to think that so many of us have accepted a very popular index, the immature:total neutrophil ratio, without question, although the test is subject to considerable interobserver variation. Because segmented and band neutrophils exist on a continuum of cellular maturation, the use of discrete boundaries is artificial and subject to observer bias. In 1993, the College of American Pathologists surveyed 6600 hematology technicians in a band neutrophil identification exercise *(82)*. Because of poor reproducibility of band neutrophil identification in this large sample, the College no longer tests laboratory proficiency in the differentiation of segmented and band neutrophils *(82)*.

In view of this dynamic behavior, do we also need age-specific cutoff values for differentiating symptomatic newborns with infection from those with no infection? In 1980, Philip and Hewitt *(83)* suggested that, for simplicity, the same laboratory cutoffs for markers used in diagnosing or ruling out neonatal sepsis should be applied during the first postnatal week. Two decades later, there are even updated systematic reviews on the accuracy of modern laboratory tests for the diagnosis of sepsis in the newborn that support this contention *(29)*. Perhaps the most surprising thing is that such methodologic flaws have also plagued the very latest applications of CRP. Philip and Mills *(84)* recommended that at any neonatal

age a CRP value ≥10 mg/L in the presence of one (or more) clinical sign(s) or one (or more) risk factor(s) for infection should be the clinical pathway for transferring a neonate from the well-baby nursery to the NICU and starting antimicrobial therapy. Franz et al. *(38, 39)*, on the other hand, considered a conventional CRP value >10 mg/L, in the presence of one (or more) clinical sign(s) compatible with infection, as a criterion to make a diagnosis of clinical septicemia at any neonatal age in NICU babies. Against this background, we have recently shown *(85)* that failure to recognize specific cutoff values by a given test for each time point of evaluation over the first 48 h of life may confound the interpretation of what constitutes a "true negative" and a "true positive" value in the diagnosis of neonatal infection.

ROLE OF NEW MARKERS

In recent years, the search for diagnostic tests for sepsis in newborn infants has turned to cytokines as well as to other substances associated with the inflammatory response, in some cases induced by cytokines, as possible indicators of infection. A few new markers remain promising, of which interleukin (IL)-6 is the most intensively studied. In addition, procalcitonin (PCT) appears to show considerable promise as a diagnostic test for neonatal sepsis.

Data pertaining to reference intervals for the new markers are very limited. We have recently shown that both IL-6 and PCT present a natural fluctuation in the immediate postnatal period *(76, 86)*, necessitating very careful adjustments in the normal ranges. This may explain the conflicting cutoff points for abnormal values that have been reported for these two markers *(28, 38, 87–92)*. We have also shown that some confounding factors, per se, should be taken into account to define "physiologic" concentrations of both IL-6 and PCT *(76, 86)*. Of note, the kinetics of IL-6 during the first 48 h of life in healthy infants are different in the near-term infant compared with kinetics in the term neonate, suggesting a gestational age-dependent effect on IL-6 values over the first 48 h of life *(76)*.

Reports in the literature about the usefulness of new indicators of infections have been conflicting. In a recent study we *(85)*, like others *(28, 88)*, found that the sensitivity of IL-6 as well as that of PCT is low, in the range of 70–80% at birth, by far the most critical decision point when evaluating a newborn to rule out sepsis. In the immediate postnatal period, however, although the sensitivity of IL-6 decreases over time, the sensitivity of PCT (as well as that of CRP) increases, making serial measurements useful for those situations in which one needs to decide how long to treat. Because the sensitivity of these markers varies over time, their use requires specific cutoff values for each time point of evaluation over the first 48 h of life. We also showed that IL-6 and PCT concentrations during the early neonatal period may relate differentially to clinical complications in the perinatal period *(85)*.

EFFECT OF ILLNESS SEVERITY AND RISK STATUS ON MARKERS

The reliability of most laboratory tests for the differential diagnosis of infectious vs noninfectious systemic inflammatory response has been assessed in highly diverse groups of ill neonates with a mixture of diagnoses and conditions and has yielded discrepant results *(30)*. In this situation, because infectious as well as noninfectious diagnoses and the conditions themselves may vary in severity, some of the variation among published reports might reflect differences in baseline severity and risk status, independently of the presence of infection. There is wide variation in clinical severity among NICUs: admission ranging from critically ill infants with multiple-organ-system failure to mildly ill term infants with transient problems related to the birth process or healthy premature infants who require technologic support until mature. Unfortunately, a major problem with the literature on the diagnostic value of laboratory aids for diagnosis of neonatal infection is its failure to evaluate the effect of overall illness severity on a given marker.

We therefore recently evaluated the influence of illness severity on CRP, IL-6, and PCT for the diagnosis of sepsis during the first 48 h of life in critically ill newborns admitted to the NICU *(85)*. To this end, we used both the SNAP and SNAP-PE scores. We found that CRP, IL-6, and PCT increase in the presence of bacterial infection and that their increases are independent of illness severity. However, we also found that illness severity has the potential to confound IL-6 concentrations in that, among babies without infection, the higher the illness severity, the higher the IL-6 concentration after birth. It is clear from the above that the diagnostic value of certain laboratory aids, such as IL-6, may be altered by physiologic severity and risk indexes.

Methodologic Issues

GENERAL CONSIDERATIONS

The value of a clinical index observed in a patient may be a useful aid for diagnosis, if the distribution of values among healthy individuals is substantially different from the distribution in true cases of the disease. At one extreme, if the two distributions are identical, clearly the value observed for a single patient can provide no information about the diagnosis, and the test is useless. At the other extreme, if the two distributions do not overlap, a cutoff value between the two distributions can provide a certain diagnosis.

The most common situation is that the two distributions overlap and the test cannot give a sure indication of the disease state of the patient. In fact, a single value may be used as a cutoff between a positive and a negative test result, and the quantitative value of the index is reduced to a dichotomy. The test is usually evaluated by calculating its sensitivity and specificity. The sensitivity of the test is the probability that a patient with the disease or condition is identified as positive by the test. The specificity is the probability that a healthy individual has a negative test result. These two probabilities may be esti-

mated by applying the test to a sample of known cases and another sample of known healthy individuals. However, the sensitivity and specificity are not of direct interest to clinicians when they are faced with the clinical problem of making a diagnosis for an individual patient. What clinicians would like to know is whether their patients have the disease, but unfortunately they cannot be certain about this. This uncertainty is also measured by a probability. Epidemiologic studies can be used to estimate the prevalence of the disease in a particular clinical setting. This is the probability that the individual has the disease before the diagnostic test is done, and is often called the prior probability of the disease. Suppose that the test is applied to a patient and the test result is positive. The clinician should now believe, even more certainly, that the patient has the disease. This degree of belief is measured by a probability called the predictive value of a positive test, which can be estimated from the sensitivity, specificity, and prevalence of the disease in the clinical setting.

This is a gross simplification of the reasoning used by a clinician faced with the problem of making a diagnosis for a single patient, however. For example, suppose that the test has been evaluated, and it is found that the predictive value of the positive test is 70%. That is, a person with a positive test has a 70% probability of having the disease. What can this tell a clinician, whose patient has a positive test result, about the disease status? It certainly does not mean that the clinician believes that the patient has a 70% chance of having the disease. This value, 70%, is based on information obtained from a sample of patients "of this type". Much more information is available for the evaluation of an individual patient, and this will have been collected by the physician in the case history. The positive test result will be just one additional piece of information. If the patient has important signs and symptoms of the disease before the test, his or her prior probability will be high, and a positive test result may almost confirm the diagnosis. On the other hand, if the patient shows few or no signs of the disease, a positive test will not imply a high probability that the patient has the disease. A diagnostic test will potentially be most useful for patients whose conditions and case histories suggest that they may have the disease.

On the basis of a review of diagnostic tests of bacterial infections in infancy *(27)*, it has been concluded that these diagnostic tests may be of limited use in the absence of other relevant clinical information about a patient. Indeed, the authors of the review suggest that when a clinician is faced with an infant with a possible serious infection, treatment may be started with antibiotics immediately. Clearly the test result will not affect the decision to start treatment if there is already strong evidence of the infection. Similarly, in the absence of indications of infection, the clinician may not start treatment even if the test were positive. The test is most likely to be useful when the case history and the condition of the patient leave the clinician in serious doubt about the presence of infection, in which case the diagnostic test may be used as a decision rule: start treatment if the test is positive; delay treatment if the test result is negative.

ACCURACY OF LABORATORY METHODS

Given the increased mobility of patients, comparable (true) test results are essential for a rational and cost-effective diagnostic approach in laboratory medicine *(93)*. From the standpoint of laboratory practice, however, when examining all of the data published to date on the laboratory aids for diagnosis of neonatal sepsis, it is obvious that differences in laboratory techniques have also been in part responsible for the conflicting opinions about the reliability of a given test during the neonatal period. Again, no discussion of the issue would be complete without consideration of the different methods used to measure CRP, the most commonly used marker for identifying neonates with sepsis.

The original test was a simple precipitin test, usually in a microcapillary tube, in which the height of the precipitant defined the amount of CRP present. A comparison of reactions obtained by different investigators using the capillary tube method revealed widely disparate results, depending on the sensitivity of the commercial antiserum used in the assay *(94)*. Not until the early 1980s did rapid and reliable quantitative immunoassays become commercially available in which monoclonal CRP-specific antibody was used. Some investigators used immunoassays that permitted direct visualization of a CRP–antibody complex through particle agglutination (i.e., latex agglutination) or through precipitation (i.e., radial immunodiffusion, immunoturbidimetry, nephelometry). Other investigators used immunoassays that enlisted a marker for detection (i.e., RIA, enzyme-multiplied immunoassay technique). Although the slide agglutination test is rapid and convenient and still used by some investigators *(95)*, it is only semiquantitative and subject to reagent variability *(96)*.

Studies comparing fully automated turbidimetric and nephelometric methods for CRP with older assays have shown superior precision, with far greater speed, sensitivity, and reproducibility. However, these assay methods still have limited sensitivity, and until recently, CRP concentrations below ~10 mg/L could not be measured precisely, leading to widespread adoption of this value, or even higher, as the upper limit of the "healthy" reference interval. This is satisfactory for some purposes in general medicine because the marked acute-phase responses that characterize bacterial infections, ischemic necrosis of tissue, and most active inflammatory conditions usually lead to much higher CRP values. However, for neonates, health-associated reference values are below conventional threshold values. Furthermore, newborns may be unable to produce high amounts of acute-phase proteins and respond to infection with a smaller increase in CRP than adults.

The clinical need for highly sensitive CRP assays was first recognized in neonatal pediatric practice *(71)*, and their recent development holds promise for a further increase in the diagnostic accuracy of neonatal infection.

However, before introduction of the new tests into routine neonatal practice, the CRP reference intervals to be established by the newly developed assays need to be compared with the traditional ones. In this context, further standardization efforts are required to ensure that high-sensitivity CRP assays have the requisite precision at low CRP concentrations *(97)*, particularly at birth when CRP upper reference limits fall below the conventional threshold values of 3–5 mg/L. On the other hand, another issue that merits discussion is the possibility of underestimating with the high-sensitivity assays the true CRP concentration because of a prozone effect *(97)*. Thus, before introduction of the high-sensitivity CRP assay as routine indicator of more severe inflammation, further investigation is required to improve agreement in the higher range among the different methods.

Further potential caveats arise from the type of assay used to quantify the biomarker. For example, use of different assay types and different antibodies in immunoassays for tumor necrosis factor-α (TNF-α) may be responsible for the discrepancies among TNF-α values *(98)*. The various immunoassays available for TNF-α measure free (bioactive) or bound plus free (total) TNF-α. Thus, immunoassays may detect not only bioactive TNF-α, but also inactive fragments, monomers, and polymers; binding of TNF-α to its soluble receptors (p55 or p75) may also interfere with its determination by immunoassay *(98)*. Other investigators have chosen to measure TNF-α by bioassay (e.g., mouse fibrosarcoma cell line WEHI 164) because it may reflect the activity of cytokine (i.e., the amount of bioactive TNF-α) in vivo better than the immunoassay *(99)*.

Another crucial point is that methods must be reproducible in time and space, with a turnover time sufficient to meet the demands of clinical practice. Some methods have automated dilution, whereas others require a manual dilution prepared off-line. In the majority of studies on the diagnostic value of cytokines in identifying or excluding the septic neonate, a manual immunoassay was used *(41, 100, 101)*. This method usually requires 2–4 h. Despite being faster, the manual method for cytokine determination is not clinically practical because technicians competent in assaying cytokines with ELISAs would need to be available 24 h a day. Even if this were possible, interobserver error would be a problem. The development of readily available automatic assays, with fast turnaround times, is essential for potential widespread clinical application of cytokines.

One seldom discussed problem is that only in recent reports are methods for calculating measures of diagnostic accuracy or making comparisons accompanied by statistical measures of their precision (i.e., 95% confidence intervals) *(40, 85)*. The issue is critical because of the relatively small number of patients in each report, with consequent possibly wide confidence intervals.

Can we learn anything from certain potential methodologic problems alluded to above? It is clear from the above discussion that the wide variations among studies on the methods (and the results) preclude any meaningful synthesis, such as metaanalysis, of all reported studies. As recently suggested by Escobar *(102)*, it is time that we begin to debate the methods we use to measure test performance, rather than just how a given test performs. This issue has been the subject of a recent report on Standards for Reporting of Diagnostic Accuracy (STARD) *(103)*. An important part of the STARD plan is to evaluate its effect on the reporting of studies. Whether the STARD report is a step in the right direction toward complete and accurate reporting of studies of diagnostic accuracy in newborn infants presents a challenging new research frontier.

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