Early Detection of Necrotizing Enterocolitis by Fecal Volatile Organic Compounds Analysis

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Objectives To test the hypothesis that fecal volatile organic compounds (VOCs) analysis by electronic nose (eNose) allows for early detection of necrotizing enterocolitis (NEC).

Study design In 3 neonatal intensive care units, fecal samples of infants born at gestational age ≤30 weeks were collected daily, up to the 28th day of life. Included infants were allocated in 3 subgroups: NEC, sepsis, and matched controls. Three time windows were defined: (1) T−5−4 (5 and 4 days before diagnosis); (2) T−3−2 (3 and 2 days before diagnosis); and (3) T−1,0 (day before and day of diagnosis). Three subgroups were analyzed by eNose.

Results Fecal VOC profiles of infants with NEC (n = 13) could significantly be discriminated from matched controls (n = 14) at T−3−2 (area under the curve ± 95% CI, P value, sensitivity, specificity: 0.77 ± 0.21, P = 0.02, 83%, 75%); the accuracy increased at T−1,0 (0.99 ± 0.04, P ≤ 0.001, 89%, 89%), VOC profiles of infants with NEC were also significantly different from those with sepsis (n = 31) at T−3−2 (0.80 ± 0.17, P = 0.004, 83%, 75%), but not at T−1,0 (0.64 ± 0.18, P = 0.216, 89%, 57%).

Conclusions In this proof-of-principle study, we observed that fecal VOC profiles of infants with NEC could be discriminated from controls, from 2-3 days predating onset of clinical symptoms. Our observations suggest that VOC-profiling by eNose has potential as a noninvasive tool for the early prediction of NEC. (J Pediatr 2015; 166: 1222–7.)

Necrotizing enterocolitis (NEC) is the most common severe gastrointestinal disease in very low birth weight infants, with reported incidence rates varying between 3% and 15%. Treatment consists of prompt cessation of enteral feeding, administration of broad spectrum antibiotics alongside supportive care. Of affected infants, 30%-40% will need surgery at some point for gut necrosis or bowel perforation. Mortality attributable to NEC remained disturbingly high over the past years, with rates varying between 15% and 30%. In survivors of NEC, neurocognitive and gastrointestinal impairments, such as short bowel syndrome, are common complications.

The pathophysiology of NEC is to be considered multifactorial. Immaturity of the gut, enteral (formula) feeding, and altered intestinal microbiota composition are the principal inducers of an excessive inflammatory response that leads to intestinal injury. This inflammatory cascade causes nonspecific clinical symptoms that may resemble sepsis, commonly leading to delayed diagnosis.

Early diagnosis and initiation of therapy are of pivotal prognostic importance and invasive diagnostic procedures may contribute to adverse neurocognitive outcome. Different biomarkers for NEC have been studied so far, mostly at the time when NEC was already suspected clinically. Unfortunately, the majority of these biomarkers lack accuracy to detect NEC in the preclinical stage and do not allow proper discrimination from sepsis. Therefore, the search for disease-specific, early and noninvasive diagnostic biomarkers for NEC remains warranted.

Because the preclinical stage of NEC is associated with alterations in gut microbiota composition, fecal volatile organic compounds (VOCs) could hypothetically serve as noninvasive biomarkers for the early detection of NEC.

Fecal VOCs are carbon-based gaseous chemicals, originating from fermentation processes of colonic microbes and from host metabolism.

Fecal VOC profile analysis can be performed by electronic nose (eNose) technology. This odor sensing method is based on pattern recognition algorithms,
mimicking the mammalian sensory system. Fecal gas analysis by eNose has previously shown potential in the (early) detection and assessment of disease activity in colorectal cancer and in pediatric inflammatory bowel disease, respectively, disorders characterized by alterations in microbiota composition.\textsuperscript{11,12}

We, therefore, hypothesized that analysis of fecal VOCs by eNose allows for detection of NEC before the onset of clinical disease. We aimed to study this in a prospective, multicenter proof of principle study in preterm infants, by comparing fecal VOC profiles of infants with NEC with matched controls and infants with sepsis.

### Methods

This prospective study was performed between September 2013 and March 2014 at the neonatal intensive care unit of the VU medical center in Amsterdam, the Emma Children’s Hospital/Academic Medical Center in Amsterdam, and the Máxima Medical Center in Veldhoven, The Netherlands. None of the centers used probiotics as prevention for NEC. The study was approved by the local institutional review boards.

Preterm infants were eligible for the study if they were born at a gestational age $\leq 30$ weeks, and written informed consent was obtained from both parents. Exclusion criteria were congenital intestinal anomalies (anal atresia, Hirschsprung disease, or short bowel syndrome) and intestinal surgery (bowel resection or stomata) during the period of stool collection because this might influence fecal VOC profiles.

In addition to standard demographic variables, the following clinical data were prospectively collected: mode of delivery, enteral and parenteral feeding pattern, medication (including antibiotics), erythrocyte transfusions, respiratory support, sepsis, and development of NEC.

All cases were reviewed independently by 2 experts (T.d.M. and H.N.) and allocated to one of the following groups: (1) infants with NEC stage IIA and higher, according to the international classification of Walsh and Kliegman\textsuperscript{13}; (2) infants with (late-onset) sepsis; and (3) matched controls. Full agreement was reached in all cases. Infants with sepsis were defined as subjects with clinical signs or symptoms of infection, combined with positive blood culture.\textsuperscript{14} Controls were defined as infants without clinical evidence of sepsis or NEC.

### Sample Size Calculation

Based on results from our previous studies on fecal gas analysis (effect size 1.340), we concluded that a sample size of 10 subjects per subgroup would be sufficient to obtain a power of 0.80 to reject the null hypothesis that no differences exist between fecal VOC profiles of patients with NEC and controls at $P < .05$ (Nquery advisor 7.0).\textsuperscript{11,12}

### Fecal Sample Collection

Fecal samples of included preterm neonates were from the diaper collected daily by the nurse, during the first 28 days of life. Approximately 0.5 g of feces was stored in a sterile plastic container and stored at $-20^\circ C$ in a freezer immediately following collection. In case the infant was discharged from the neonatal intensive care unit, or transferred to another hospital, before age of 28 days, stool sampling was terminated. If an infant passed more than 1 stool per day, only the first produced sample was stored. In case of absence of daily bowel movements, the subsequently produced fecal sample was collected.

### Sample Selection

The stored stool samples produced up to 5 days before the diagnosis of NEC and sepsis were used for fecal VOC analysis. Based on sample size calculation, the necessary number of fecal samples per time window was obtained by clustering samples into 3 time windows: (1) $T_{-5_{--}4}$ (5 and 4 days before diagnosis); (2) $T_{-3_{--}2}$ (3 and 2 days before diagnosis); and (3) $T_{-1_{--}0}$ (day before and day of diagnosis). To investigate whether fecal VOC profiles of preterm infants with NEC differed from controls and from sepsis per defined time window, each fecal NEC sample was matched with 1 control sample by center of birth, gestational age, postnatal age, number of days exposed to antibiotics, and birth weight. Furthermore, to investigate whether fecal VOC profiles of infants with NEC differed from infants with sepsis, VOC profiles of both subgroups were compared.

### VOC Analysis by eNose

VOC analysis of the selected fecal samples was performed with use of a Cyranose 320 eNose (Smiths Detections, Pasadena, California). The preparation of samples and technique of VOC measurements in fecal gas were in accordance with methods described in detail in previous studies.\textsuperscript{11,12} In short, approximately 1 g of frozen feces was transferred from the stored containers into a sealed vacutainer (BD Vacutainer; Belliver Industrial Estate, Plymouth, United Kingdom). This vacutainers were resealed and gradually heated to $37^\circ C$ for 1 hour in an incubator, to enhance vapor release from the stools. The heated vacu- tainers were subsequently connected to the eNose and analyzed in an air-tight closed loop system to prevent headspace dilution. Headspace sampling was performed using the Cyranose 320 eNose (Smiths Detections), a handheld chemical vapor analyzer, containing a fully-integrated nanocomposite array comprising 32 polymer sensors. Upon exposure to a gaseous mixture, these polymer sensors interact competitively with VOCs, causing the sensor material to swell, thereby increasing the electrical resistance of the sensor matrix. Multiple biomarkers interact with each individual sensor and individual biomarkers may interact with multiple sensors. The resulting alterations in resistance depend on the chemical characteristics of both the sensor material and interacting VOCs and are combined into a so-called smell print. This smell print can subsequently be used to differentiate clinical groups by pattern recognition analysis.\textsuperscript{13} Samples were analyzed in random order, using www.randomizer.org. The investigators performing the eNose analysis were blinded to clinical data.
Statistical Analyses
Basic patient demographic data were tabulated and compared by independent t test, χ² test, or nonparametric tests where appropriate.

The eNose provides a raw data output consisting of resistance changes of the sensors upon exposure to the VOC mixture. Principle component analysis was performed on the raw data to recombine the variance of the original dataset into a set of orthogonal principle components or factors. This unsupervised method captures the highest amount of variability of the original dataset in the lowest number of variables thereby reducing the risk of overfitting our diagnostic algorithm.

As previously described, samples were pooled into 3 time periods. Principle components differentiating between cases and controls at this time point (eg, NEC vs controls) were selected by means of Student t test (P < .05). These principle components were used in a supervised canonical discriminant analysis internally validated by a leave one-out method in view of the relatively low sample size. This method builds a diagnostic algorithm using data from all but one of the subjects. The remaining subject is subsequently introduced to the algorithm and classified providing a probability of disease for that case. This iterative process is repeated until each subject has been excluded from the primary algorithm once. The combined disease probabilities for all cases are used to construct a receiver operator characteristic curve and compute single point sensitivity, specificity, and negative and positive predictive values. The overall accuracy of the algorithm was assessed by the area under the curve (AUC) and associated 95% CI.

Results
In the study period, 128 infants (13 NEC, 31 sepsis, 84 controls) were included, accounting for a total of 2110 stored stool samples. Fecal samples of all NEC and sepsis cases (and of 14 controls were used for fecal gas analysis to strictly match each fecal NEC sample per defined time window with 1 control sample. An overview of the number of fecal samples per subgroup and per time window used for VOC analysis is given in Table I (available at www.jpeds.com). Patient characteristics of the 3 clinical subgroups are depicted in Table II. Individual characteristics of neonates who developed NEC are depicted in Table III (available at www.jpeds.com). Seven (54%) of 13 infants with NEC died (5 within 1 day following diagnosis, 1 after 3 days, and 1 after 13 days). An overview of the isolated pathogens from blood cultures in the sepsis group is given in Table IV (available at www.jpeds.com).

Patients with NEC could not be discriminated from matched controls at time window T₁₅₋₅₋₄ (AUC ± 95% CI, P value, sensitivity, specificity; 0.65 ± 0.25, 0.257, 60.0%, 60.0%). At T₁₅₋₃₋₂, VOC analysis discriminated infants with NEC from controls with an accuracy of 0.77 ± 0.21, 0.024, 83.3%, 75.0%. This further increased to a high accuracy at T₁₋₁₀ (0.99 ± 0.04, 0.001, 88.9%, 88.9%). A scatter plot for the discrimination of infants with NEC and controls is shown in Figure 1.

Patients with sepsis could be differentiated from patients with NEC at 2-3 days prior to clinical onset: 0.80 ± 0.17, 0.004, 83.3%, 75.0%. This discrimination was not possible at T₁₋₁₀ (0.64 ± 0.18, 0.216, 88.9%, 56.5%) and T₁₋₅₋₄ (0.52 ± 0.23, 0.886, 50.0%, 40.0%).

Detailed test characteristics for all classification algorithms are depicted in Table V. Corresponding receiver operator characteristic curves for diagnosis of NEC compared with controls and NEC vs sepsis are displayed in Figure 2.

Discussion
In this prospective multicenter study, we hypothesized that fecal VOC profiles measured by eNose could serve as an early diagnostic biomarker for NEC. We observed that 2-3 days...
before the clinical onset of NEC, fecal VOC profiles of affected infants could be discriminated significantly from controls, and the difference in the VOC profiles of the 2 groups increased even further toward onset of NEC. Besides, infants with NEC could be discriminated from subjects with sepsis 2-3 days before diagnosis, but not at time window $T_{-1,0}$.

The limited available information in the literature on fecal gas analysis in the early detection of NEC is derived from a pilot study using gas chromatography mass spectroscopy (GC-MS). Garner et al described in this relatively small-scale study (6 NEC cases vs 7 controls), specific differences in VOC composition next to a decreased total number of present VOCs in subjects with NEC in the 4 days before clinical onset of disease, compared with controls. Our present study describes eNose technology in the search for novel diagnostic biomarkers for NEC. Our findings underline that VOC analysis might be an effective tool to detect NEC in the preclinical stage. Benefits of eNose devices over GC-MS include lower costs, high-throughput capacities, user friendly hardware and software, all allowing for the future applicability in clinical practice. We performed the fecal gas analysis following gradually heating of the fecal samples to 37°C, to enhance vapor release from the stools. This particular temperature was chosen to evaluate whether eNose devices have potential as bedside screening tool, when using freshly produced fecal samples. In a previous study, we have performed validation sessions on fecal gas analysis by eNose, in which we observed no statistically significant effects of thawing and refreezing and of different temperatures of fecal samples (varying from 15°C to 35°C) on measured VOC profiles.

The major reservoir of fecal VOC source is considered to be microbes residing in the intestinal tract. The microbiome plays an essential factor in the development of NEC and has been subject to numerous microbial studies analyzing its composition in NEC. Recent reports on microbiota composition in NEC described significant differences in species mainly belonging to the phylum Proteobacteria, detectable up to 2 weeks prior to onset of NEC, next to an increase in Citrobacter, and a reduced diversity and depletion of enterococcus. Current opinion is that these microbiota alterations in infants with NEC result from manipulation by environmental factors linked to the development of NEC, such as the antenatal or early postnatal use of antibiotics and feeding type. Here, we observed that differences in VOC profiles between NEC and controls gradually increased from 3 days before, toward the onset of clinical diagnosis of NEC. The observed VOC shifts can, however, not solely be assigned to shifts in microbial presence. First, VOCs with a microbial origin reflect, at least in part, functional metabolic aspects of these bacteria. Many of these VOCs are, therefore, nonspecies specific. Fecal gas analysis by eNose may be able to detect sudden shifts in microbial activity not observed by microbiome analysis such as the sudden increase in the production of hydrogen, associated with pneumatosis intestinalis, a pathognomonic radiologic sign of NEC. Second, VOCs are not merely produced by the gut microbiota, but at least partly result from the intestinal mucosal inflammatory process preceding the clinical onset of NEC.

Although fecal VOCs measured in the days preceding clinical NEC were highly discriminative from controls, this does not automatically implicate that observed smell prints are disease-specific. This is illustrated by the finding that VOC profiles of infants with NEC and sepsis were distinguishable at $T_{-3-2}$, but not at $T_{-1,0}$. Possible explanation for this apparent discrepancy might be that both disorders have their unique microbial and metabolic shifts predating clinical diagnosis, reflected by the early observed differences in

### Table V. Performance characteristics of fecal VOC analysis for the discrimination of NEC, sepsis, and controls

<table>
<thead>
<tr>
<th>Time window</th>
<th>AUC ± 95% CI</th>
<th>P value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+LR</th>
<th>−LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEC vs control</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{0-1}$</td>
<td>0.99 ± 0.04</td>
<td>&gt;.001</td>
<td>88.9</td>
<td>88.9</td>
<td>8.1</td>
<td>0.1</td>
</tr>
<tr>
<td>$T_{2-3}$</td>
<td>0.77 ± 0.21</td>
<td>.024</td>
<td>83.3</td>
<td>75.0</td>
<td>3.3</td>
<td>0.2</td>
</tr>
<tr>
<td>$T_{4-5}$</td>
<td>0.65 ± 0.25</td>
<td>.257</td>
<td>60.0</td>
<td>60.0</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>NEC vs sepsis</td>
<td></td>
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<tr>
<td>$T_{0-1}$</td>
<td>0.64 ± 0.18</td>
<td>.216</td>
<td>88.9</td>
<td>56.5</td>
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<tr>
<td>$T_{2-3}$</td>
<td>0.80 ± 0.17</td>
<td>.004</td>
<td>83.3</td>
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<td>50.0</td>
<td>40.0</td>
<td>0.8</td>
<td>1.3</td>
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+LR, positive likelihood ratio; −LR, negative likelihood ratio. Sensitivities, specificities, and positive and negative likelihood ratios are reported for the optimum cut-points.

Figure 1. Scatter plot for the discrimination of infants with NEC (squares) and controls (circles) at time window $T_{0-1}$ by eNose. Axes depict 2 orthogonal linear recombinations of the original 32 sensor data, designed to capture the highest amount of original data variance, by means of principal component analysis. These variables are called factors.
VOC profiles. Subsequent merging of VOC profiles might result from increased intestinal permeability by gut barrier failure in NEC cases, leading to bacterial translocation (with or without sepsis) and by production of nonspecific biomarkers of inflammation. Further analysis by GC-MS might shed light on this subject by identification of those molecular compounds that are present at different stages and different underlying causes of inflammation. In addition, infants of the sepsis group had significantly lower postnatal age at time of sepsis onset compared with onset of NEC, which hypothetically could have influenced our observations. To obtain detailed insight in the potential of fecal VOC analysis in (early) discrimination between sepsis and NEC, understanding of the course of VOC profiles in sepsis compared with controls is indispensable. We performed a post hoc analysis to compare VOC profiles of infants with sepsis and controls. Here, fecal VOC profiles of controls were used from similar time windows as in the NEC vs controls analysis (Table 1). No significant differences were observed between these subgroups at all defined time windows (range AUC 0.64–0.67; range P value .08–.13). However, our study design does not allow reliable comparison between these 2 subgroups because infants with sepsis were not strictly matched with controls.

A strength of this study is the multicenter, prospective design including infants with sepsis as a separate subgroup, allowing to compare (early) VOC profiles of sepsis and NEC, diseases which are typically difficult to distinguish in clinical practice, especially at an early or preclinical stage. Furthermore, the control group was strictly matched and consisted of infants belonging to the intention-to-diagnose group.

This study has limitations. Because the number of NEC cases was limited, samples were clustered in 2-day windows, preventing detailed day-to-day description of VOC course. At window $T_{-1,0}$ a total of 9 samples of subjects with NEC were available for VOC analysis, 2 of them collected at $T_0$, before diagnosis of NEC was established. VOC profiles of those 2 infants closely resembled profiles of 7 samples at $T_{-1}$. Obviously, results of this study have to be externally validated.

Figure 2. Receiver operator characteristic curve with 95% CI for diagnosis of NEC compared with controls and NEC vs sepsis, at time windows A, $T_{-1,0}$, B, $T_{-3,2}$, and C, $T_{-5,4}$.
validated in a study comprising larger number of subjects with NEC. Because different gut pathogens have been reported to different institutional outbreaks of NEC, next to the presence of seasonal variation, validation studies should preferably be performed in a multicenter setting, in different countries, and compromising all seasons. Furthermore, findings per race, ethnicity, and sex may not make the findings applicable to all populations, especially to populations in developing countries. Future studies are needed to ascertain detailed insight in how metabolic, immunologic, and inflammatory processes in NEC shape fecal VOC composition, and whether found differences between NEC and controls are NEC-specific or not. Because the eNose method is a noninvasive and cost-effective technique allowing for real-time and bedside analysis of fecal VOC profiles, it is an interesting candidate for future application in daily clinical practice, especially in countries with fewer resources.

Because fecal VOC profiles could be identified several days before clinical onset of NEC, this technique may offer opportunities for early intervention strategies.

In conclusion, fecal VOC profiles of infants with NEC could strongly be discriminated from controls, from 2-3 days predating the onset of clinical symptoms. Our observations imply that VOC profiling has large potential as a noninvasive bedside tool for the early prediction of NEC.

References

### Table I. Number of fecal samples (n) in each group per selected time windows used for VOC analysis

<table>
<thead>
<tr>
<th>Window</th>
<th>NEC</th>
<th>Control</th>
<th>Sepsis</th>
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<tr>
<td>T&lt;sub&gt;-1.0&lt;/sub&gt;</td>
<td>9</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>T&lt;sub&gt;-3.2&lt;/sub&gt;</td>
<td>12</td>
<td>12</td>
<td>20</td>
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<tr>
<td>T&lt;sub&gt;-5.4&lt;/sub&gt;</td>
<td>10</td>
<td>10</td>
<td>18</td>
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</table>

### Table III. Performance characteristics of fecal VOC analysis for the discrimination of NEC, sepsis, and controls

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<tr>
<td>T&lt;sub&gt;-1.0&lt;/sub&gt;</td>
<td>0.99 ± 0.04</td>
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<td>T&lt;sub&gt;-3.2&lt;/sub&gt;</td>
<td>0.77 ± 0.21</td>
<td>.024</td>
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<td>T&lt;sub&gt;-1.0&lt;/sub&gt;</td>
<td>0.64 ± 0.18</td>
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<td>88.9</td>
<td>56.5</td>
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<td>0.20</td>
</tr>
<tr>
<td>T&lt;sub&gt;-3.2&lt;/sub&gt;</td>
<td>0.80 ± 0.17</td>
<td>.004</td>
<td>83.3</td>
<td>75.0</td>
<td>3.3</td>
<td>0.2</td>
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<tr>
<td>T&lt;sub&gt;-5.4&lt;/sub&gt;</td>
<td>0.52 ± 0.23</td>
<td>.886</td>
<td>50.0</td>
<td>40.0</td>
<td>0.8</td>
<td>1.3</td>
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+LR, positive likelihood ratio; -LR, negative likelihood ratio. Sensitivities, specificities, and positive and negative likelihood ratios are reported for the optimum cut-points.

### Table IV. Isolated pathogens n (%) from blood cultures in 31 sepsis patients

- **Staphylococcus aureus**: 2 [6]
- **CNS (Coagulase negative Staphylococcus)**: 21 [68]
- **Staphylococcus epidermidis**: 10 [32]
- **Staphylococcus capitis**: 5 [16]
- **Staphylococcus haemolyticus**: 1 [3]
- **Staphylococcus warneri**: 2 [6]
- Combination of 2 different CNS: 3 [10]*
- **Escherichia coli**: 3 [10]
- **Enterococcus faecalis**: 1 [3]
- **Serratia marcescens**: 1 [3]
- **Candida albicans**: 1 [3]
- **More than 1 pathogen cultured**: 2 [6†]

*CNS, Coagulase negative Staphylococcus.
†2 × Staphylococcus capitis and Staphylococcus epidermidis; 1 × Staphylococcus capitis and Staphylococcus haemolyticus.

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- **Candida albicans**: 1 [3]
- **More than 1 pathogen cultured**: 2 [6†]

CNS: Coagulase negative Staphylococcus.

*2 × Staphylococcus capitis and Staphylococcus epidermidis; 1 × Staphylococcus capitis and Staphylococcus haemolyticus.

†1 × Enterobacter cloacae and Staphylococcus warneri; 1 × Acinetobacter baumanii and Staphylococcus capitis.

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