Aspects on early diagnosis of neonatal sepsis

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ANDREAS OHLIN

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Table of contents

ABSTRACT 9

ABBREVIATIONS 10

ORIGINAL PAPERS 11

INTRODUCTION 12
Epidemiology of neonatal sepsis 12
Early onset sepsis 14
Late onset sepsis 14
Diagnosis of neonatal sepsis 16
Blood culture 17
Haematological markers 18
Cytokines and acute phase proteins 18
Cell surface markers 20
Clinical scoring systems and heart rate analysis 21
Polymerase chain reaction 21
Staphylococcus epidermidis 25
Bacterial typing 26
Virulence factors and cell wall anchored proteins 27

AIMS OF THE THESIS 28

MATERIAL AND METHODS 29
Design 29
Patients 29
Blood cultures 30
EDTA samples 31
DNA preparation 31
C-reactive protein values 32
Clinical signs 32
Polymerase chain reaction 33
Pulsed field gel electrophoresis 36
Statistics 36
Ethics 37

RESULTS 38
Clinical signs (I) 38
Signs associated with sepsis 38
Signs not associated with sepsis 38
The effect of gestational age 38

Real-time PCR of plasma samples (II) 39
Real-time PCR of plasma samples (III) 40
Comparison between the specific probes and blood culture 41
Sequencing of PCR amplicons 41
Case studies of samples with conflicting results 42
Typing of S. epidermidis (IV) 42
sdrG 42
sdrF 42
sesE 42
aap 43
PFGE 43
Discriminatory index 43

DISCUSSION 45
Strengths and limitations 52
Future research 53

CONCLUSIONS 54

ACKNOWLEDGMENTS 55

SWEDISH SUMMARY 57
Sammanfattning 57
Bakgrund 57
Målsättning med studien 57
Material och metoder 57
Resultat 57
Slutsatser 58

REFERENCES 59

ORIGINAL PAPERS I–IV 77

The baby was astonishing. He had little cloth ears, floppy as cats. The warmth of his round stomach could heat the world. His head smelled like a sacred flower. And his fists held mysterious, tiny balls of fluff from which he could not bear to be parted.

Babyn var förunderlig. Han hade små mjuka öron som gick att böja på alla ledder, precis som en katts. Hans lilla runda mage var varm nog att värma hela världen. Hans hjässa doftade som en helig blomma. Och i sina små knutna nävar hade han mystiska små fjunbollar som han inte alls ville skiljas från.

–Monica Ali, Brick Lane

Till Oliver och Linnea mina två fantastiska barn som en gång i tiden också hade en liten rund mage som kunde värma hela världen.

ABSTRACT

This thesis presents four studies, all designed to improve the problematic diagnostic situation concerning infants with suspected sepsis.

Study I included 401 neonates with suspected sepsis. Nine signs of sepsis and C-reactive protein were prospectively recorded and logistic regression was used to assess associations between these signs and a subsequently confirmed diagnosis of sepsis. C-reactive protein and five of the clinical signs were statistically significantly associated with a positive blood culture. When the material was stratified by gestational age, differences between premature and full term infants were detected.

Studies II and III were prospective studies that used samples collected from neonates with suspected sepsis to evaluate a novel real-time polymerase chain reaction (PCR) method. The results where compared with simultaneously collected blood cultures. Study II used plasma samples and resulted in a sensitivity of 42% and specificity of 95%. In study III, the protocol was improved and adapted to whole blood samples which resulted in a sensitivity of 79% and specificity of 90%. Both protocols included species-specific probes and study III indicated that PCR has the potential to detect bacteria in culture-negative sepsis.

Staphylococcus epidermidis is the most common pathogen in neonatal sepsis, but there is still a lack of typing methods suitable for large materials of *S. epidermidis*. In *Study IV* we therefore evaluated a new *S. epidermidis* genotyping method based on PCR for the repeat regions of four genes that encode for cell wall anchoring proteins. The method was applied to 49 well-defined neonatal blood isolates of *S. epidermidis*. The combination of *sdrF* and *aap* seemed to be optimal, resulting in a diversity index of 0.92.

Conclusions

- Bradycardia, apnoea, low blood pressure, feeding intolerance and distended abdomen are obvious early signs of neonatal sepsis. Premature and full-term infants differ in terms of the signs they display in neonatal sepsis.
- Blood is superior to plasma for developing PCR methods for bacterial DNA detection. The PCR method described in study III can detect neonatal bacteraemia, but it can be further improved before it is used in routine care.
- There has been a lack of useful typing methods for *S. epidermidis*. We can now present PCR of the genes for the cell wall anchoring proteins *sdrF* and *aap* as a novel and feasible approach when there is a need to type a large number of *S. epidermidis* isolates.

ABBREVIATIONS

bp	base pairs
CD64	Clusters of differentiation 64
CoNS	coagulase-negative *Staphylococcus*
CRP	C-reactive protein
CI	confidence interval
CWA	cell wall anchored proteins
D-index	discrimination index
EDTA	ethylenediaminetetraacetic acid
EOS	early onset sepsis
FcγRI	Fcγ Receptor I
GBS	group B *Streptococcus*
IL	Interleukin
IL-1 ra	IL-1 receptor antagonist
LOS	late onset sepsis
LLOS	late late onset sepsis
MLST	multi locus sequence typing
NEB	New England Biolabs Incorporated
NICU	neonatal intensive care unit
OR	odds ratio
PCT	procalcitonin
PFGE	pulsed field gel electrophoresis
S. epidermidis	*Staphylococcus epidermidis*
TNF-α	tumour necrosis factor α
USÖ	Örebro University Hospital
VLBW	very low birthweight

ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their respective Roman numerals (I-IV):

I. Ohlin A, Björkqvist M, Montgomery SM, Schollin J. Clinical signs and CRP values associated with blood culture results in neonates evaluated for suspected sepsis. Acta Paediatr. 2010;99(11):1635-1640.

II. Ohlin A, Bäckman A, Björkqvist M, Mölling P, Jurstrand M, Schollin J. Real-time PCR of the 16S-rRNA gene in the diagnosis of neonatal bacteraemia. Acta Paediatr. 2008;97(10):1376-1380.

III. Ohlin A, Bäckman A, Ewald U, Schollin J, Björkqvist M. Diagnosis of neonatal sepsis by broad range 16S real-time PCR. Submitted.

IV. Ohlin A, Bäckman A, Söderquist B, Wingren S, Björkqvist M. Rapid typing of neonatal *Staphylococcus epidermidis* isolates using polymerase chain reaction for repeat regions in surface protein genes. Eur J Clin Microbiol Infect Dis.29(6):699-704.

INTRODUCTION

Epidemiology of neonatal sepsis

Sepsis has always been one of the most common complications affecting newborn infants. It is normally divided into three categories, depending on time of onset: early onset sepsis (EOS) at < 3 days of age, late onset sepsis (LOS) at 3-28 days of age, and late late onset sepsis (LLOS) at 29-120 days of age. Of these, LOS is the most common infection, especially in very low birth weight (VLBW) infants.

Sepsis is normally defined as bacteraemia in combination with systemic inflammatory response syndrome, but there is no widely accepted definition for neonatal sepsis[2]. Since blood culture has a low sensitivity in neonatal sepsis[3], many studies also include infants with clinical signs of sepsis but a negative blood culture. This condition is normally referred to as clinical, probable or suspected sepsis[4-6], but has not been sufficiently defined; in addition, the clinical signs that that are used vary greatly and are poorly evaluated.

The reported incidence of sepsis varies between 1 and 10 per 1000 live births, but large population-based studies are few, and most of the studies available are focused on high-risk infants such as premature or VLBW children in the industrialised world[7-14]. It is even harder to assess the incidence of neonatal sepsis in the developing world, but rates between 2 and 50 per 1000 live births have been reported for early onset sepsis. Many of these infants have limited access to adequate therapy; for this reason, among others, 99% of the world's yearly 4 million neonatal deaths occur in the developing world, 26% of these deaths being caused by severe infections[15,16].

In the industrialised world, neonatal sepsis is a cause of both neonatal death and neonatal morbidity[8,17,18]. The exact impact of having a neonatal infection is difficult to define, since many of these infections affect infants with many other risk factors and complications, but in two large American studies and one Israeli study, the all-cause mortality was approximately two to three times higher for patients with early or late onset sepsis. For both early and late onset sepsis, the risk of death is higher with gram-negative pathogens than with gram-positives, with the highest risk attributed to late onset *Pseudomonas* infection[8,18,19] (Fig 1).

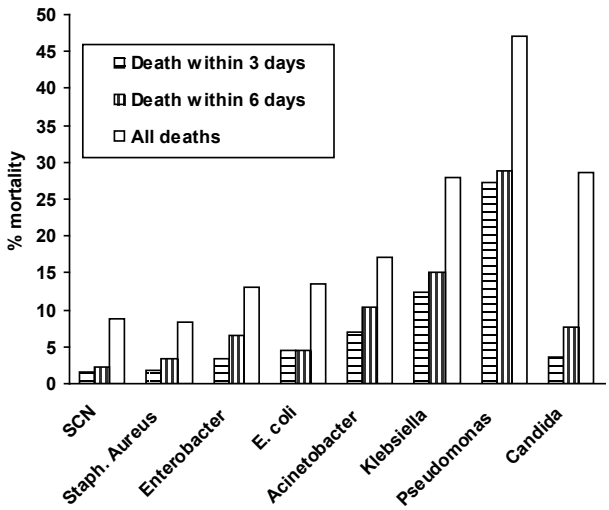

Fig 1. Mortality in late onset sepsis divided by pathogen, from a national study of 5555 VLBW infants in Israel (in this figure the abbreviation for Coagulase-negative staphylococci is SCN). Reproduced with permission from Pediatrics, 109, 34-39, 2002 by AAP[19].

Sepsis is also associated with increased costs and a high risk of neonatal morbidity. A 2004 study from the Vermont Oxford Network estimated that one septic episode prolongs length of hospital stay by approximately 7 days at a cost of 10 000 US Dollars, while Chen et al. reported a similar increase in length of stay (but at a lower cost) from a study in China[20,21]. To avoid these costs and mortalities, there is an increasing interest in preventing neonatal infections, and there is evidence that strict hygiene routines can be effective measures to prevent nosocomial LOS[22-27], and prophylactic intrapartum antibiotics can prevent early onset GBS sepsis[28,29].

Early onset sepsis

EOS is normally considered as a vertical transmission passed on from mother to child during labour and delivery. In most early onset infections this is caused by pathogens present in the maternal genital flora ascending to the foetus through ruptured or intact amniotic membranes. Risk factors for EOS include maternal factors such as premature rupture of membranes, maternal fever, maternal urinary tract infection, and colonisation with GBS; and offspring factors such as prematurity, asphyxia/low Apgar scores, low birth weight, and male sex[30,31]. However, transplacental haematogenous transmission of bacteria can also occur, primarily involving *Listeria monocytgenes*[30].

During the last 40 years EOS has been dominated by Group B Streptococci (GBS) that caused an estimated 1000 deaths per year in the United States during the 1970s[32]. This high mortality rate has now been controlled with widespread use of intrapartum antibiotic prophylaxis which has an efficacy of over 85%[33,34]. To identify the mothers that will benefit from intrapartum antibiotic prophylaxis both screening based and risk factor based programs have been suggested. The screening based method has shown to be more effective[35], but at the cost of a higher antibiotic consumption[32]. The American centre for disease control has issued consensus guidelines in 1996 and 2002 and recommends the screening method[29,36] but other countries like Sweden recommend the risk factor method[37]. These guidelines have lowered the American infection rate from 1.7/1000 live births in 1990 to 0.4/1000 live births in 2005[28], even if higher numbers are still a problem in risk-populations[32]. This increasing use of intrapartum antibiotics is changing the incidence in EOS that is now shifting toward gram-negatives[9,38].

Late onset sepsis

The most common cause of LOS is nosocomial infection as a complication of neonatal intensive care. LOS mainly affects premature or low birth weight infants. The incidence of LOS in VLBW infants has been reported at 17–30%, but in a large national study including all patients born in Sweden before 27 weeks of gestational age, 41% of the surviving infants had at least one episode of septicaemia; and in an even larger recent

American study including infants born at an gestational age of 22-28 weeks, the rate of EOS was 2% and the rate of LOS was 36%[8,14,18,19,39,40]. These infections most commonly occur at a postnatal age of approximately 2-3 weeks[8,12,13,41] (Fig2).

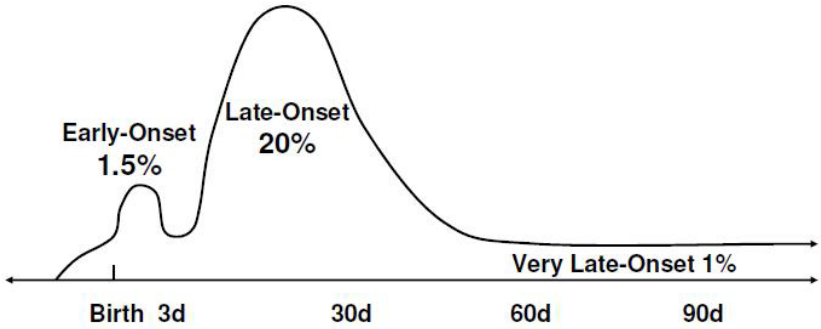

Fig 2. Graph showing the timing of bacterial and fungal sepsis in VLBW infants. Percentages indicate the approximate risk for an VLBW infant to contract sepsis during the NICU stay. Clin Microbiol Rev. 2004;17(3):638-680 reproduced with permission from the American Society for Microbiology[30].

The risk factors normally associated with LOS are prematurity, low birth weight, male sex, low serum IgG levels, low Apgar scores, young mother, mechanical ventilation, treatment with dexamethasone, prolonged use of intravascular catheters, total parenteral nutrition, and delayed enteral feedings[8,12,13,18,42-44]. The most common cause of LOS is coagulase-negative *Staphylococci* (CoNS), which in many materials contributes more than 50% of infections; the next most common bacteria are *Staphylococcus aureus*, Group B *Streptococcus, Enterococcus*, *Escherichia coli, Klebsiella*, and *Pseudomonas*[8,12,19,45]. Among the CoNS, *Staphylococcus epidermidis* is the most common cause of neonatal sepsis[46-52]. CoNS are considered to cause a non-fulminant type of neonatal sepsis with lower CRP levels and only a marginal increase in mortality[8,10,41,53,54] compared with other LOS pathogens[55,56]. However CoNS is associated with an increased morbidity such as increased rate of BPD[56,57] and poor neurodevelopmental outcome[17,58]. It has been suggested that this increased morbidity is caused by the inflammation that occurs when CoNS triggers the immature immune system of preterm infants[59].

Diagnosis of neonatal sepsis

Neonatal sepsis is a serious and potentially dangerous condition which can develop rapidly and cause death or morbidity if not treated promptly and correctly. The quest for optimal diagnostic tools has been ongoing for decades[60], but despite all efforts the basic problem still prevails; many infants, both full term and preterm, present with vague and unspecific symptoms, and the clinician in charge has to decide whether or not to start empirical antibiotic treatment. This decision must be made directly, as the available tests are imprecise and time consuming. This dilemma has actually been unchanged since the discovery of penicillin[61]. The presenting signs vary slightly between preterm and full term infants, but in all groups the signs seem to have a low positive predictive value, though they seem to be more effective in low income settings[18,62-65]. In clinical practice, the recommended approach is therefore to liberally start intravenous antibiotics and then perform a ruling-out procedure that normally lasts for several days. If all tests are negative and the infant has recovered, the antibiotics can be discontinued and the patient can be discharged from the neonatal intensive care unit (NICU). This rule-out procedure normally includes cultures (blood, cerebrospinal fluid, urine and possibly skin cultures), x-rays, and a combination of laboratory tests; while these are performed, the patient is closely monitored for additional signs of sepsis. If it were possible to decrease the time taken by this investigation, the benefits would be obvious in terms of reduced costs, antibiotic consumption, parental worry, and infant suffering. Hence, there is a great need for new and fast diagnostic methods. The ideal prerequisites for such a test were published in 2004, and still apply very well (Table 1)[66]. The same article presented a list of 58 different laboratory tests that had already been evaluated as diagnostic tests for neonatal sepsis. In addition, a recent review by Pierrakos et al. reviewed 3370 references covering 178 biomarkers[67].

Table 1 Characteristics of an ideal infection marker

Clinical characteristics

1. A well defined optimal cut off that is comparable between different NICUs
2. Favourable diagnostic utilities:
   - sensitivity (approaching 100%)
   - specificity (>85%)
   - positive predictive value (>85%)
   - negative predictive value (approaching 100%)
3. Detects infection at an early stage
4. Differentiates between different types of pathogen (viral *v* bacterial)
5. Guides antibiotic use (type and duration)
6. Monitors progress of treatment
7. Prognostication

Laboratory characteristics

1. Stable compound
2. Adequate time window for specimen sampling (sustained increase or decrease in level for at least 48 h after the onset of clinical manifestations)
3. Quantitative measurement
4. Small volume of specimen
5. Easy method of measurement
6. Quick laboratory turnover time
7. Results comparable between laboratories
8. Low cost

NICU, Neonatal intensive care unit.

Table 1. Characteristics of an ideal infection marker. Reproduced from Arch Dis Child Fetal Neonatal Ed. 89, 229-235, 2004 with permission from BMJ Publishing Group Ltd[68].

Blood culture

Blood culture is the gold standard test to diagnose neonatal sepsis. Blood from arterial or venous puncture can be used, as well as blood from newly inserted umbilical catheters. The skin should be prepared with an antibacterial solution before venepuncture, but care must also be taken so that the applied solution does not harm the vulnerable skin of extremely preterm infants[69-72]. Kellogg et al. reported in 1997 that low level bacteraemia (<10 colony forming units/ml) was common in infants; to optimise sensitivity, they recommended a sample volume of 6 ml. However, this would represent approximately 4.5% of an infant's blood volume and hence many others recommend that only 1 ml should be taken and that the full volume should be used for aerobic cultures, since anaerobic bacteria are rare in neonatal intensive care[71,73,74]. Despite this, for practical reasons (to minimise skin punctures, blood loss, and pain) even

smaller volumes are often used, which could lead to a suboptimal sensitivity[75]. Even if optimal blood volumes are used, blood culture has obvious limitations in sensitivity, and a negative blood culture alone cannot support withdrawal of antibiotic therapy if the patient's clinical condition indicates ongoing sepsis[31,73]. In addition to the limited sensitivity of blood culture, the method is time consuming, and most microbiology laboratories will wait 5-7 days before delivering a full report even though the majority of clinically important bacteria can be detected within 48 hours[76]. Cultures from superficial sites like the axilla, umbilical stump, and ear correlate very poorly with blood culture results, and should therefore not be used either to diagnose neonatal sepsis or as guidance for optimal antibiotic treatment[77].

Haematological markers

Several haematological markers (e.g. white blood cell count, absolute neutrophil count, immature/total ratio, etc) have been suggested and evaluated as diagnostic tests for neonatal sepsis[31,60,78,79]. The interpretation of these tests is complicated, by the fact that the normal values are affected by various conditions such as post-gestational age, asphyxia, and maternal factors such as fever and hypertension[78,80,81]. This could be one reason why these tests show fairly poor results in large clinical surveys[82]. The results are better when several tests are merged together into a scoring system, but the sensitivity and specificity are still not high enough to recommend this method for routine clinical use[60,66,73,79,83]. In contrast to these tests, there is one study of granulocyte colony-stimulating factor in neonates with suspected sepsis that shows excellent sensitivity and acceptable specificity. Unfortunately, a large group of infants were excluded from the final calculations since they had suspected but not proven sepsis, and furthermore this study has not yet been repeated[84,85].

Cytokines and acute phase proteins

Cytokines are endogenous chemical mediators that carry information between different cells and are important factors in the human inflammatory response. They are regulated by a complicated web of regulatory mechanisms including several different cell types[86] (Figure 3). In case of infection, both pro-inflammatory and anti-inflammatory cytokines are upregulated according to a specific time schedule, and so by studying this upregulation in blood samples we can conclude whether systemic inflammation is present or not. This inflammation may be caused by sepsis, but can also be triggered by trauma, tissue damage, or even the normal

birth process[87-90], and so the diagnostic potential of most cytokines is limited to a good sensitivity. To achieve an optimal specificity, a cytokine that is specific to sepsis-related inflammation still needs to be defined.

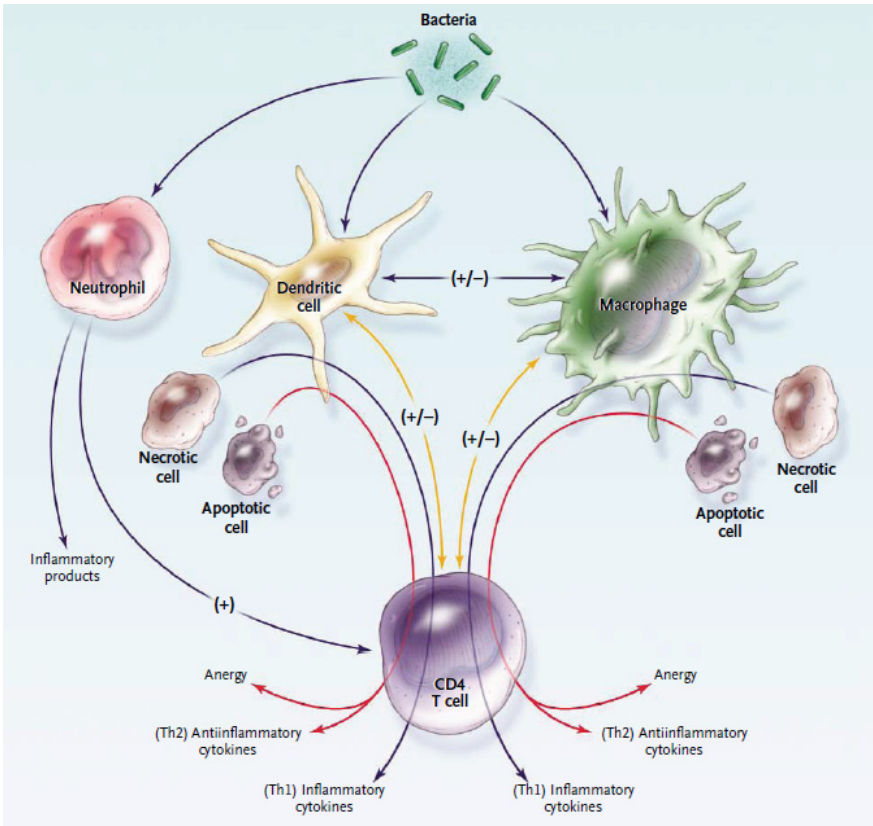

Figure 3. The response to pathogens in sepsis, involving "cross-talk among many immune cells, including macrophages, dendritic cells and CD 4 T Cells[86]. Copyright © [2003] Massachusetts Medical Society. All rights reserved.

The most thoroughly studied acute phase protein is C-reactive protein (CRP), which is also commonly used in routine care both in Sweden and in other parts of Europe. CRP is induced by interleukin-6 (IL-6), and is hence not the earliest marker to rise in the case of infection; rather, it rises within 6-8 hours after onset of infection and peaks 24-48 hours later. It has a half life of 19 hours, and has the capacity for a 1000 fold increase[91]. This means that CRP is not a good screening test to detect sepsis at an early

stage, but it is a suitable ruling-out test that can support the discontinuation of antibiotics when repeated measures over a 48 hour period remain negative[92]. It has also proven to be a useful test in monitoring the progress of a disease and guiding alterations in therapy[93]. Furthermore, CRP levels are correlated with organ dysfunction and severity of infection[94]. IL-6, IL-8, tumour necrosis factor α (TNF-α), and procalcitonin (PCT) have also been suggested as routine tests to diagnose sepsis, which seems logical as they are precursors to CRP and hence would add sensitivity early in the sepsis course. Several authors have suggested that a cytokine kit including both early and late markers should be used[4,66,94-97].

Since IL-6 and TNF-α are precursors to CRP in the inflammatory cascade and respond very quickly to infection, it seems logical to evaluate them as diagnostic tests instead of or together with CRP[98]. Several groups have performed such evaluations, demonstrating that both IL-6 and TNF-α appear to be more sensitive than CRP in detecting sepsis at an early stage[87]. Kuster et al. even found increased levels of IL-6 and IL-1 receptor antagonist (IL-1ra) 1-2 days before the clinical diagnosis of preterm neonatal sepsis was made[99]. Although this article was published in 1998, it is still the only article evaluating IL-1ra as a diagnostic tool for neonatal sepsis[100-102]. IL-8 has similar kinetics to IL-6, and subsequently also performs well as a diagnostic marker; a multicentre randomised controlled trial showed that IL-8 and CRP in combination can reduce the number of infants receiving unnecessary antibiotic therapy[97,103-105].

PCT is produced in the liver and macrophages and responds faster than CRP in neonatal sepsis, but also responds to non-infectious complications in the newborn period such as respiratory distress, asphyxia, and intracranial haemorrhage. Some concerns have therefore been raised about its specificity as a diagnostic tool in suspected neonatal sepsis[106-110].

Cell surface markers

When the human body is challenged by an invading microorganism, the immune system responds by activating neutrophils and natural killer cells. One important part of this activation is to upregulate the number of cell surface antigens, which act as receptors for antibodies and thus play a crucial roll in phagocytosis and the host versus microbe response. Several of these receptors have been evaluated as markers for neonatal sepsis and CD64 (FcγRI) has proved to be superior to CD25, CD45RO, and CD11b[111,112]. Evaluations of CD64 indicate that it is a selective marker for

bacterial infections that is not upregulated by respiratory distress syndrome, premature rupture of membrane, or surgery. However no large-scale randomised trials have yet been performed, costs have not been studied and CD64 is also upregulated in DNA virus infections[89,111,113-128].

Clinical scoring systems and heart rate analysis

Sepsis is defined as bacteraemia together with signs of systemic inflammation. There have been attempts to evaluate the early clinical signs of sepsis to construct an algorithm that can separate signs of sepsis from all the other signs that newborns can display[129]. These studies might be of some help to the clinician in charge, but the sensitivities and specificities reported are currently not high enough to justify changing the current practice of liberal use of antibiotics[54,62-65,83,130-133].

Studies of heart rate analysis have been more thoroughly developed, and although this method is more technically demanding, it has the potential to become a routine tool in many NICUs. The method has mainly been described by Griffin and Randall, though a recent publication from France also evaluated the method[134]. Griffin and Moorman are currently conducting a large randomised trial that will hopefully conclude the question of whether it is feasible to use this technique in standard routine care[62,135-143]. The method uses microcomputers to collect 4096 consecutive cardiac interbeat (RR) intervals from which a heart rate characteristics index is calculated. The group has shown that a pathological heart rate characteristics index of reduced variability and transient decelerations precedes the clinical signs of sepsis[138,141], predicts neonatal infection and death[136], and is associated with mortality[140], neurodevelopment outcome[144], and abnormal laboratory tests[137].

Polymerase chain reaction

Polymerase chain reaction is a standard molecular technique based on the discovery of heat-stable DNA polymerases that can continue to duplicate genomic material even after the DNA has been denaturated by heat[145,146] (Figure 4). The method was first described in 1985, and was immediately adapted for numerous medical problems. PCR has played a fundamental role in many important medical landmarks such as HIV diagnosis[147] and the HUGO project[148], as well as great discoveries in forensic medicine[149] and archaeology[150]. The first application of PCR described in the literature was the diagnosis of sickle cell anaemia by detecting the gene mutation, but very soon the method was also used to detect foreign DNA in the human body to diagnose viral or bacterial infections[148,151]. However, this knowledge has not yet been transformed into a widely accepted broad

range PCR method that can be used in NICUs to detect the DNA from bacteria causing neonatal sepsis.

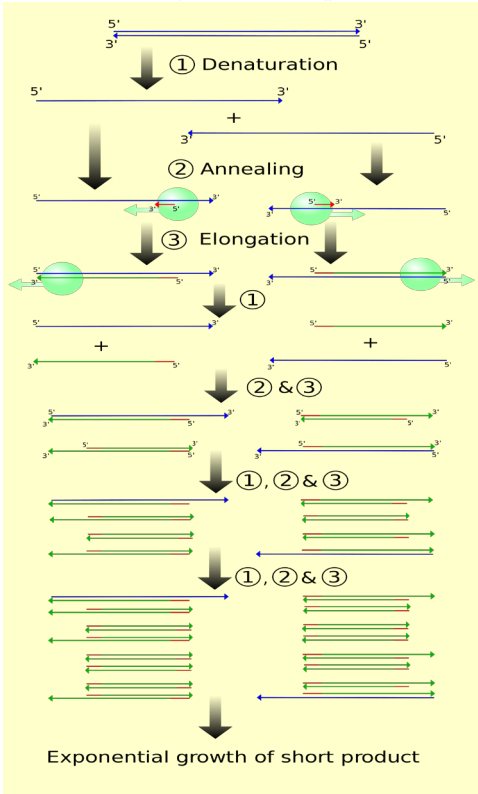

Fig 4. Schematic drawing of the principle of polymerase chain reaction.

The first attempt at such an approach was made by Laforgia et al., who successfully detected DNA encoding for the 16 S subunit of the bacterial ribosome in four out of four blood culture positive infants with early onset sepsis[152]. The rationale for targeting the gene encoding for the 16 S subunit is that the eukaryote ribosome has a different structure which does not contain a 16 S subunit. Hence if 16 S is detected, it must originate from an invading prokaryote[153]. Furthermore, the gene encoding for 16 S contains both conservative regions that are suitable targets for universal PCR primers and variable regions that can be sequenced to allow species identification.

Since the first publication by Laforgia, only a few other studies have been published[152]. Harris et al. published one study that included different types of samples such as pus and biopsies, while Shang et al. published another study with an elegant microarray approach that would probably be

difficult to implement in routine care[154,155]. The first large studies with good test characteristics were achieved using a protocol first described by Jordan, including a 5 hour preculturing step in which the blood sample is cultured in tryptic soy broth in order to enhance the number of bacteria[156-159]. This approach is very appealing, since the preculturing improves sensitivity, but on the other hand time is lost during culturing, and speed is one of the most important advantages of PCR. Another drawback is that the culture requires viable bacteria, while PCR can detect dead bacteria, at least in vitro[159,160].

In order to construct a PCR method with good sensitivity that functions without preculturing, the DNA extraction must be very efficient. The amount of bacterial DNA in a blood sample from a patient with neonatal sepsis is minimal compared to the large amount of human DNA, hence it is vital that no bacterial DNA is lost in the extraction process. Many protocols therefore include enzymes that can break down the bacterial cell wall and expose the DNA to the chemicals in the DNA extraction kit[3,161]. Since the PCR method is designed to detect limited amounts of DNA, the risk of false positive samples due to contamination is substantial, meaning that all reagents and laboratory equipment must be filtered to remove external DNA that could work as a template for the DNA process, or bought from a manufacturer that can guarantee that the products are DNA-free.

Jordan was also the first to publish an article describing the use of a PCR method without preculturing. The study used discarded whole blood samples originally collected for complete blood count, and found a 94% overall agreement between PCR and culture[161]. This study was followed by eight other studies reporting sensitivities ranging from 42% to 100% and specificities from 88% to 99%[6,159,160,162-165](Table 2).

Study	*n Blood culture positive*	*n Blood culture negative*	*Collected Consecutive ly*	*Sensitivity*	*Specificity*	*Patients*	*Method*	*Specific probes*
Laforgia 1997	4	29	Yes	100%	93%	NICU Italy Early onset	PCR 861 bp agarose gel	Not applicable
Jordan 2005	53	32	No	96%	100%	NICU Pittsburgh?	Real-time PCR 380 bp	No
Yadav 2005	9	91	?	69%	100%	Level II unit India?	PCR, 380 bp agarose gel	Not applicable
Ohlin 2008	50	245	Yes	42%	95%	Level III unit Sweden	Real-time PCR Plasma samples380 bp	1 universal and 4 specific probes
Wu 2008	34	566	?	100%	97%	NICU China	Real-time PCR	Gram stain specific probes
Reier-Nilsen 2009	6	42	Yes	67%	88%	>1000g, first week Norway	3 PCR reactions 1500, 1100 and 500 bp	Not applicable
Dutta 2009	52	190	Yes	96%	96%	Level III NICU India	PCR, 380 bp agarose gel	Not applicable
Chan 2009	42	176	Yes	79%	97%	Preterms, >72 h old NICU Hong Kong	Real-time PCR 228 bp	Gram stain specific probes
Elwan 2009	39	34	Yes	67%	79%	NICU Cairo	PCR 861 bp	Not applicable
Ohlin 2010 (submitted)	56	312	Yes	79%	90%	Two level III units Sweden	Real-time PCR 380 bp	1 universal and 6 specific probes

Table 2 Studies describing PCR methods for detection of neonatal sepsis without preculturing.

Several of these studies also benefited from the advantages of real-time PCR, by adding specific probes to the protocol. These probes are designed to be selective and only detect a defined subgroup of bacteria such as a specific species or a specific gram staining entity. If such probes can be used effectively, the PCR will not only detect the bacteria but also give detailed information about which bacteria the patient is infected with and thus indicate which treatment might be suitable. Although no evaluations have yet been performed, there has been some speculation on the possibility of creating specific probes designed to detect genes encoding for different pathogenetic factors such as antibiotic resistance[166-168].

Furthermore PCR has the advantage that it can (at least in theory), detect non-viable bacteria, which would be a great advantage in cases where cultures are collected after initiation of antibiotic therapy or in cases of suspected early onset sepsis where the mother was treated with intra-partum antibiotics. This theory was tested as a secondary outcome by Dutta et al. and described in a case report from an adult patient by Sakka et al.[159,169]. Dutta et al. unfortunately used a protocol requiring pre-culturing; this was probably the reason why they only managed to detect bacterial DNA in 12% of the samples 12 hours after antibiotic therapy.

Staphylococcus epidermidis

Staphylococci are a type of coccus (round-shaped bacteria) that congregate together to form clusters resembling clusters of grapes (Fig 5); the name comes from "staphylos", the Greek word for "grape". They are facultative anaerobic gram-positive cocci of the family Micrococcaceae, measuring 0.5 -1.5 µm in diameter. Staphylococci are divided into two groups, distinguished by their phenotypic ability to produce the enzyme coagulase, which causes clotting of blood plasma. The main coagulase-positive staphylococcus species is *Staphylococcus aureus*. More than 30 different coagulase-negative species have been described, but only half of them are found in humans[170]; the most common human-infective species by far is *S. epidermidis*[46-52]. As the name implies, *S. epidermidis* often colonises the human skin; however, it can also be found on mucous membranes and in the faecal microflora of newborn infants[46,48,171,172].

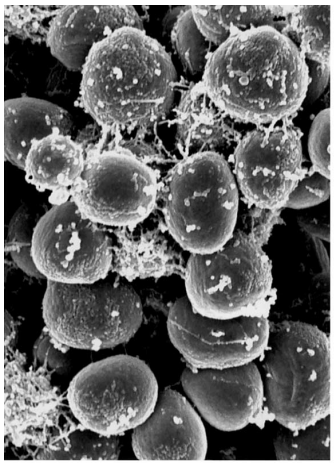

Fig 5. Electron microscopy picture of S. epidermidis[1] *from Vuong ,J INFECTIOUS DISEASES. 188:5 (2003) with permission from University of Chicago Press*

Bacterial typing

In most individual cases of sepsis, detection and identification of the pathogen along with information on antibiotic resistance is sufficient to optimise the treatment and make a prognosis of the course of the disease. However, when there is an outbreak with several cases of late onset sepsis in one unit, or when one patient is plagued by repeated episodes of sepsis, there can be a need for typing of the causative isolates beyond the species level, in order to map routes of infection, discover reservoirs, or track down the origin of the infectious organism. Since *S. epidermidis* is the most common cause of late onset neonatal sepsis in the developed world, and most *S. epidermidis* isolates are multiresistant to antibiotics, the need for typing methods for these microorganisms is particularly imperative[173,174]. When developing or evaluating a new typing method, the goal is to find a stable technique with a high degree of reproducibility even over long periods of time. The optimal typing technique should also have a high discriminatory power, low cost, and a short turnaround time; and if possible should not have complicated technical requirements. The output data should be easy to store and share, so that databases can be constructed and comparisons can be made between laboratories and researchers.

Typing systems are classically divided into phenotypic typing systems, which divide bacteria according to their phenotypic characteristics such as resistance to antibiotics or biotyping of biochemical characters, and genomic typing systems, which are based on detection of the differences in the bacterial genome. Phenotype-based typing methods for CoNS have

only limited discriminatory power, making them unsuitable for scientific studies[175,176]. The current gold standard for typing of CoNS and *S. epidermidis* is pulsed field gel electrophoresis (PFGE)[177,178], but recently some authors have referred to multi locus sequence typing (MLST) as the gold standard[179-181]. Both these methods are time consuming and expensive which is a problem since epidemiological studies of *S .epidermidis* tend to involve large numbers of isolates due to the fact that each included human subject is normally colonised with several different isolates. There is therefore an ongoing search for a faster and cheaper typing tool for *S. epidermidis*[182,183]. The increasing numbers of *S. epidermidis* infections in neonates together with the spreading of glycopeptide resistant strains is making this search even more important[174,184].

Virulence factors and cell wall anchored proteins

S. epidermidis normally does not infect the immunocompetent host, but those with a compromised immune defence such as premature infants or patients on cytostatic drugs are at risk. Many of these infections are linked to intravascular catheters or other foreign materials. The unique ability of *S. epidermidis* to attach to different materials and form a protective biofilm layer via quorum sensing is one major reason why it is the most common finding in catheter-related bloodstream infections[1,185]. Several virulence factors have been described and proposed to be of importance for *S. epidermidis*, and it is known that endemic clusters demonstrating biofilm production and other pathogenetic factors can be found in NICUs[52,186-191], but until today no single virulence factor that can discriminate between invasive and non-invasive has been identified. To facilitate further research on virulence factors and invasive routes, studies including large numbers of isolates, typed in detail, needs to be performed.

Recently there has been an increasing interest in the surface proteins of *S. epidermidis,* and 11 such proteins have been described to date[192]. These cell wall anchored proteins (CWA) often interact with different targets in the host; for example, the earliest-described CWAs in *S. epidermidis* have been shown to bind to fibrinogen, fibronectin, collagen, and elastin[193]. There has also been some speculation regarding whether some of these CWAs could be important for biofilm production, which is a well-known pathogenic factor in *S. epidermidis*. However, some of the newly described CWAs still have unknown functions, even though it has been shown that they are expressed both in vivo and in vitro[186,192].

AIMS OF THE THESIS

1. To identify the clinical signs at presentation which are most predictive of neonatal sepsis and to investigate whether the signs varies with gestational age.

2. To develop and evaluate a new and fast PCR assay that can detect bacterial DNA in blood samples from neonates with suspected sepsis.

3. To develop and evaluate a new feasible PCR-based typing method for *S. epidermidis* that can be applied to large numbers of isolates with a limited workload.

MATERIAL AND METHODS

Design

Study I included 401 neonates with suspected sepsis. Nine signs of sepsis and C-reactive protein were prospectively recorded, and logistic regression was used to investigate associations between these signs and subsequently confirmed diagnoses of sepsis.

Study II is a prospective study that during 1999-2005 included 295 plasma samples collected from neonates with suspected sepsis to evaluate a novel real-time PCR method, with the results being compared with simultaneously collected blood cultures.

Study III is a prospective study that evaluated a new and improved version of the PCR protocol in study II. Study III included 368 blood samples and cultures collected at two NICUs from October 2007 to November 2009.

Study IV evaluated a new method of typing *S.epidermidis* based on PCR of surface protein genes. The method was applied to 49 well-defined neonatal blood isolates of *S. epidermidis*.

Patients

In studies I and II, all patients were recruited from the neonatal intensive care unit at Örebro University Hospital (USÖ), Örebro, Sweden. This is a level 3 unit offering neonatal intensive care to the inhabitants of the county of Örebro. The unit mainly admits patients from the in-house delivery unit, but also handles transfers from the level 1 unit at Karlskoga Hospital, which is located 40 km west of Örebro. There are approximately 3500 deliveries in the county per year, and the unit admits approximately 450 patients per year. A limited number of patients from other neighbouring counties are also transferred to the unit both in and ex utero, mainly because of prematurity and lack of available NICU cots at the transferring unit. USÖ does not provide neonatal surgery, and hence all patients in need of surgery are transferred to Drottning Silvias Hospital (Gothenburg) for thoracic surgery or Uppsala University Hospital for general surgery and neurosurgery. The unit consists of five cots for intensive care and twelve cots for medium intensive care. The service is provided in two large intensive care rooms and three single rooms where family care can be provided. During the study period developmental supporting care was available throughout, as was an increasing frequency of kangaroo mother care.

In study III, patients were also recruited from the NICU at Uppsala University Hospital. This unit has a capacity of ten intensive care patients located in three large intensive care rooms, and ten medium intensive patients, mainly in single rooms where family-centred care is provided. Uppsala offers level 3 care to inhabitants of the county of Uppsala as well as referrals from six other counties in mid and north Sweden, which are transferred mainly because of premature birth or need of neonatal surgery. To enable this, the unit has a 24-hour on-call air transport team. The Uppsala NICU has approximately 700 admissions per year, and aims to offer 24-hour kangaroo mother care to as many patients as possible.

Studies I and II included infants under 28 days of age in which clinical signs of infection led to blood culture and sepsis treatment with intravenous antibiotics. Study III included all patients under 3 months of age that were subjected to blood culture. In study I, the infants were only eligible for inclusion once, while in studies II and III infants were eligible to re-enter if they had multiple episodes of suspected sepsis.

Blood cultures

All blood cultures were collected by venepuncture or from a newly inserted central catheter. Before venepuncture, the skin was disinfected with an ethanol-based chlorhexidine solution (5mg/ml). Nurses were instructed to first collect 1.0 ml for blood culture and then to collect the study sample. Blood collected in Örebro was immediately inserted into paediatric BACTEC® blood culture bottles (Becton Dickinson, MD, USA) and sent to Örebro University Hospital, where a non-radiometric BACTEC® 9240 system was used to detect bacteria and fungi.
Study III also included blood cultures from Uppsala University Hospital; these were collected by the same procedure as described above, but analysed at the clinical microbiology laboratory in Uppsala. Samples were injected into Pedi-Bact® blood culture bottles (Biomerieux, Marcy l'Etoile, France), and a BacT ALERT® non-radiometric system was used to detect bacteria and fungi. All detected bacteria were identified at the respective laboratory by routine accredited laboratory methods. Growth of CoNS was considered relevant irrespective of the time point when the growth was confirmed.

EDTA samples

The EDTA samples in studies II and III were collected as described above, with 0.5-1.0 ml of whole blood being inserted into 3 ml EDTA Vacutainer® tubes (Becton Dickinson and Greiner Bio-One). Smaller volumes were accepted if this volume could not be obtained. Samples were stored at +4° C pending transport to the laboratory, and transported at room temperature. In study II plasma was used, and hence the samples were centrifuged at 150 x g for 10 min and the supernatants (0.05-0.2 ml) were transferred to 1.5 ml screw cap tubes (Sarstedt GmbH, D-51588, Numbrecht, Germany) and then stored at -70° C until DNA preparation. In study III, the samples were transferred to 1.5 ml screw cap tubes (Sarstedt GmbH, D-51588, Numbrecht, Germany) and stored at -80° C until DNA preparation.

DNA preparation

In study II, DNA was prepared with the QIAamp DNA Mini Kit 02/2003 (QIAGEN GmbH, D-40724, Hilden, Germany). The eluating buffer was filtered with a 0.2 µm filter. The protocol for isolation of bacterial DNA from biological fluids was used.

In study III, we modified the DNA preparation protocol slightly to allow the use of mutanolysin to facilitate degradation of the cellular walls of gram-positive bacteria[161,164]. The samples were frozen at -80°C before preparation and then centrifuged at 14000 g for 5 min; 200 µl of the sample was kept and the pellet dissolved in 10 µl (100 units) mutanolysin (Sigma-Aldrich, Schnelldorf, Germany). After incubation at 37° C, 20 µl ProtK and 200 µl AL buffer were added and incubated at 56° C. Next, 200 µl 99.5% ethanol was added and the lysate was loaded on the Qiagen column and centrifuged and washed as previously described. The filter was then eluated twice with 180 µl filtered AE buffer, and the eluate stored at +4° C. The ethanol and the eluating and washing buffers were all filtered with a 0.2 µm filter.

In study IV we again used a QIAamp DNA Mini Kit to isolate DNA from the strains, but with the following modifications. Bacteria obtained from the blood agar plate were dissolved in sterile saline and pelleted, dissolved in 180 µL ATL buffer (QIAGEN) with 10 µL mutanolysin 10 U/µL (Sigma, St. Louis, MO, USA) added, and incubated at 37° C for 30 min. The mix was treated with proteinase K and AL buffer (QIAGEN) at 56° C for 30 min. DNA-purified according to the tissue protocol using ethanol and wash buffers supplied in the kit, and finally eluted in 50 µL AE buffer (QIAGEN) and stored at 4° C.

C-reactive protein values

CRP was measured in all infants in study I at inclusion and once daily for three consecutive days by a non-competitive immunochemical method for routine clinical use (Vitros^{R} 950, Jonson and Jonson, USA) at the clinical chemical laboratory at Örebro University hospital. The method had a lower detection limit of 7 mg/l and a turnaround time of approximately 1 hour.

Clinical signs

Nine signs known to be associated with sepsis[18,130,194] were prospectively collected on standardised study forms together with medical history and laboratory values, by the NICU nurse responsible for the care of the baby. Since scoring of clinical signs can be somewhat subjective, we only collected data at one unit (USÖ), to ensure that high-quality data collection was possible. This unit has a stable staff of doctors, nurses, and nurses' assistants; the nurses were particularly crucial to this study since they performed the 24 h observations of the infants and filled out the forms. Of the nurses at this NICU, 95-100% have an academic degree in paediatric nursing, and no nurse with less than 5 years of experience in neonatology handles the infants in intensive care. To further increase data quality and ensure that no signs were missed, all medical records of the infants were later re-examined by the authors.

The nine clinical signs were defined as follows:

- Feeding intolerance – difficulty in accepting enteral feedings presenting as repeated vomiting or increased gastric retention, leading to a decrease in the frequency of enteral feeds.
- Tachypnoea – breathing rate > 60 breaths/min, persisting for > 60 minutes and not responding to standard nursing procedures such as altered incubator temperature and intensified analgesic therapy.
- Apnoea – repeated episodes of apnoea (lack of breathing movements > 20 s) leading to active intervention.
- Bradycardia – heart rate < 100 heartbeats/min registered over a minimum of 20 seconds at least 3 times within a 3 hour period leading to active intervention.
- Increased oxygen need – any increase in the amount of oxygen needed to obtain an oxygen saturation of 88-92% persisting > 60 minutes. Rapid fluctuation in oxygen need around a stable baseline was not considered as increased oxygen need.

- Irritability or seizures – clinical or EEG-verified seizures or irritability not judged to be caused by pain.
- Patent ductus arteriosus – presence of a clinically-relevant ductus arteriosus with a left to right shunt, verified by echocardiogram and clinical judgement.
- Distended abdomen – abdomen distension defined as a visually obvious abdomen distension with or without glossy skin and with or without visual bowel movements or increased abdominal circumference.
- Hypotension or impaired peripheral circulation – a mean arterial pressure in mmHg < gestational age in weeks or a significant decrease in the patient's blood pressure (defined as a decrease reported to the physician that could result in alterations to therapy) or use of inotropic drugs or pallor.

Polymerase chain reaction

PCR was used in studies II-IV. In studies II and III, a short turnaround time was very important and hence real-time PCR was used. Study IV was focused on typing of isolates and hence turnaround time did not matter; here conventional PCR was chosen instead. All real-time PCR reactions were run on a LightCycler 2.0 (Roche Diagnostica GmbH, 68298 Mannheim, Germany), while for the conventional PCR we used a Mastercycler gradient 5331 (Ependorf; Netheler- Hinz GmbH, 22331 Hamburg, Germany).

In studies II-III the PCR primers (RW01: 5´-ACTGGAGGAAGGTGGGGAT-3´ and DG74: 5´-AGGAGGTGATCCAACCGCA-3´) and the universal bacterial Taqman probe (RDR245: 5´-(6FAM)- TACAAGGCCCGGGAACGTATTCACCG-(TAMRA)-3´) were adapted from Jordan[161].
In study II we used four additional probes that were modified versions of probes described by Greisen[195]:

- the Taqman probe RDR 278: Gram-negative probe 5´-(6FAM) GTAAGGGCCATGAGGACTTGACGT-(TAMRA)-3´.

- the Taqman probe RDR 327: *S. aureus* probe, 5´-(VIC)GCCGGTGGAGTAACCTTTTAGGAGC-(TAMRA)-3´.

- the CoNS I probe: *S. epidermidis*,

5´-(6 FAM)-CGACGGCTAGCTCCAAATGGTTACT-(TAMRA)-3´.

- the CoNS II probe: Staphylococcus probe II, 5´-(VIC)-CGGCTAGCTCCAAAAGGTTACTCTA-(TAMRA)-3´.

In study III, six species-specific probes were used in pairs to limit the number of capillaries necessary to four:

- Capillary 1 – RDR245 probe (general bacterial probe)[161,164]
- Capillary 2 – Gram-negative RDR278 and *Staphylococcus aureus* RDR327 probes[164,195]
- Capillary 3 – CoNS and CoNS II probes[164,195]
- Capillary 4 – *Escherichia coli* RDR140 and Group B *Streptococcus* (GBS) KG0001 probes. The GBS probe was slightly modified by adding an extra C at the 5´-end[195]

All primers in studies II and III were purchased from Scandinavian Gene Synthesis (SGS AB, Box 91, SE-731 22 Köping, Sweden), and probes from Applied Biosystems UK (Warrington, England). All primers and probes were tested for specificity and cross-reactions both manually on reference strains and with the NCBI BLAST tool (www.ncbi.nlm.nih.gov/BLAST).

In study IV, the repeat regions for *aap* and *sdrG* were amplified as previously described by Monk and Archer[196], using Taq PCR Master Mix Kit (QIAGEN, Hilden, Germany) (25 µL/50 µL reaction), 2 µM of each primer (Applied Biosystems, Warrington, UK), and 2 µL DNA preparation. The primers for *sdrF* and *sesE* (Scandinavian Gene Synthesis AB, Köping, Sweden) were designed using the PRIMER3 program and compared with published sequences on BLAST. To facilitate fragment analysis of the products one primer in each pair was purchased labelled with VIC and 6-FAM, respectively.

Gene	Type of repeat*	Primers	Restriction enzyme	Fragment size Range (approx)
aap	B	[196]	-	414–514 bp
sdrG	serine-aspartate	[196]	HpyCH4III	700–1200 bp
sdrF	serine-aspartate	SDRF1 (5´-CTGATGGGGAAGATGTTCGT-3´) SDRF2 (5-´TCATGATCTTCATTTGCTCCTG-3´)	BtsCI	250–1200 bp
sesE	B	SE1L (5´-CATCTTTATCTGTACCGCCTGT-3´) SE1R (5´-ATCCAACTGCCACTGAACCT-3´).	BstYI	800–1200 bp

Table 3. Details of the 4 PCR methods used to study the 49 S. epidermidis isolates in study IV.

**B repeat – repeat region following the A (ligand) region. Serine-aspartate repeat – repeat region consisting of serine and aspartate that is located downstream from the B-region. Nomenclature adapted from Bowden et al[192].*

The *sdrF*, *sdrG*, and *sesE* amplicons (6 µL) were treated with restriction enzymes for 2 hours. *SdrG* was treated with HpyCH4III (New England Biolabs Inc. (NEB), Ipswich, MA, USA) 5 U at 37°C, *sdrF* with BtsCI (NEB) 10 U at 50°C, and *sesE* with BstYI (NEB) 5 U at 60°C; *aap* was analysed without any enzyme treatment. Enzymes were selected using the NEB cutter computer program.

The PCR products and restriction fragments were separated and visualised on a 1.5% agarose gel (Agarose 1000, 1 × TBE, 100 V, 60 min) stained with ethidium bromide. The stained bands were compared to a size marker on each gel (100 bp DNA ladder, Gibco BRL, Carlsbad, CA, USA).

The product sizes of all *aap* PCR products and the enzyme-treated fragments from *sdrF*, *sdrG*, and *sesE* that yielded similar visual results were confirmed by fragment analysis, using ABI 310 or ABI 3130xl, and compared to LIZ-1200 (GeneScan 1200 LIZ, ABI, Foster City, CA, USA) size standard in Peakscanner 1.0/GeneMapper 4.0 (ABI).

Pulsed field gel electrophoresis

PFGE was used as the gold standard, for all isolates included in study IV, using data from an earlier study[186]. PFGE is a gel-based typing method where digested chromosomal DNA fragments are separated in an agarose gel by applying electrical fields in various directions. We used the *Sma1* restriction enzyme, which is the most commonly used enzyme for CoNS and has a well documented discriminatory power for *S. epidermidis*[50,187,188,197], and prepared the isolates with the GenePath Group 1 Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

The data in study I data were analysed with logistic regression using SPSS 14.0 for Windows. The blood culture result was used as the dependent variable in logistic regression, the association with each of the clinical signs was evaluated separately and the adjusted model simultaneously included all measures. All of the independent measures were modelled as binary dummy variables. Logistic regression analysis of CRP at inclusion and maximum CRP revealed that CRP at inclusion was more closely associated with positive blood culture than was CRP maximum; for this reason, CRP at inclusion was used in all calculations.

Stratified analysis was used to investigate whether prematurity (< 37 weeks vs. ≥ 37 weeks gestational age), modified the association of positive blood culture with the included signs. Effect modification by gestational age was further assessed using interaction testing. Interaction terms for gestational age with each relevant sign were generated and their association with a positive blood culture assessed with adjustment for the main effects (gestational age, sex, and the relevant sign) using logistic regression[198].

In studies II and III confidence intervals for sensitivity, specificity, positive and negative predictive value were calculated using biconf.exe a stand-alone MS-DOS program constructed by Martin Bland which calculates exact confidence intervals for binomial proportions.

In study IV, we calculated a discrimination index (D-index) to evaluate the discriminatory capacity of different PCR methods[199]. Briefly, the D-index represents the likelihood that two randomly picked strains from the sample will be separated by the method and recognised as two different strains. This means that a method with a high D-index (close to one) divides the sample into many small groups, whereas a low D-index (close to zero) indicates that the sample is only divided into a few large groups. In addition 95% CI of were calculated as described by Grundmann et al.[200].

Ethics

The local ethics committee approved studies I-III, and all parents of the included children gave their informed consent. Since study IV only included bacterial isolates and no human or animal subjects, no ethical approval from the local ethic committee was sought.

RESULTS

Clinical signs (I)

Study I included 401 neonates of whom 68% were included during the first 48h of life (early onset) and 32% were included between 2-28 days of age (late onset), no infants with late late onset sepsis was included. Blood cultures were positive in samples from 83 infants and the most common bacterial findings were coagulase negative staphylococci, *Staphylococcus aureus* and Group B Streptococci.

Signs associated with sepsis

Five of the nine clinical signs (bradycardia, apnoea, low blood pressure, feeding intolerance, and distended abdomen) were statistically significantly associated with a positive blood culture and the associations remained after adjusting for sex and gestational age. After additional adjustment for CRP at inclusion and the other signs, only apnoea and low blood pressure remained as independent predictors for a positive blood culture. Female sex was independently associated with a positive blood culture. CRP > 10 mg/l at inclusion was strongly and independently associated with a positive blood culture.

Signs not associated with sepsis

In the unadjusted analysis, four of the nine symptoms (increased oxygen need, patent ductus arteriosus, irritability/seizures and tachypnoea) showed no statistically significant association with positive blood culture. However, in the model adjusted for sex, gestational age, CRP at inclusion, and the other signs, the association with tachypnoea reached statistical significance.

The effect of gestational age

Stratification by gestational age with a cut-off at 37 weeks showed that tachypnoea was negatively associated with a positive blood culture in preterm infants but positively associated with a positive blood culture in full-term infants To verify that this was statistically significant, we performed an interaction test which produced a statistically significant odds ratio for interaction of 0.24 (95% CI: 0.080-0.74: p=0.013). Bradycardia was associated with a positive blood culture in preterm but not in full-term infants producing an odds ratio for the interaction test of 5.21 (95% CI: 0.95-28.4: p=0.056). For irritability/seizures we identified a more complicated relationship with gestational age and found evidence of

effect modification. When the preterm infants were divided between 32-36 weeks (preterm) and 24-31 weeks (extremely preterm) irritability/seizures was only notably associated with sepsis among those in the preterm group, with an OR of 15.9 (95% CI: 1.33-190.8). Although not statistically significant, an interaction test provided further evidence of effect modification by this division of gestational age (preterm compared with full-term), with an OR for interaction of 3.71 (95% CI: 0.84-16.4; p=0.08). No differences between preterm and full-term infants were detected for CRP, and sex or any of the other signs,

Real-time PCR of plasma samples (II)

Study II included 295 blood cultures originating from 288 infants, 50 were positive with regard to bacterial findings. The correlation between PCR and blood culture is presented in table 4 showing a sensitivity of 42% (28%-57%) a specificity of 95% (92%-97%) a positive predictive value of 64% (45%-80%), and a negative predictive value of 89% (84%-92%): 95% confidence intervals are given in brackets.

Study II		**Culture**		
		Positive	Negative	Total
PCR	Positive	21	12	33
	Negative	29	233	262
	Total	50	245	295

Table 4. Comparison of results from blood culture and real-time PCR in neonatal plasma samples 1999-2005.

Of the 21 samples positive in both blood culture and PCR, one PCR sequence was not interpretable due to double sequence, leaving 20 samples available for comparison. The sequenced PCR product was in accordance with the culture findings in 17 cases. The specific probes were correct in 16

of 21 cases when probe results were compared with culture findings, and correct in 19 of 20 when compared with sequence data.

Real-time PCR of plasma samples (III)

In study III 368 samples from 317 newborns (207 boys, 110 girls) were included during the study period. Blood culture was positive in 56 of the 368 samples analysed, and the bacterial spectrum was similar to that in study II with CoNS as the most common finding. The PCR results were compared with the simultaneously drawn blood cultures to enable calculations of sensitivity and specificity (including 95% confidence intervals) with blood culture considered as the gold standard (Table 5). The sensitivity was 79% (66%-88%), the specificity was 90% (86%-93%), the positive predictive value was 59% (47%-70%), and the negative predictive value was 96% (93%-98%).

Study III		**Culture**		
		Positive	Negative	Total
PCR	Positive	44	31	75
	Negative	12	281	293
	Total	56	312	368

Table 5. Comparison of results from blood culture and real-time PCR in neonatal blood samples 2007-2009.

PCR detected 29/35 (83%) of the samples positive for CoNS, 3/3 of the samples with *Enterobacter* species, 5/7 (71%) of the samples with multiple isolates, 2/2 of the samples with *E. coli*, 2/2 of the samples with *S. aureus*, 2/2 of the samples with *Pseudomonas aerginosa*, 1/2 (50%) of the samples with GBS, 0/1 (0%) of the samples with *Enterococcus faecalis*, and 0/2 (0%) of the samples with *Micrococcus* species.

Comparison between the specific probes and blood culture

When applied to the 368 samples, the general (RDR245) probe was positive in 19 samples, 10 of which had a positive blood culture (8 CoNS, 1 *Enterobacter* species, and 1 *Pseudomonas aerginosa*). All of these 10 samples were simultaneously positive in one or more other probes.

The CoNS I probe was positive in 56 samples, 32 of which had a corresponding blood culture positive for CoNS. The CoNS II probe was positive in four samples, all showing CoNS in the corresponding blood culture. Three out of four samples positive for CoNS II were simultaneously positive for the CoNS I probe. The *S. aureus* probe was positive in four samples, all of which were positive for *Staphylococci* (3 *S. aureus* and 1 CoNS) in blood culture. The GBS probe was positive in one sample with GBS in the corresponding blood culture, but also failed to detect one GBS-positive specimen. The *E. coli* probe was positive in six samples, two of which had negative cultures and the remaining four of which were positive for *E. coli* or *Enterobacteriaceae*.

The correlation was weaker for the gram-negative probe, which was positive in 14 samples of which 3 had a blood culture positive for gram-negative pathogens, 3 had growth of gram-positive bacteria, and 8 were negative. In eight of the eleven false-positive samples, probe sequencing indicated that contaminating *Pseudomonas* DNA was the cause.

Sequencing of PCR amplicons

All PCR-positive samples were sequenced to verify the bacterial origin of the amplicons. In the samples that were positive in both PCR and blood culture, the sequence results were identical with the blood culture result at the species level in 36 out of the 44 samples. In the 8 samples with conflicting results, the DNA sequences matched either *Pseudomonas* DNA or uncultured *Marinobacterium*.

The sequences from the 31 samples positive in PCR but negative in blood cultures showed that the DNA originated from *Pseudomonas* or *Marinobacterium* in 12 samples, *Staphyloccus* in 7 samples, *E. coli/Enterobacter* in 6 samples, and uncultured bacteria in the remaining 6 samples. The high number of samples positive for *Pseudomonas* DNA could be traced to the DNA extraction kit, which was positive for *Pseudomonas mendocina*.

Case studies of samples with conflicting results

Seven of the infants with a positive PCR and negative blood culture had definite or suspected sepsis. Five of these cases had ongoing sepsis diagnosed with a positive blood culture 31-120 h prior to inclusion, and three of them had ongoing antibiotic treatment when they were re-cultured and included in the study

Two cases with positive PCR and negative blood culture had clinical signs indicating a suspected sepsis; one of these had antibiotics before inclusion. Both received antibiotics (for 9 and 15 days respectively) and had PCR findings consistent with the clinical picture. The remaining 24 samples with positive PCR and negative blood culture were judged to be contaminated.

In twelve cases, the PCR was negative and the blood culture positive (Table 4). Five of these originated from patients with definitive bacterial sepsis (with CoNS, GBS, and *Enterococcus faecalis*), but in six cases the positive blood culture was judged to originate from skin contamination. One infant had *Enterobacter cloacae* sepsis, but the CoNS (1/2 bottles) detected in the blood culture drawn at the same time as the study sample was probably a contaminant.

Typing of *S. epidermidis* (IV)

The *sdrG, sdrF*, and *sesE* repeats were present in all 49 isolates, whereas *aap* was detected in only 38/49 isolates.

sdrG

After treatment with HpyCH4III restriction enzyme, the *sdrG* samples showed 2–4 distinct bands of a size ranging from 100 to 300 base pairs (bp), representing 13 different genotypes

sdrF

After treatment with BtsCI restriction enzyme, the *sdrF* samples showed 1–3 clear distinct bands in the size range of 250–1,000 bp, representing 17 different genotypes.

sesE

After treatment with BstYI restriction enzyme, the *sesE* samples showed 1–5 distinct bands in the size range of 100–700 bp, representing 8 different genotypes (Table 2).

aap

Since the *aap* repeat was found in 38/49 isolates, one genotype consisted of 11 isolates without *aap*. The remaining 38 isolates were analysed with fragment analysis showing a size range of 414–514 bp and 7 genotypes.

PFGE

Six isolates were indistinguishable by PFGE, and 17 were classified as single isolates. The remaining isolates were assigned to various genotypes with 2 or 3 isolates.

Discriminatory index

All the calculated D-indices are presented in table 6. The highest D-index for a single gene was found for *sdrF* (0.82), followed by *aap*, *sdrG*, and *sesE*, respectively. When combining two genes, the highest D-index was found in *aap/sdrF* (0.92) followed by *aap/sdrG*. When a third gene was added, the D-index was only marginally enhanced (*aap/sdrG/sdrF,* D-index 0.94). The corresponding figure for PFGE was 0.98.

Gene	Number of genotypes	No. of isolates with most common genotype (%)	D-index	95% confidence interval
aap	8	13 (27)	0.81	0.77–0.86
sdrG	13	25 (51)	0.72	0.59–0.85
sdrF	17	19 (39)	0.82	0.73–0.92
sesE	8	24 (49)	0.70	0.59–0.80
aap sdrG	18	13 (27)	0.89	0.84–0.94
aap sdrF	21	11 (22)	0.92	0.87–0.96
aap sesE	16	11 (22)	0.88	0.83–0.93
sdrG sdrF	20	15 (31)	0.88	0.81–0.95
sdrG sesE	17	21 (43)	0.80	0.69–0.91
sdrF sesE	22	15 (31)	0.88	0.81–0.95
aap sdrG sdrF	23	8 (16)	0.94	0.91–0.97
aap sdrG sesE	21	11 (22)	0.91	0.87–0.96
aap sdrF sesE	24	11 (22)	0.92	0.88–0.97
sdrG sdrF sesE	22	12 (24)	0.91	0.86–0.96
PFGE	34	6 (12)	0.98	0.96–0.99

Table 6. Number of genotypes and discrimination index (D-index) for the repeats and combinations of repeats in the S. epidermidis neonatal blood isolates (n = 49).

DISCUSSION

Neonatal sepsis is a dangerous condition that affects between 0.1-1% of, newborns but among extremely preterm infants incidence rates as high as 30-40% have been reported. Neonatal sepsis causes increased mortality and morbidity[17,18,56], with consequences such as poor neurological outcome, bronchopulmonary dysplasia and necrotizing enterocolitis, leading to prolonged hospital stays and increased costs[20,21].

Diagnosis of neonatal sepsis is based on bacteraemia demonstrated by a positive blood culture, a method with well-known limitations in turnaround time, sensitivity, and specificity. There is no widely accepted definition for neonatal sepsis, but most definitions demand bacteraemia together with clinical signs of sepsis or increased inflammatory parameters[2]. Since blood culture has a poor sensitivity, sepsis treatment is often administered to patients with a clinical picture of sepsis but negative blood cultures; this condition is normally called clinical or suspected sepsis. The standard treatment for neonatal sepsis is intravenous broad spectrum antibiotics together with supportive intensive care. If neonatal sepsis was easier to diagnose, fewer infants would receive antibiotic treatment and the overall antibiotic consumption in neonatal intensive care could diminish.

The basis of this thesis was the ambition to develop a faster method to detect bacteraemia and sepsis in the newborn. With this ambition we initiated the first PCR study. The results from this study were suboptimal, and so we conducted a follow-up study with an optimised sample collection and an improved PCR protocol to gain some indication whether PCR could become a routine method to detect bacteraemia. During the course of these two studies we expanded our ambition to making a scientific contribution to the whole diagnostic process from the start of sepsis symptoms, through the detection of the bacterium, and finally to a detailed typing of the pathogen that enables tracking of the isolate and hopefully discovering the origin of the infection.

To limit the scope of this thesis, we chose a funnel-like design. At the top of the funnel the symptoms of all infants with suspected sepsis were studied (I). The focus then shifted toward those infants with a positive culture in study II and III, since the problem was to achieve a PCR method that could detect all culture-positive cases. Finally at the tip of the funnel (IV), the focus narrowed further to cultures positive for *S. epidermidis*, which is by far the most common pathogen in modern NICUs[8,12,19,43].

Clinical signs of sepsis are the fundamentals of all sepsis work-ups, and will continue to be so until a better "rule-out sepsis" test is developed. Such a test would need to be so sensitive that a negative result could support withdrawal of antibiotic therapy no matter what symptoms the patient was displaying. With this knowledge in mind, it is provocative that there are thousands of articles on biomarkers for sepsis but only a handful on clinical signs[67], even though clinical signs are the method used by neonatologists when deciding whether to administer antibiotics. It becomes even more provocative when one considers that the infants in NICUs in the developed world are becoming more and more premature[12,40], meaning that the historic references on neonatal signs are becoming obsolete and less adequate.

Against this background, the first study included in this thesis is of great interest. We studied nine clinical signs and one laboratory test for sepsis in a large population evaluated for suspected neonatal septicaemia. The study was performed on a well-selected population in which all included infants had suspected sepsis, and the results can only be used to define predictors of a positive culture in this specific group; the associations differ from those that would be found in healthy infants, or even in a population of patients for whom sepsis was not strongly suspected. This is an obvious strength of this study, since this is the population that neonatologists constantly struggle with, in their aim to use antibiotics wisely and selectively, but it must not be forgotten that the results only concern this population, and should not be generalised to support decisions on all infants.

Since this study focuses on clinical signs, we could not use clinical signs to separate sepsis cases from contaminated blood cultures. We were therefore forced to include all positive blood cultures, keeping in mind that the material was diluted with an unknown number of false positive blood cultures. This dilution probably resulted in weaker associations than would have been the case if only definite sepsis cases were included. The results showed that evaluated CRP is associated with bacteraemia, which is not surprising. Several previous studies have already shown that CRP is a sensitive marker of inflammation, but it responds with a lag time of 24-48 h. Repeated measurement of CRP has therefore been suggested as a method to rule out sepsis, rather than a method of detection[90,92,201].

The associations between the five common neonatal signs and positive blood culture are more interesting. Two of these signs (apnoea and hypotension) were associated with a positive blood culture independent of gestational age and each other, and three signs (feeding intolerance, distended abdomen, and bradycardia) were not independent from the other signs, often appearing together with them. This implies that great care should be taken before antibiotics are discontinued in a patient with bradycardia, apnoea, low blood pressure feeding intolerance or distended abdomen. This is not a controversial result, but it gives scientific support to widely-adopted treatment strategies that have until now been based more on tradition than science. Unfortunately, our study was too small and limited to give guidance on the four signs that were not associated with a positive blood culture. One or more of these signs might not actually be signs of sepsis, and patients with these signs might receive unnecessary antibiotics and sepsis treatment. The question of whether these speculations are true or not cannot be answered without conducting a larger study on sepsis signs.

Study I also suggests that full-term and preterm infants display clinical signs differently, which is a valuable result since there are very few previous studies on this subject[65]. The results imply that bradycardia is a sign for sepsis in preterm but not in full-term infants, which is very interesting since the ongoing randomised trial on heart rate analysis includes only premature infants[135]. Given the results of study I, the findings of this randomised trial should not be extrapolated to all infants. Furthermore, tachypnoea seemed to be a weak sign for sepsis in full-term infants, but was clearly not a useful sign among preterm infants. The explanation for this is unknown. We have speculated that preterm infants might not have the energy to display tachypnoea when affected by sepsis; instead, they lose their physiological control and both heart rate and respiration rate drop. This is further supported by the strong association between sepsis and apnoea (OR 7.1; 95% CI 3.4-14.7) in preterm infants. However, we cannot exclude the possibility that these differences are explained by the fact that preterm and full-term infants are infected with different pathogens, and hence that the differences originate from pathogen factors rather than host factors.

All the patients in this thesis were included between 1997 and 2009. This is a fairly limited time span, and so the results are still adequate for the population of newborns that are admitted to NICUs today. However it remains the case that both infants and pathogens vary over time, and so

studies on factors such as clinical signs must be repeated at regular time intervals.

The problem of contaminated samples is an important issue in sepsis research. Studies II and III were designed to detect bacteraemia, but whether or not this bacteraemia represents sepsis can only be determined after evaluation of other laboratory tests such as CRP and a clinical judgement of the patients condition. When a sample is collected for bacterial detection (blood culture or PCR), the intention is to detect a pathogen that originates from the patient´s presumed bacteraemia, but there is always a risk that the sample may be contaminated by another bacterium originating from the patient´s skin or the environment around the patient (sample contamination). Sample contamination can affect blood culture as well as PCR (Fig 6). When the sample is subsequently processed in the laboratory, there is a risk of additional contamination (laboratory contamination). The risk of laboratory contamination is far greater for PCR methods, since these samples are subjected to a more complicated laboratory process. In addition, PCR has the ability to detect dead bacteria or even just fragments of bacterial DNA that can act as templates for the PCR process.

Bacteraemia + sample contamination + laboratory contamination = All positive samples

Fig 6. The equation of positive PCR samples.

Hence when conducting PCR studies it is not enough to use sterile laboratory equipment and solutions; they must either be DNA-free upon purchase or be thoroughly filtered to remove all traces of DNA[202-205]. This problem becomes extra evident in broad range PCRs that can detect many different species, including environmental bacteria that never act as pathogens in humans.

Another important issue when conducting research on sepsis in the newborn is that there is no perfect gold standard test to compare the new results with. When calculating sensitivity and specificity the new results will be compared with blood culture; a method with widely-known limitations in both sensitivity and specificity. With this design, a new

method can never perform better than blood culture, and can never prove that the blood culture was incorrect. To circumvent this problem, we chose in studies II and III, to present the cases with conflicting results as case reports, to allow the readers to judge for themselves on a case to case basis which method is the most accurate.

In study II we presented twelve cases with positive PCR and negative blood culture. It was obvious that in all twelve the blood culture was accurately negative and the PCR was falsely positive. In study III, which used a more developed PCR method, the case reports were more interesting. In five cases, the PCR seems to have detected a pathogen that blood culture failed to detect. Furthermore, we presented twelve cases that were negative in PCR and positive in blood culture, and in at least six of these cases the blood cultures were obviously contaminated and the PCR was correctly negative. We believe that this is the first article that actually demonstrates that PCR has the potential to outperform blood culture, or at least to add clinically important information when used in conjunction with blood culture.

The decision to collect plasma and not whole blood samples for the first PCR study was made in an attempt to obtain specimens with minimum amounts of human DNA. This decision was based on the knowledge available at the time but it proved to be a mistake that resulted in the poor sensitivity of 42% in study II.

To improve the sensitivity, we decided to conduct study III where we collected whole blood, added mutanolysin and included additional specific probes. Mutanolysin is an enzyme that has the ability to break down the gram-positive cell wall, and was added with the expectation that it would facilitate DNA extraction and hence increase the sensitivity, especially concerning *Staphylococci*. This strategy was successful; we saw a statistically significant increase in sensitivity from 42% to 79% ($p<0.001$, chi2) at the cost of a somewhat lower specificity, and the sensitivity for staphylococci increased from 8/26 (31%) in study II to 31/37 (84%) in study III. This was of course vital for the improved results in study III, since staphylococci comprised 37/49 (76%) of the single isolates included in this study.

In study III we included patients from two hospitals. Both centres showed similar test results, proving that the bacterial DNA in EDTA samples is stable enough to be transported 200 km at room temperature. Study III

also showed that the method is applicable to larger NICUs where surgical patients are cared for. To our knowledge, study III is the first two-centre evaluation of a PCR method for neonatal sepsis.

The overall results from study III (sensitivity 79%, specificity 90%) are in accordance with the results of other groups (Table 2), with the exception of the 2008 article from Wu et al.[162], which reports a sensitivity of 100%. The method of Wu et al. is based on gram-stain-specific probes which can discriminate between gram-positive and gram-negative samples. This setup is appealing and clinically relevant, though it does not provide the same highly detailed information as the species-specific probes used in the present thesis. Hopefully this study can be repeated, and the gram-stain-specific-probes can be complemented with more specific probes such as the CONS 1 probe used in study III.

It is not unlikely that more detailed primers and probes will be developed and used in the future, so that the PCR-result also could include information on antibiotic susceptibility, pathogenetic factors, detailed species typing and so on. However, in the near future PCR will complement rather than replace culture. PCR has the potential to be developed into a faster and more sensitive method than culture, and a method that can detect dead bacteria where culture fails, however PCR can only extract genetic information. To acquire the phenotypic requisites of a pathogen we will still need to rely on positive cultures. With a positive culture, the clinical microbiology laboratory can extract detailed information on antibiotic susceptibility, pathogenetic factors, and detailed typing that can be used to study the relationship between different isolates as well as to track a specific isolate and conduct research concerning pathogenetic factors and spreading patterns. This epidemiological surveillance is becoming increasingly important with the emergence of glycopeptide resistant strains among *S. epidermidis*, which is the most common neonatal pathogen today[174,184]. Unfortunately, there is a lack of good typing methods for *S. epidermidis*, antibiograms lack discriminatory power, and the currently used gold standards PFGE and MLST are too laborious and expensive[52,181,186,196,206]. We therefore evaluated PCR of repeat regions in four genes (*sdrF*, *sdrG*, *sesE*, and *aap*) encoding different cell-wall-anchored proteins.

These specific genes were chosen because previous studies have shown that they have a high prevalence in sepsis strains, contaminants, and skin flora,

as well as a proven stability when recultured[192,193,207]. In our study, we found that 100% of the isolates were positive for *sdrF*, *sdrG* and *sesE*, and 78% of the isolates were positive for *aap*. These proteins play important roles in the attachment and interaction between bacteria and human cells, and there is evidence that some of these proteins are expressed during clinical infections with *S. epidermidis*[192,207]. It has been suggested that these repeat regions correlate to coil-like structures in the protein that span the cell wall, or distances the A region of the protein away from the cell wall[192].

To ensure a sufficient discriminatory power we evaluated four different genes in our study. The highest D-index for a single gene was found for *sdrF* (0.82), followed by *aap* (0.81), *sdrG* (0.72), and *sesE* (0.70). When two genes were combined the highest D-index was found in *aap/sdrF* (0.92), followed by *aap/sdrG* . The addition of a third gene increased the D-index only marginally. To detect the amplicons, we used a gel-based approach, because this limits the workload involved. A sequence-based approach have other advantages, for example it facilitates storing and sharing of data, but also increases the resources required to an extent close to that required for PFGE or MLST. In summary we conclude that the presented method has a lower discriminatory power compared with PFGE or MLST, but also a lower workload. We therefore suggest that this method is suitable for an initial typing of a large material to trim down the number of interesting isolates to a subgroup which if necessary can be further investigated with PFGE or MLST.

Several publications indicate that late onset nosocomial sepsis is a preventable disease[22-26,208]. If prevention can be achieved, there will be no need for diagnostic methods. For this to happen, hygiene routines in NICUs must be taken to another level, especially when handling intravascular catheters[209]. Furthermore, we must initiate a mind shift towards a mental state where every case of LOS is unacceptable, and health care providers must accept the costs associated with sepsis prevention.

Strengths and limitations

Study I is unique in that it is an observational study including prospectively collected clinical signs from more than 400 infants with suspected sepsis. The use of logistic regression analysis on these data allowed us to extract knowledge that was not previously known. The results concerning gestational age differences are unique, and present data that have never before been published from a modern NICU.

The PCR studies (II and III) are among the largest studies ever published on PCR without preculturing. The samples were prospectively collected, and the studies present detailed information on the samples with conflicting results. They show the benefits with mutanolysin use and DNA extraction from whole blood. Study III demonstrates the benefits of mutanolysin use and DNA extraction from whole blood, and presents strong indications that broad-range PCR can actually detect non-viable bacteria; this is of great interest, since it has long been speculated upon but the scientific evidence has been lacking[159,210].

Study IV presents a new typing method for *S. epidermidis* that is less complicated than PFGE and MLST, and hence is suitable for typing of large materials of *S .epidermidis* strains.

The limitations of this thesis are that only one of the studies, study IV, presents a method that can be directly implemented for clinical or research purposes. The method in study III could also be used in clinical practice, but since other equally promising studies have been published recently, it seems more logical to consider the strength of these studies and hopefully merge them together into one optimal method. Another limitation is that study III was the only one which included material from more than one hospital; this increases the risk that the results only reflect the local situation, and this must be taken into consideration before the results are generalised to other locations.

Future research

Future research in the area of neonatal sepsis diagnosis is highly warranted for the benefit of the health care professionals who deal with infected infants and their parents. The following proposals offer some suggestions for how this research could be conducted:

- Heart rate analysis is promising; the published articles by Griffin and Moorman are convincing, and the results from their ongoing multicentre trial will provide data on the benefits of the methods when applied to VLBW[136-144]. However since this study only includes VLBW infants with a gestational age less than 32 weeks, another study including fullterm infants is warranted.
- Neutrophil cell surface antigens such as CD64 seem to be more sepsis-specific than cytokines. However costs must be considered and it would be worth conducting a randomised controlled study where patients with negative CD 64 values are left without antibiotic therapy[89,111,117-119,122,124-128].
- Real-time PCR has obvious potential, and if the study by Wu et al. can be repeated this could be the perfect "rule-out sepsis test" since the reported sensitivity is 100%[162].
- There is no need for any more evaluations of cytokines such as CRP, IL-6, and IL-8 in suspected sepsis, at least not in blood samples. On the other hand, cytokine levels in urine are virtually unexplored, and have the advantage that urine is constantly produced and can be collected without pain or risk of anaemia[211-214]. Cytokines in urine are therefore suitable for daily screening, and studies evaluating the utility of this monitoring method should be of widespread interest.
- There is a need for a large scale prospective multicentre trial focused on describing neonatal signs at different gestational ages. Such a study must be conducted by one of the existing large neonatal networks.

CONCLUSIONS

- Bradycardia, apnoea, low blood pressure, feeding intolerance and distended abdomen are obvious early signs of neonatal sepsis. Premature and full-term infants differ in terms of the signs they display in neonatal sepsis.
- Blood is superior to plasma for developing PCR methods for bacterial DNA detection. The method PCR described in study III can detect neonatal bacteraemia, but it can be improved further before it is used in routine care.
- There has been a lack of useful typing methods for *S. epidermidis*. We can now present PCR of the genes for the cell wall anchoring proteins *sdrF* and *aap* as a novel and feasible approach when there is a need to type a large number of *S. epidermidis* isolates.

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SWEDISH SUMMARY

Sammanfattning

Bakgrund

Nyfödda barn har en ökad risk att drabbas av blodförgiftning (neonatal sepsis) jämfört med vuxna och äldre barn. Förtidigt födda och barn som vårdas på neonatal intensivvårdsavdelning är extra utsatta. Neonatal sepsis kan ha ett snabbt och dramatiskt förlopp och de initiala symtomen är diskreta och svårtolkade, dessutom är de diagnostiska metoderna som finns till hands inexakta och tidskrävande. Detta gör att behovet att utveckla nya diagnostiska metoder är stort.

Målsättning med studien

- Att beskriva vilka symtom som är mest associerade med en positiv blododling i ett stort material av nyfödda som alla behandlades för misstänkt neonatal sepsis.
- Att utveckla och utvärdera en ny och fungerande PCR-metod som kan påvisa DNA från bakterier i blodprover från nyfödda med misstänkt sepsis (delstudie II och III).
- Att utveckla och utvärdera en lätt använd typnings metod för *Staphylococcus epidermidis* med hjälp av en PCR-metod riktad mot bakteriens serine-aspartat dipeptid regioner.

Material och metoder

Studie I utvärderade associationen mellan 9 kliniska symptom, CRP nivåer och positiv blododling med logistisk regressions analys.
Studie II utvärderade en realtids PCR metod riktad mot bakteriens 16 S region på ett material av 295 plasma prover från 288 nyfödda med misstänkt neonatal sepsis.
Studie III utvärderade en realtids PCR metod riktad mot bakteriens 16 S region på ett material av 368 prover från 317 nyfödda med misstänkt neonatal sepsis.
Studie IV utvärderade PCR av generna *aap, sdrG, sdrF* och *sesE* på 49 väl karaktäriserade stammar av *Staphylococcus epidermidis.*

Resultat

Metoden i studie II resulterade i en sensitivitet på 42% och en specificitet på 95%, detta resultat förbättrades till en sensitivitet på 79% och en specificitet på 90% i studie III som också visade att PCR kan detektera

bakterier i vid odlingsnegativ sepsis. Studie I visade att apné och lågt blodtryck är oberoende prediktorer för positiv blododling i den studerade populationen, vidare fann vi indikationer på att för tidigt födda inte uppvisar samma symptom som fullgångna vid neonatal sepsis. Studie IV visade att den studerade metoden var användbar för att typa stora material av *Staphylococcus epidermidis* och att kombinationen av generna *sdrF* och *aap* var mest lämplig.

Slutsatser

- Blod är överlägset plasma vid utveckling av PCR-metoder för detektion av bakterie DNA vid misstänkt neonatal sepsis, den studerade metoden i studie III är lovande och har potential att även upptäcka odlingsnegativ sepsis men det finns potential för ytterligare förbättringar innan metoden sätts i kliniskt bruk.
- Bradycardi, apné, lågt blodtryck, matproblem och utspänd buk är tydliga tecken på neonatal sepsis och det finns en skillnad i vilka symptom för tidigt födda och fullgångna visar vid neonatal sepsis.
- PCR typning av *sdrF* och *aap* är en väl användbar metod när det finns ett behov att typa ett större antar isolat av *Staphylococcus epidermidis*.

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