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de Meij, T.G.J.

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CHAPTER 12

Detection of sepsis in preterm infants by fecal volatile organic compounds analysis: a proof of principle study

Daniel J.C. Berkhout
Hendrik J. Niemarkt
Martin Buijck
Mirjam M. van Weissenbruch
Paul Brinkman
Marc A. Benninga
Anton H. van Kaam
Boris W. Kramer
Peter Andriessen
Nanne K.H. de Boer
Tim G.J. de Meij

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ABSTRACT

Objectives

Several studies associated altered gut microbiota composition in preterm infants with late-onset sepsis (LOS), up to days prior to clinical onset of sepsis. However, microbiota analysis as early diagnostic biomarker is in clinical practice currently not feasible because of logistic aspects and high costs. Therefore, we hypothesized that analysis of fecal volatile organic compounds (VOC) may serve as non-invasive biomarker to predict LOS at a preclinical stage, since VOC reflect the composition and activity of intestinal microbial communities.

Methods

In a prospective multicenter study, fecal samples were collected daily from infants with a gestational age of < 30 weeks. VOC signatures of fecal samples from infants with LOS, collected up to five days before diagnosis, were analyzed by means of an electronic nose technology (Cyranose 320®) and compared to matched controls.

Results

Fecal VOC profiles of infants with LOS (n = 36) could be discriminated from controls (n = 40) at three days (AUC [± 95% CI], p-value, sensitivity, specificity; 70.2 [52.2-88.3], 0.033, 57.1%, 61.5%), two days (77.7 [62.7-92.7], 0.050, 75.0%, 70.8%) and one day (70.4 [49.6-91.3], 0.037, 64.3%, 64.3%) before the onset of LOS.

Conclusions

Fecal VOC profiles of preterm infants with LOS could be discriminated from matched controls, up to three days before clinical onset of the disease, underlining the hypothesis that intestinal microbiota may play an etiological role in LOS. Notably, VOC profiling is clinically feasible and the potential of this technique in the early detection of LOS needs to be confirmed in future studies.

INTRODUCTION

Despite advanced technology and specialized care at neonatal intensive care units, incidence rates of late-onset sepsis (LOS, onset > 72 h after birth) in premature infants has increased over the past decades towards 20%.[1,2] LOS is associated with prolonged hospital admission and associated morbidities including patent ductus arteriosus, bronchopulmonary dysplasia, and necrotizing enterocolitis (NEC), and most importantly, impaired neurodevelopmental outcome.[3,4] Although overall mortality rates in premature infants have declined over the past years, sepsis-related mortality rates remained unchanged.[5,6]

Although intravascular catheters are considered the major source of LOS,[7] recent studies on LOS have demonstrated genetically incongruity between organisms isolated from the blood culture and bacteria cultured from the intravascular catheter tip.[8,9] The genetic similarity between cultured LOS pathogens and isolates from the gastro-intestinal tract points to the origin from the gut.[10-16] These isolated pathogens from blood cultures could be detected in the gut already several days prior to the clinical onset of LOS.[10,13,14,16] These findings have led to the hypothesis that in at least a part of the LOS cases, the gut instead of foreign bodies serves as the primary source of infection.[10-17] The precise mechanisms are not understood. The translocation from the gut into mesenteric lymph nodes and ultimately into the bloodstream may be mediated by intestinal hypoperfusion leading to a disturbed mucosal barrier function of the already immature gut, low bacterial species diversity supporting overgrowth of potentially invasive microbes, suboptimal nutritional condition and immaturity of the immune system.[18-21]

Therefore, intestinal microbiota analysis may have potential as an early non-invasive diagnostic biomarker for LOS. Since traditional culture techniques are usually insufficient to detect the common LOS pathogens in fecal samples (strains of *Coagulase-negative Staphylococcus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*), novel molecular techniques are needed to unravel the highly complex intestinal microbiota composition. However, implementation of such techniques into daily clinical practice is hampered by the scarcity of necessary equipment, inefficiency in time and high costs and the inability to generate results within acceptable time frames from a clinical point of view. The search for new, preferably non-invasive biomarkers therefore continues.

Fecal volatile organic compounds (VOC) may offer a solution in this dilemma. VOCs are primarily generated by microbial metabolic processes and host metabolism and are therefore

considered to, in part, reflect gut microbiota composition. VOCs are carbon-based chemicals originating from both physiological and pathophysiological metabolic processes in the human body.[22] They can be analyzed by either chemical analytical techniques, including gas chromatography-mass spectrometry (GC-MS), enabling identification of individual VOCs, or handheld electronic nose (eNose) devices.[23] We hypothesized that LOS is preceded by dysbiosis in the gut and therefore may be detected by fecal VOC analysis. The aim of the present study was to compare VOC profiles obtained from fecal samples collected of preterm infants with LOS in the days prior to clinical onset of disease, with VOC profiles of matched controls, as measured by eNose technology.

METHODS

Subjects

Preterm infants with a gestational age of < 30 weeks and born between September 2013 and June 2015 at the Neonatal Intensive Care Units (NICU) of the VU Medical Centre and Academic Medical Centre in Amsterdam, and the Máxima Medical Centre in Veldhoven, were eligible to participate in this prospective case-control study. Subjects with congenital intestinal anomalies (e.g. anus atresia, Hirschsprung's disease) and surgery of the gastro-intestinal tract were excluded. In addition, preterm infants who developed NEC (any stage) during the first 28 days of life were excluded from this study. Standard demographic and clinical data, including mode of delivery, enteral and parenteral feeding pattern, presence of central venous catheter, medication, respiratory support, clinical condition (including sepsis and NEC) was prospectively collected. None of the participating NICU's routinely administered probiotics to the studied population. The study was approved by the local Institutional Review Boards of all participating centers, and written informed consent was obtained from all parents of included patients.

Sample size calculation

Based on the results of our former studies on fecal VOC analysis, we concluded that per selected time-interval a minimal of ten subjects per study group was required to obtain a power (β-error) of 0.80 in order to reject the null hypothesis that no differences between the VOC profiles of patients with LOS and matched controls exists, with an p-value (α-error) at < 0.05.[24-26]

Sample collection

From birth until the postnatal age of 28 days, fecal samples were collected daily from the diaper by a nurse. When an infant was discharged from the NICU, transferred to another hospital or deceased before the age of 28 days, stool collection was stopped. If a subject passed more than one fecal sample per day, only the first produced stool was collected. If bowel movements were absent during a day, the subsequently produced fecal sample was collected. Fecal samples were stored in a stool container (Stuhlgefäß 10 ml, Frickenhausen, Germany) at a temperature of -20°C.

Patient cohort and sample selection

Preterm infants were allocated to the LOS group if the following Vermont Oxford criteria were met; 1) presence of clinical signs of systemic infection (such as apnea, temperature instability, feeding intolerance, worsening respiratory distress, hemodynamic instability), 2) a positive blood culture, which was obtained ≥ 72 hours after birth, and 3) antibiotic treatment based on the cultured pathogen.[27] Controls were defined as healthy preterm neonates without any clinical symptoms or signs of systemic infection for which appropriate antibiotic treatment was administered.

Fecal samples produced from one day up to five days prior to the day of clinical onset of LOS (T_0) were correspondingly distributed over five 24-h time intervals (T_{-1} up to T_{-5} respectively). Samples produced at T_0 were not used for further analysis to minimize the risk of type I error, since it could not reliably be ascertained whether these samples were produced prior to start of antibiotics. Each fecal LOS-sample was matched to one control sample based on: center of birth, gestational age, birth weight, postnatal age, feeding pattern and number of days exposed to antibiotics prior to T_0.

VOC analysis by eNose

Fecal samples were analyzed by means of Cyranose 320® (Smiths detection, Pasadena, California USA). For a detailed description of the sampling and measurement procedure, we refer to our previous study.[24] In brief, approximately 0.5 gram of frozen feces was put in a sealed vacutainer (BD vacutainer 3 ml, Franklin Lakes, USA) and placed in a 37°C stove for one hour, allowing VOCs to fill the headspace. Afterwards, two needles were placed into the cap of the sealed vacutainer, which were subsequently incorporated into an airtight system attached to the eNose. After a baseline reference was created by connection of a VOC-filter

(A1, North Safety, Netherlands), the actual measurement was performed. Fecal VOCs were led across an array of 32 sensors, all comprising conductive carbon black material which is blended throughout a specific non-conducting polymer.[28] These polymer coatings swell when VOCs are presented, subsequently increasing the electrical resistance by augmenting the distance between the conductive carbon black material particles.[28] Each individual VOC interacts with multiple sensors and each sensor interacts with different fractions of the VOC mixture. Each polymer coating being unique, eventually leads to 32 unique resistance changes, resulting in a so-called 'smellprint'.[28] Based on pattern recognition algorithms, these smell-prints can be used to differentiate between clinical subgroups.

Data analysis

Clinical and demographic data were compared by independent t-test, non-parametric test or chi-square test where appropriate. Data analysis was performed using R-script (Rstudio® (version 0.99.489) engine by R (version 3.2.2), using R packages: MASS, stats, sva, pROC). To minimize variance based on systematic non-biological differences between analyzed groups (batches), ComBat algorithm was applied prior to the analysis. This function allows for removal of batch effects and other unwanted sources of variation.[29] After batch correction, principal component analysis (PCA) was used to reduce data from 32 individual sensors into a set of four principal components (PC), capturing the largest variance in the dataset. An independent t-test was performed to assess discriminating PCs. These PCs were subsequently used in a internally cross-validated canonical discriminant analysis (CDA) based on the leave one out method, in order to calculate the probability of belonging to the LOS group. A ROC-curve with associated 95% CI, p-value, sensitivity (%) and specificity (%) was constructed for the test-set and after internal validation was performed. A p-value of < 0.05 was considered statistically significant.

RESULTS

Patient population

A total of 248 preterm infants were consecutively included during the study period (80 sepsis, 168 subjects without sepsis). Based on the strict matching criteria, we could subsequently select 76 preterm infants for further VOC analysis (36 sepsis cases matched to 40 controls), together providing 156 fecal samples (Table 12.1). In six infants allocated to the LOS

Table 12.1 Number of fecal samples in each group per selected time-point used for VOC analysis. Selected time-points are one (T_{-1}), two (T_{-2}), three (T_{-3}), four (T_{-4}) and five (T_{-5}) days prior to diagnosis.

Time-point	Sepsis (n = 36 cases)	Controls (n = 40 cases)
T_{-1}	14	14
T_{-2}	22	22
T_{-3}	17	17
T_{-4}	13	13
T_{-5}	12	12

group (four *Staphylococcus capitis*, two *Staphylococcus epidermidis*) treatment consisted of central line removal followed by 72-96 hours of antibiotic treatment, in all other cases antibiotics was administered for a minimum of five days. In the LOS cohort, 78% of isolated pathogens were *coagulase-negative Staphylococcus* (CoNS) species (Table 12.2). Two (6%) of 36 neonates with LOS died within the first 28 days of life and in both cases *Escherichia coli* was isolated from the blood culture. None of the infants assigned to the control group died during the study period. An overview of patient characteristics of the two subgroups is depicted in Table 12.3.

Fecal gas analysis

Based on their fecal VOC profiles, patients with LOS could statistically significant be discriminated from strictly matched controls, at T_{-3} (AUC [± 95% CI], p-value, sensitivity,

Table 12.2 Isolated pathogens (n [%]) from blood cultures in 36 sepsis patients

Coagulase negative Staphylococcus (CoNS)	28 [78]
Staphylococcus epidermidis	13 [50]
Staphylococcus capitis	8 [22]
Staphylococcus warneri	1 [3]
Staphylococcus haemolyticus	1 [3]
Combination of more than 1 CoNS†	5 [14]
Staphylococcus aureus	5 [14]
Escherichia coli	2 [6]
Combination of more than 1 pathogen‡	1 [3]

† 1x *Staphylcoccus haemolyticus* & *Staphylococcus capitis* & *Staphylococcus warneri*.
2x *Staphylococcus epidermidis* & *Staphylococcus haemolyticus*.
1x *Staphylococcus capitis* & *Staphylococcus epidermidis*.
1x *Staphylococcus hominis* & *Staphylococcus epidermidis*.
‡ 1x *Acinetobacter baumannii* & *Staphylococcus capitis*.

Table 12.3 Subject characteristics of the two subgroups sepsis and controls

	Sepsis n = 36	Controls n = 40	Significance† [p-value]
Sex			
Male (n [%])	21 [58]	20 [50]	NS [0.473]
Birth weight, (median [IQR]), g	1025 [344]	1148 [350]	NS [0.254]
Gestational age, (median [IQR]), weeks + days [days]	27 + 6 [19]	28 +2 [13]	NS [0.684]
Way of delivery (n [%])			
Vaginal delivery	19 [53]	22 [55]	NS [0.372]
Caesarean section	17 [47]	18 [45]	
Feeding pattern (n [%])			
Breast milk ± formula	34 [94]	37 [93]	NS [0.737]
Exclusive formula	2 [6]	3 [7]	
Postnatal age at T_0, (median [IQR]), days	9 [4]	NA	NA
AB use before T_0 (n [%])	35 [97]	35 [88]	
Days AB use before T_0, (median [IQR]), days	3.5 [3.3]	3 [1]	NS [0.133]
Central line present at T_0 (n [%])	13 [36]	NA	
Deceased (n [%])	2 [6]	0 [0]	NS [0.134]
Age deceased, days (median)	22	NA	

† A p-value < 0.05 was considered statistically significant.
AB = antibiotics; IQR = inter-quartile range; NA = not applicable; NS = not significant; T_0 = day of diagnosis.

specificity; 70.2 [52.2-88.3], 0.0329, 57.1%, 61.5%), T_{-2} (77.7 [62.7-92.7], 0.0496, 75.0%, 70.8%) and T_{-1} (70.4 [49.6-91.3], 0.0369, 64.3%, 64.3%). This discrimination was not possible for T_{-4} (60.9 [38.0-83.8], 0.5968, 45.5, 46.7) and T_{-5} (63.2 [39.8-86.6], 0.3443, 70.0, 64.3). An overview of the performance characteristics for the discrimination of LOS from healthy controls is given in Table 12.4.

Notably, not all preterm infants with LOS did pass stools on each day prior to LOS onset. We performed a post-hoc analysis to assess whether fecal VOC-profiling could be of value to predict LOS, regardless of the number of days that the last fecal sample was produced/collected prior to LOS onset. Each LOS infant and matched control with fecal samples collected on either three, two or one day prior to clinical onset was included. Fecal samples closest to T_0 were selected for analysis. A total of 32 LOS samples and matched controls were available, the number of samples per time-interval used is depicted in Supplemental Table 12.1. VOC profiles of LOS subjects could statistically significantly be discriminated from controls, irrespective of the day of last stool passage prior to LOS onset (66.8 [53.2-80.4], 0.0242, 64.5%, 63.6%).

Table 12.4 Performance characteristics with corresponding sensitivity and specificity of fecal VOC-analysis for the discrimination of late-onset sepsis and controls. Accuracy, sensitivity, specificity, positive and negative likelihood values were obtained after internal validation.

Time-point	Sepsis samples† (n =)	AUC ± 95% CI	p-value	Accuracy (%)	Sensitivity (%)	Specificity (%)	+ LR	- LR
T_{-1}	14	70.4 (49.6-91.3)	0.037	64.3	64.3	64.3	1.80	0.55
T_{-2}	22	77.7 (62.7-92.7)	< 0.050‡	72.7	75.0	70.8	2.57	0.35
T_{-3}	17	70.2 (52.2-88.3)	0.033	58.8	57.1	61.5	1.48	0.70
T-4	13	60.9 (38.0-83.8)	0.600	46.2	45.5	46.7	0.85	1.17
T_{-5}	12	63.2 (39.8-86.6)	0.344	66.7	70.0	64.3	1.96	0.47
T_{-3} to T_{-1}	32	66.8 (53.2-80.4)	0.024	64.1	64.5	63.6	1.77	0.56

† corresponding number of fecal samples from controls were analyzed; ‡ exact p-value = 0.04964.
AUC ± 95% CI = area under the curve with 95% confidence interval; + LR = positive likelihood ratio; - LR = negative likelihood ratio; VOC = Volatile Organic Compound.

A post-hoc analysis was also performed to assess whether differences in VOC profiles of the two groups changed when only CoNS cases were selected. Preterm infants with CoNS sepsis (n = 28) could be discriminated from strictly matched controls at T_{-1} (70.4 [48.4-92.5], 0.0377, 66.7%, 64.3%), but not at all other preselected time intervals (see Supplemental Table S12.2).

DISCUSSION

In the present study we have compared fecal VOC profiles of infants with LOS, up to five days prior to clinical onset, with VOC profiles of matched controls. We found statistically significant differences in fecal VOC-profiles between the two subgroups, from up to three days, but not earlier at four and five days before clinical onset of LOS.

Over the past decades there has been a rapidly increasing interest in the potential of VOCs as diagnostic biomarker for several diseases, including malignancies, inflammatory, metabolic and infectious diseases.[30-37] Several human and animal studies have evaluated the value of VOC as diagnostic biomarker for sepsis, using blood, exhaled breath or tracheal aspirate.[38-42] In only one study, including preterm ventilated infants, samples were collected prior to onset of sepsis.[38] In two of eight subjects with bloodstream infections, tracheal aspirate samples were collected prior to and shortly after diagnosis and subsequently analyzed by an electronic nose (Cyranose320®). Remarkably, VOC profiles from samples obtained prior to diagnosis clustered with samples from infants without sepsis, whereas samples obtained shortly after diagnosis clustered within the sepsis group. This may implicate that, although number of included subjects was very low, VOCs analysis of tracheal aspirates do not have the potential to detect LOS in preclinical stage.

In a recent study from our research group on fecal gas analysis in preterm infants we observed that VOC signatures of infants with NEC (n = 13) could be discriminated from strictly matched controls (n = 14) as well as from infants with LOS (n = 31), up to three days prior to clinical onset of NEC.[26] However, study design did not allow for reliable comparison between LOS and controls, since both subgroups could not strictly be matched as groups were primarily matched to NEC cases.

The observed differences in fecal VOC profiles between LOS cases and healthy matched controls in our study are consistent with findings from studies on altered intestinal microbiota composition before LOS onset.[10-16] This may be explained by the fact that fecal VOCs are

considered to largely reflect microbiota composition and its interaction with the host.[43] In these studies, causative pathogens could be isolated from the gut ranging from ten up to one day preceding LOS onset,[10,14] with detection rates varying between 54-82%.[10,13,14,16] Furthermore, microbial diversity in LOS subjects was found to be lower preceding a sepsis episode compared to controls,[11,12] next to an acquired predominance for the phyla *Firmicutes* and *Proteobacteria*.[11-13] In healthy controls, microbial diversity and abundance of obligate anaerobes, including *Clostridium*, *Klebsiella* and *Veillonella* species, increases over time.[11,13]

In the current study, fecal VOC analysis allowed for statistically significant discrimination between controls and LOS in a preclinical phase, but the clinical significance of this outcome may be questioned. Obviously, observed accuracies are currently insufficient to introduce VOC analysis as a diagnostic biomarker for LOS in daily clinical practice. There are several causes for the relatively modest accuracy to discriminate both subgroups.

Firstly, results of our study may be influenced by the origin of the pathogens in LOS; the assumption that the majority of LOS cases are caused by indwelling devices has been debated in several recent studies.[8,9] In 30% of blood stream infection (BSI), the causative pathogen could not be isolated from the central venous catheter (CVC)(8, 9), whereas another study demonstrated that the route of acquisition could not be determined in 70% of BSI cases.[44] As LOS can probably not solely be attributed to the presence of indwelling devices nor to bacterial translocation from the gut, early diagnostic biomarkers for LOS entirely focusing on the gut, like fecal VOC analysis in the present study, will lead to suboptimal results.

Secondly, another possibility for the modest accuracy was the relatively small samples size in combination with the high heterogeneity of the study group, reflected by many different isolated pathogens, hampering better classification. Although majority of LOS cases were caused by CoNS species, apparently creating a homogenous population, it has been demonstrated that each CoNS strain produces a unique combination of VOCs.[45] However, presence of species-specific metabolic fingerprints may offer opportunities to identify causative pathogens based on their VOC profiles.[46]

Thirdly, since microbial composition is highly variable within the first months of life,[20] differences between subjects in postnatal age at day of diagnosis may augment inter-individual differences in VOC profiles within both study groups, which may have contributed to the only modest accuracy. Future studies, including larger number of subjects, are needed to longitudinally assess day-to-day changes in VOC profiles in early life, including influences

of environmental factors, like diet, medication and variation in microbial colonization on VOC-outcome.

Strengths of this study are the prospective multicenter design and the inclusion of cases and controls matched for center and clinical demographics. This study has also several limitations. First, the study was designed as a feasibility study for VOC profiling of neonatal sepsis and represents a relatively small sample size. Second, fecal samples were not available from all subjects at every time-point, leading to even smaller sample sizes at predefined time-points. It would therefore be interesting to evaluate whether VOC profiles from rectal swabs could be used instead of fecal samples, since these can be harvested at any time. Future studies, comprising larger number of subjects are needed to externally validate our observations. Since intestinal microbiota colonization and consequently VOC fingerprints are influenced by many different environmental factors, including medication and feeding pattern, this external validation study should preferably be performed in multi-center setting to minimize the risk of type I error.[16]

Next to validation of our observations, future studies should aim at identifying LOS-specific VOCs, using chemical analytical techniques, like GC-MS. While such techniques are expensive, time-consuming and require highly-trained personnel, limiting its use as screening tool in clinical practice, this step will allow for development of tailor-made eNose devices that can be implemented in daily NICU practice. In contrast to GC-MS, a primed, disease -specific eNose can be applied in clinical practice because of its relatively low costs and high-throughput capacities, enabling real-time and bedside analysis. This study demonstrates that analysis of fecal VOCs may be able to reflect/detect gut dysbiosis which is not only present before the development of NEC but also before onset of late onset sepsis.[26] Therefore, fecal VOC analysis by an eNose holds promise to become a non-invasive, time and cost effective method in the NICU to detect gut dysbiosis. As the altered VOC profiles are found before onset of LOS, this opens a window of opportunity to prevent sepsis by altering gut microbiota by preventive strategies such as probiotics administration or individualized, targeted antibiotic treatment.

In conclusion, we observed in this proof of principle study that fecal VOC analysis by eNose allowed for discrimination between preterm infants with LOS and matched controls, up to three days prior to clinical onset of LOS. These findings underline the hypothesis that, at least in a selection of LOS, intestinal microbiota plays an etiological role in LOS. The potential of fecal VOC profiling in the early detection of LOS needs to be explored in future studies.

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SUPPLEMENTAL MATERIAL

Supplemental Table S12.1 Number of fecal samples per time-point selecting the samples closest to the day of diagnosis (T_0)

Time-point	Sepsis (n =)	Controls (n =)
T_{-1}	14	14
T_{-2}	15	15
T_{-3}	3	3

Supplemental Table S12.2 Performance characteristics with corresponding sensitivity and specificity of fecal VOC-analysis for the discrimination of late-onset sepsis with coagulase negative staphylococcus isolated from blood culture and controls. Accuracy, sensitivity, specificity , positive and negative likelihood values were obtained after internal validation.

Time-point	Sepsis samples† (n =)	AUC (± 95% CI)	p-value	Accuracy (%)	Sensitivity (%)	Specificity (%)	+ LR	-LR
T_{-1}	13	70.4 (48.4-92.5)	0.037	65.4	66.7	64.3	1.87	0.52
T_{-2}	18	78.1 (60.7-95.4)	0.093	75.0	80.0	71.4	2.80	0.28
T_{-3}	13	66.2 (44.5-88.1)	0.123	61.5	58.8	66.7	1.76	0.62
T_{-4}	8	73.4 (44.9-100.0)	0.151	62.5	60.0	66.7	1.80	0.60
T_{-5}	9	66.7 (39.3-94.1)	0.259	66.7	66.7	66.7	2.00	0.50

† Corresponding number of fecal samples from controls were analyzed.
AUC ± 95% CI = area under the curve with 95% confidence interval; + LR = positive likelihood ratio; - LR = negative likelihood ratio; VOC = Volatile Organic Compound.