Outbreak of Yersiniabactin-producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit

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Background: The Gram-negative bacterium *Klebsiella pneumoniae* is a frequent pathogen causing outbreaks in neonatal intensive care units. Some *Enterobacteriaceae* can acquire the ability to sequester iron from infected tissue by secretion of iron-chelating compounds such as yersiniabactin. Here we describe an outbreak and clinical management of infections because of a highly virulent yersiniabactin-producing, nonmultiresistant *K. pneumoniae* strain in a neonatal intensive care unit. Outbreak investigation and effectiveness assessment of multidisciplinary infection control measurements to prevent patient-to-patient transmission of highly pathogenic *K. pneumoniae* were undertaken.

Methods: Outbreak cases were identified by isolation of *K. pneumoniae* from blood or stool of infants. Clinical data were abstracted from medical charts. *K. pneumoniae* isolates were genotyped using whole genome sequencing, and yersiniabactin production was evaluated by luciferase assay.

Results: Fourteen cases were confirmed with 8 symptomatic and 6 colonized patients. Symptomatic patients were infants of extremely low gestational and chronologic age with fulminant clinical courses including necrotizing enterocolitis and sepsis. Whole genome sequencing for bacterial isolates confirmed the presence of an outbreak. All outbreak isolates produced yersiniabactin.

Conclusions: Yersiniabactin-producing *K. pneumoniae* can display a high pathogenicity in extremely premature infants with low chronologic age. This outbreak also underlines the considerable potential of today's infection control systems for recognizing and controlling nosocomial infections in highly vulnerable populations.

Key Words: preterm neonate, yersiniabactin-producing *Klebsiella pneumoniae*, outbreak, necrotizing enterocolitis, neonatal sepsis

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The Gram-negative bacterium *Klebsiella pneumoniae* is a well-recognized pathogen of nosocomial infections in intensive care units and has been reported to be responsible for at least 15% of Gram-negative nosocomial infections.[1,2] One characteristic of *K. pneumoniae* is the capacity to silently colonize the gastrointestinal tract of patients and hospital staff without causing symptoms.[3] Such carriers often remain unrecognized, resulting in impaired infection and outbreak control.[4]

In recent years, outbreaks with multiresistant *K. pneumoniae* have been reported worldwide with different sources and reservoirs such as bath soap,[5] breast milk[6] and ultrasonography gel.[7] In the majority of outbreaks, an epidemiologically proven environmental source was missing, suggesting the possibility of patient-to-patient and staff-to-patient transmission. Because *K. pneumoniae* can survive for hours on human skin, the likelihood of transmission through skin contact is high.[8]

In recent years, different *Klebsiella* strains have shown the potential to acquire pathogenic properties as well as multidrug-resistance.[9,10] One pathogenic factor is the production of siderophores. Iron is an important bacterial micronutrient needed in numerous biological processes including metabolic cycles, gene regulation and DNA synthesis.[11] Iron-deprivation mechanisms are an important metabolic adjustment during the host immune response against invading pathogens. Thus, by production of siderophores, bacteria possess the ability to sequester and uptake iron from infected tissues. Members of the *Enterobacteriaceae* family, including *K. pneumoniae*, have been found to produce different siderophores.[12] The expression of the siderophore yersiniabactin is associated with virulence in *Yersinia* species and was found to spread horizontally with high frequency among *Enterobacteriaceae* species.[13] However, the potential contribution of the yersiniabactin production to pathogenicity in *K. pneumoniae* and other *Enterobacteriaceae* causing extraintestinal infections remains undefined. Premature infants, especially extremely premature infants below 1000 grams birth weight, are highly susceptible to severe bacterial infections resulting in high mortality and morbidity.[14]

In May 2016, an outbreak of yersiniabactin-producing *K. pneumonia*e occurred in the neonatal intensive care unit (NICU) at the Medical University of Vienna. Here we report the clinical course, genomic profiling of the *K. pneumoniae* strain as well as the multidisciplinary management leading to immediate control of the outbreak.

METHODS

Study Center and Data Collection

The Department of Neonatology at the Medical University Vienna/General Hospital Vienna is a tertiary care academic center consisting of 2 NICUs (22 beds, level IV) and 2 intermediate care wards (24 beds). The outbreak primarily occurred in the NICU connected to the prenatal ward and obstetric department. This NICU consists of 12 beds separated into 3 bays with 4 beds each and is geographically separated from the other neonatal wards. In general, the average nurse:patient ratio is 1:1.7 during day-time and 1:2.4 during night shifts. The probiotic preparation Infloran (Laboratorio Farmaceutico S.I.T. S.r.l., ITA) (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) is routinely used in our institution for prevention of necrotizing enterocolitis (NEC). We do not use enteral antibiotics for NEC prevention. Demographic and clinical data of affected patients were retrieved from the patient *data* management system ICCA (Philips Healthcare, Hamburg, GER). *K. pneumoniae*

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culture results and antibiotic resistance profiles were analyzed using the monitoring of microorganism (MOMO) database of Medexter Healthcare as described previously.[15] NEC was classified according to modified Bell's staging criteria.[16]

Luciferase Assay

Production of the siderophore yersiniabactin was quantified indirectly using a luciferase reporter assay as described elsewhere.[17,18] Briefly, bacterial strains were cultivated in nutrient broth medium (NBD) medium for 24 hours at 37°C. Next, bacteria were pelleted by centrifugation, and the supernatant was added to the indicator strain WR 1542 harboring plasmid pACYC5.3L. All the genes necessary for yersiniabactin uptake, that is, *irp6, irp7, irp8, fyuA, ybtA,* are located on this plasmid. Furthermore, the reporter plasmid is equipped with a fusion of the *fyuA* promoter region with the luciferase reporter gene. The amount of yersiniabactin can be quantified semi-quantitatively, as yersiniabactin-dependant upregulation of *fyuA* expression is determined by luciferase activity of a *fyuA-luc* reporter fusion.

Infection Control—Outbreak Investigation

After the rapid clinical deterioration of 2 extremely premature infants in the same night, the infection control (IC) team was consulted, and microbiological cultures of blood and stool samples of index patients were sent for microbiological evaluation. A case was defined as the occurrence of *K. pneumoniae* isolated from culture of any specimen collected during the outbreak period. Active surveillance of all patients at the NICU was initiated to prospectively identify additional cases. Infants with rectal colonization with *K. pneumoniae* were immediately isolated and cohorted in 1 room. Working on patient beds was only allowed using barrier precautions with single-use gowns and gloves. During the epidemiologic investigation, all staff members were reeducated on proper hand hygiene by the IC team. IC practices in the NICU were reviewed including an reassessment of all invasive procedures. Direct observations of routine work flow were conducted by the IC team. The reprocessing of medical equipment was reevaluated, and all special equipment identified as potential outbreak sources used in the NICU was examined. Environmental cultures were collected from all surfaces in patient care areas, from sinks, ultrasound probe gels and medical equipment. Environmental cleaning as well as reprocessing of infant beds was reassessed. From day 1 to 7, the NICU was closed for new admissions. Thereafter, newly admitted infants were cohorted with surveillance culture-negative infants in a separated ward room. Additionally, all patients were cared for under barrier precautions using single-use gowns and gloves until the last patient with *Klebsiella* spp. colonization was discharged home. After the implementation of the immediate outbreak management, we did neither observe novel *K. pneumoniae* related infections nor colonizations of the pathogen. Affected infants were isolated during the entire hospital stay, and the last colonized patient was discharged after 4 months of hospitalization.

Microbiological Analysis

All isolates were identified to species level using standard microbiological methods for culture and identification in our clinical microbiology laboratory. Antimicrobial resistance testing was performed according to European Committee on Antimicrobial Susceptibility Testing recommendations (www.eucast.org).

Whole Genome Sequencing and Data Analysis

High-quality genomic DNA was extracted from 14 *K. pneumoniae* isolates from overnight cultures by using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and quantified with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) using the dsDNA BR Assay Kit (Thermo Fisher Scientific). Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) was carried out according to the manufacturer's protocol to obtain ready to sequence bacterial genomic libraries. The libraries were paired end sequenced (2×300 bp) on an Illumina Miseq instrument. Genome assemblies were performed de novo using Velvet (Version 1.1.07),[19] and whole genome sequence (WGS) data interpretation was carried out with the SeqSpheree$^+$ (Ridom, Münster, GER) analysis software.[20]

The multilocus sequence type (MLST)[21] and the core genome MLST (cgMLST) were extracted from the WGS data. The *K. pneumoniae* sensu lato cgMLST scheme (defined by John Rossen and Dag Harmsen) comprising 2358 core and 1946 accessory genes was used for outbreak analysis with the defined cluster threshold of 15 allelic differences and for the calculation of minimum spanning trees (MSTs). For detection of virulence genes, the allelic library of genes associated with virulence in *K. pneumoniae* from BIGSdb (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) was integrated into SeqSphere$^+$. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions PHGE00000000 to PHGR00000000. The versions described in this article are versions PHGE01000000 to PHGR01000000.

Statistical Analysis

Patient characteristics are presented as mean ± standard deviation or as frequency within the study group if not stated otherwise. Continuous variables were analyzed using 2 sample *t* test, and categorical variables were analyzed using Fisher exact test. A $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 24.0 (IBM, Armonk, NY).

RESULTS

Demographic Data of Infants

A total of 14 patients were affected by the *K. pneumoniae* outbreak in May 2016. Eight of those infants had a symptomatic course: the 2 index patients developed a fulminant NEC and died within 24 hours; 3 infants developed NEC with conservative treatment (2 cases of NEC stage Ib, 1 case of NEC stage IIa) and 3 further infants suffered from sepsis. Asymptomatic patients were colonized with the *K. pneumoniae* strain but did not develop signs of infections during the hospital stay. Symptomatic patients displayed lower gestational age (25.11 ± 0.90 vs. 26.90 ± 2.44 weeks of gestational age [WOG], $P = 0.07$), lower birth weight (709 ± 157 vs. 966 ± 241 gram; $P = 0.03$) and were significantly younger at time of diagnosis of colonization with *K. pneumoniae* (17.75 ± 12.57 vs. 43.00 ± 20.56 days postpartum; $P = 0.01$). Demographic data are summarized in Table 1.

Time Course of the Outbreak

Data of involved patients are summarized in Figure 1. Within 1 night, 2 extremely premature infants with an uncomplicated previous course acutely deteriorated from spontaneous breathing on continuous positive airway pressure to the full-blown picture of fulminant NEC within hours. Index patient 1 (25 + 4 WOG, 5 days old) had a blood culture taken and intraabdominal swabs (obtained during emergency laparotomy); all were positive for *K. pneumoniae*. Within an hour, Patient 2 (24 + 4 WOG, 8 days old, triplet III, also uncomplicated on continuous positive airway pressure until that night), located in another room, developed fulminant pan-NEC and died. On the following day, patient 3 (24 + 4 WOG, 9 days old, triplet II) and 4 (24 + 4 WOG, 9 days old, triplet I), located in the same room as patient 2, exhibited signs of sepsis. Blood cultures were positive for *K. pneumoniae.* Bacterial cultures from vaginal swabs and the amniotic membrane tissue taken from the triplet's mother showed negative results. On day 4 of the outbreak, patient 5 (24 + 6 WOG, 13 days old) displayed clinical signs of sepsis and showed a

TABLE 1. Demographic Data of Symptomatic (n = 8) and Asymptomatic (n = 6) Patients Colonized With *Klebsiella pneumoniae*

	Symptomatic Patients, n = 8	Asymptomatic Patients, n = 6	*P*
Gestational age, wk (mean ± SD; minimum–maximum)	25.11 ± 0.90; (24.14–26.86)	26.90 ± 2.44; (24.14–31.28)	0.07
Birth weight, g (mean ± SD; minimum–maximum)	709 ± 157; (440–915)	966 ± 241; (625–1370)	0.03*
Age at disease onset, d (median; minimum–maximum)	12.5; (5–41)	n.r.	n.r.
Age at first detected colonization, d (median; minimum–maximum)	12.5; (5–41)	43 (19–74)	0.01*
Male, n (%)	4 (50)	3 (50)	1.00
Multiples, n (%)	4 (50)	1 (16.7)	0.30
Necrotizing enterocolitis, n (%)	5 (62.5)	0 (0)	0.03*
Sepsis, n (%)	3 (37.5)	0 (0)	0.20
Blood culture positive, n (%)	4 (50)	0 (0)	0.08
Death, n (%)	2 (25)	0 (0)	0.47

Continuous variables were analyzed using 2 sample *t* test and categorical variables were analyzed using Fisher exact test.
*$P < 0.05$.
n.r. indicates nonrelevant; SD, standard deviation.

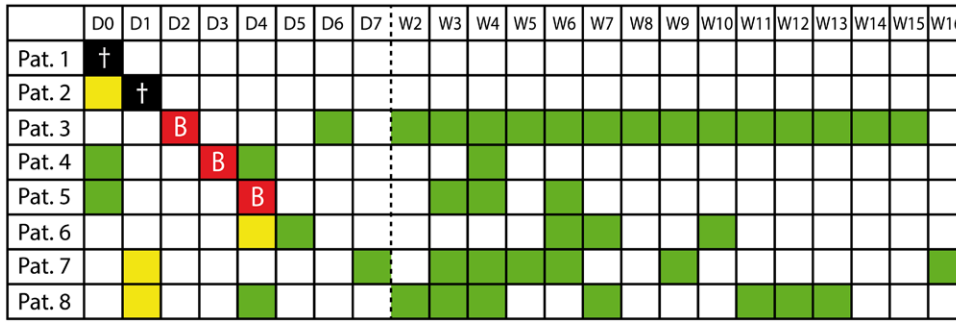

FIGURE 1. Timeline of the outbreak in the neonatal ward. The first week of the outbreak is shown on a daily basis (D = day). After the first week, culture results from the conducted rectal surveillance cultures are shown in weekly frequencies (W = weeks; dotted line). Black box indicates case of death; green box, positive surveillance culture; Pat., patient; red box, first detection in blood culture; yellow box, onset of disease. full color online

positive blood culture with *K. pneumoniae*. Patient 6 (25+6 WOG, 22 days old) developed NEC stage II with a positive rectal swab for *K. pneumoniae*. Both infants were located in the bay next to patients 2 to 4. Patient 7 (24+1 WOG, 31 days old) and Patient 8 (26+6 WOG, 41 days old) showed subtle signs of NEC stage 1 with occult and gross blood in stool but stable vital signs on day 1 of the outbreak. Both patients were assigned later to the outbreak as rectal surveillance cultures were positive for *K. pneumoniae*. Immediately after the fulminant death of the index cases, all admitted infants at our four neonatal wards (2 NICUs, 2 neonatal intermediate care (NIMCs)) were screened for rectal colonization with *K. pneumoniae*. We identified 6 colonized asymptomatic infants at 1 of our intermediate care wards showing no clinical symptoms of *K. pneumoniae* infection during their entire hospital stays.

IC

Lack of space between and around beds as well as a very high turn-over rate of patients in the NICU were identified during the IC assessment as contributing factors. No breaches in reprocessing procedures were recognized. All environmental cultures were negative for *K. pneumoniae*; therefore, no environmental source as cause of the outbreak could be identified. Nevertheless, multiuse gel containers for ultrasound probes were replaced against single-use containers. Inconsistencies in the environmental cleaning procedures of patient care areas including bed reprocessing could be observed and led to retraining sessions for cleaning personel. Hand hygiene teaching sessions for all health care workers were reinforced during the outbreak and regular hand hygiene audits implemented.

WGS Outbreak Analysis

All 14 *K. pneumoniae* isolates were sequenced with a minimum of 98% of good cgMLST targets and an average coverage of 84-fold. All isolates were assigned to classical MLST 664 and cgMLST 1306. Based on the cgMLST, the group including symptomatic patients (n = 8) had a maximum allelic difference of 3 (average distance 0.96), the group including asymptomatic patients had a maximum allelic difference of 5 (average distance 3.53), and between these two groups the maximum allelic difference was 5 (average distance 2.29). Based on the cgMLST and the accessory genome, the group of symptomatic patients had a maximum allelic difference of 3 (average distance 1.68), the group including asymptomatic patients had a maximum allelic difference of 8 (average distance 5.67) and between these two groups the maximum allelic difference was 7 (average distance 3.67) (Fig. 2).

Via the integrated virulence database from BIGSdb, all alleles (n = 11) belonging to yersiniabactin were detected (Table 2). The locus *irp*1 was identified as new allele, submitted to the Bacterial Isolate Genome Sequence Database (BIGSdb) database and a new allele number was assigned (irp1_new). The new combination of yersiniabactin loci revealed a new yersiniabactin sequence type (YbST = new).

DISCUSSION

To the best of our knowledge, this is the first description of an outbreak of a yersiniabactin-producing *K. pneumoniae* strain in a NICU. Using WGS for bacterial isolates, we were able to confirm

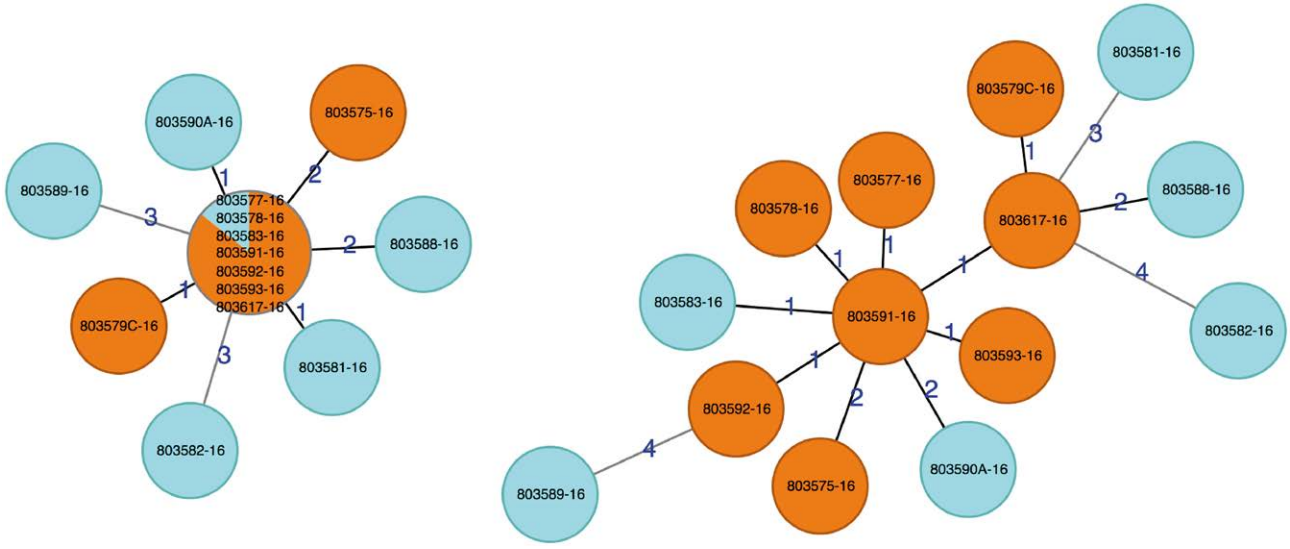

FIGURE 2. MST based on the core genome and the accessory genome of *Klebsiella pneumoniae*. Fourteen outbreak isolates are shown as circles; allelic differences between isolates are represented by blue numbers between isolates. The outbreak cluster was identified with a maximum allelic difference of 15, all 14 isolates lie within this definition. Symptomatic patients are colored in orange; asymptomatic patients in light blue. MST based on core genome Multilocus Sequence Typing (cgMLST) comprising 2358 core target genes (left) and MST based on cgMLST plus 1946 accessory target genes (right). full color online

TABLE 2. Typing Scheme for Yersiniabactin Virulence Operon

YbST new	Loci	*ybtS*	*ybtX*	*ybtQ*	*ybtP*	*ybtA*	*irp2*	*irp1*	*ybtU*	*ybtT*	*ybtE*	*fyuA*
	Allele	6	4	20	61	1	253	new	2	76	4	2

YbST indicates yersiniabactin sequence type.

the presence of an outbreak among the respective *K. pneumoniae* isolates. Inclusion of the pan genome (cgMLST plus accessory genome analysis) did not reveal major differences in comparison to the cgMLST analysis and thus confirmed the outbreak cluster.

Invasive infection with the described bacterial strain led to fulminant NEC and sepsis courses in extremely premature infants. The clinical course of infected infants appeared to be influenced by gestational age as well as chronologic age. Only extremely premature infants with low chronologic age developed fulminant disease upon infection with yersiniabactin-producing *K. pneumoniae*. The premature gut, especially of infants born before 28 weeks of gestation, exhibits various immaturities rendering the intestine vulnerable to exogenous factors leading to dysbalance in microbial colonization. This can result in exaggerated inflammatory response of the immature intestinal epithelium to luminal bacteria and poor intestinal microperfusion.[16,22] Thus, the "leaky gut" of extremely premature infants and the higher virulence of siderophore producing bacteria might predispose those infants to higher epithelial damage with increased bacterial translocation leading to an excessive systemic inflammation. This hypothesis is underlined by the asymptomatic clinical courses of affected patients with higher gestational and chronologic age where a more mature gut was not affected at all by yersiniabactin-producing *K. pneumoniae*. In our setting, a protective isolation strategy prevented the transmission of *K. pneumoniae* to newly admitted premature infants.

Over the last decade, numerous publications delivered novel insights into the relationship between colonization of the gut and health/disease status.[23] In premature infants, a lower diversity of the premature gut colonization seems to be a risk factor for NEC.[24] These findings correlate with clinical observations that infants who receive antibiotics for a longer duration display a higher risk for developing NEC.[25] On the other side, oral decontamination with antibiotics in the first weeks of life resulted in a lower NEC incidence in low birth weight infants.[26] Thus, the combination of diversity and dysbiosis seems to be important to develop a stable gut homeostasis in preterm infants. The lower diversity can be tackled by probiotics, which show promising results in NEC prevention.[27] Several randomized controlled trials as well as consequently conducted meta-analyses showed a clear benefit for probiotics in premature infants.[28,29] Interestingly, a large multicenter randomized placebo-controlled trial using *Bifidobacterium breve* alone showed no effect on the NEC incidence in very low birth weight infants.[30] Thus, the optimal probiotic strain and dosage in premature infants needs to be elucidated.

Whether routine screening for rectal colonization can prevent outbreaks in NICUs is controversially discussed in the literature. Whereas some authors concluded that routine mucosal cultures are inefficient for the prediction of late-onset sepsis in NICUs[31] or might even be harmful by enticing to overuse of antibiotics,[32] others have found beneficial effects of a once-weekly screening strategy for multi-drug resistant organism (MDRO).[33,34] Likewise, whereas in the United Kingdom, guidelines claim that "There is currently insufficient evidence on clinical effectiveness to recommend weekly screening in NICUs",[35] the Robert-Koch-Institute (RKI) in Germany stipulates at least once weekly rectal screening cultures in Very low birth weight infant (VLBWI) in German NICUs.[36] All guidelines and authors arguing in favor of a routine screening strategy, however, recommend screening for particular pathogens only, such as MDRO or pathogens particularly relevant in NICUs such as *Serratia marcescens*, but not screening

for all gut pathogens. Although we had performed routine rectal MDRO screening at our NICU, the yersiniabactin-producing *K. pneumoniae* strain was not recognized as an harmful strain because of an normal antibiogram. Hence, we only realized that transmission inside the ward had been going on when the first 2 patients simultaneously developed fulminant NEC, and subsequent routine screening and genotyping unveiled colonization of 6 additional patients with the same—microbiologically "harmless"—pathogen. As a result of the outbreak experience, we switched to a routine once-weekly screening strategy for all pathogens, irrespective of antibiogram, although there are no data in the literature supporting this approach.

In conclusion, we describe the first outbreak with a yersiniabactin-producing *Klebsiella* strain in a NICU. Extremely low gestational age and birthweight as well as colonization in the first weeks of life were significant risk factors for fulminant and invasive infections with this pathogen. Although not suspicious by conventional microbiology, this pathogen has to be added to the list of pathogens with particular virulence in chronologically young extremely premature infants. Whether routine screening for all pathogens (including microbiologically "harmless" strains) and/or protective isolation of extremely premature infants in the first weeks of life to prevent "unphysiologic" colonization can prevent fulminant infections as described in this report will have to be tested in future trials.

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