

## Full-length article

**Nosocomial colonization due to imipenem-resistant *Pseudomonas aeruginosa* epidemiologically linked to breast milk feeding in a neonatal intensive care unit**

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**Key words**

neonatal intensive care unit; *Pseudomonas aeruginosa*; metallo- $\beta$ -lactamase; breast milk

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**Abstract**

**Aim:** We describe a one-year investigation of colonization by imipenem-resistant, metallo- $\beta$ -lactamase (MBL) producing *Pseudomonas aeruginosa* in a neonatal intensive care unit (NICU) of the University Hospital of Palermo, Italy. **Methods:** A prospective epidemiological investigation was conducted in the period 2003 January to 2004 January. Rectal swabs were collected twice a week from all neonates throughout their NICU stay. MBL production by imipenem-resistant strains of *P aeruginosa* was detected by phenotypic and molecular methods. Pulsed field gel electrophoresis (PFGE) was carried out on all isolates of *P aeruginosa*. The association between risk factors and colonization by imipenem-resistant, imipenem-susceptible *P aeruginosa* isolates and other multidrug-resistant Gram negative (MDRGN) organisms was analyzed for variables present at admission and during the NICU stay. Data analysis was carried out by the Cox proportional hazards regression model. **Results:** Twenty-two of 210 neonates were colonized with imipenem-resistant, MBL-producing *P aeruginosa* isolates and 14 by imipenem-susceptible *P aeruginosa* isolates. A single pulsotype, named A, was shared by all imipenem-resistant isolates. Colonization by *P aeruginosa* of pulsotype A was positively correlated with breast milk feeding and administration of ampicillin-sulbactam, and inversely correlated with exclusive feeding by formula. In the Cox proportional hazards regression model, birthweight of more than 2500 g and breast milk feeding were independently associated with an increased risk of colonization by MBL-producing *P aeruginosa*. **Conclusion:** The results strongly support an association between colonization by a well-defined imipenem-resistant, MBL producing *P aeruginosa* strain and breast milk feeding. Such a study may highlight the need for implementation of strategies to prevent expressed breast milk from becoming a vehicle of health care-associated infections.

**Introduction**

*Pseudomonas aeruginosa* is one of the main organisms responsible for drug-resistant health care-associated infections<sup>[1–3]</sup>. Its ability to survive and replicate in hospital in a wide range of ecological niches, sometimes hostile to other microorganisms, may create tenacious environmental reservoirs, such as faucets, tap water, sinks,

respiratory-therapy equipment and even detergents and disinfectants<sup>[4,5]</sup>. Well known as a multiresistant organism, it is posing new therapeutic and public health challenges because of the emergence and widespread dissemination of strains producing metallo- $\beta$ -lactamases (MBL). Indeed, these enzymes can confer resistance to all  $\beta$ -lactams except monobactams and are not susceptible to the clinically available  $\beta$ -lactamase inhibitors<sup>[3,6]</sup>. Several types of

these enzymes have been detected; namely, IMP, VIM, SPM, GIM and SIM, mostly in *P aeruginosa* and other Gram-negative nonfermenters<sup>[7]</sup>. The IMP- and VIM-type enzymes are currently the most widespread and in Europe, where they were first detected, the VIM-type enzymes are overall the most prevalent<sup>[8,9]</sup>. Colonization and infection by *P aeruginosa* in premature neonates rise commonly from an environmental source, though health-care workers have proved to be involved as the reservoir or vehicle of transmission<sup>[10,11]</sup>. Preterm neonates are potentially at higher risk for infection with *P aeruginosa* because they may be immunocompromised, often undergo invasive procedures and require prolonged hospitalization<sup>[1,2,10,12]</sup>. Outbreaks of illness traced to contaminated feeding in neonatal intensive care units (NICU) are also increasingly reported in association with formula products and, though less frequently, expressed human milk<sup>[13,14]</sup>.

We describe a one-year investigation of colonization by imipenem-resistant, MBL-producing *P aeruginosa* in the NICU of the Dipartimento Materno-Infantile at the University Hospital of Palermo, Italy and the study of cross-transmission and risk factors for colonization with this pathogen in the NICU.

## Materials and methods

**Setting** This study was part of a larger prospective study that examined surveillance cultures in the NICU of the University Hospital of Palermo, Italy, from 2003 January 7 to 2004 January 6<sup>[12]</sup>. The unit is a section of the Dipartimento Materno-Infantile, which is also a referral centre for congenital malformation in Sicily. The department includes units of infertility and assisted reproduction and maternal-fetal medicine.

Approximately 200 neonates are admitted annually in the NICU under study. The NICU is divided into 2 rooms connected by sliding doors. Hand washing facilities are available in each room. Use of a liquid detergent with 4% chlorhexidine gluconate is strictly enforced for all staff and visitors. Gloves are used routinely in aseptic procedures.

During the study period, the average patient-to-nurse ratio was 3:1 and 2:1 in the intermediate care and intensive care section, respectively. Prematurity was the most common reason for admission to the NICU (approximately 70%). Other diagnoses for admission included respiratory distress, congenital malformation, surgical conditions and infections.

Whenever possible, neonates were exclusively or predominantly fed human milk. Alternatively, sterile

ready-to-feed formula was preferred, but some specialized formulas were also used and prepared from powdered formula products. In the case of expressed milk feeding, mothers were given a reusable personal collection kit to use with the breast pump. Milk was then chilled and transported to the hospital, where it was labeled and refrigerated until use within a maximum of 48 h. Warming in water bath and handling of breast milk before feeding was carried out in a designated area in the formula preparation room. As the first-line therapy for suspected early-onset sepsis a combination of ampicillin-sulbactam plus gentamicin was adopted.

**Patients** All neonates admitted during the period under study, who remained hospitalized for at least 48 h in the NICU and from whom at least one rectal swab culture could be obtained, were enrolled.

The following parameters were recorded for all neonates: demographic characteristics, gestational age, birthweight, inborn or outborn condition, delivery type, APGAR score, length of stay, co-morbidity conditions. During the NICU stay, data about use and duration of the following devices and procedures, that could be considered as possible risk factors were also collected: antimicrobial therapy, central venous and peripheral catheterization, nasogastric and endotracheal tube insertion, and type of feeding (*ie*, parenteral, breast milk or formula).

A rectal swab culture yielding Gram-negative bacilli in the absence of clinical symptoms and signs was considered evidence of colonization. Nosocomial infection was identified according to the recommendations of the Centers for Diseases Control, Atlanta, GA, USA<sup>[15]</sup>. Congenital anomalies were defined as conditions listed under the International Classification of Disease (ICD-9) codes 740 to 759. The study protocol was reviewed and approved by the Ethical Committee of the University of Palermo, and a written parental consent was obtained for each neonate.

**Microbiological surveillance** Rectal swabs were collected twice a week (every Tuesday and Friday) from all neonates throughout their NICU stay. Isolation and identification of *P aeruginosa* and other Gram-negative organisms were carried out as previously described<sup>[12]</sup>. After biochemical identification by API20E or API20NE (BioMerieux, Marcy-l'Etoile, France), susceptibility to a panel of 10 antimicrobial substances was assessed by disk diffusion on Mueller-Hinton agar plates, according to the Clinical Laboratory Standards Institute (CLSI) guidelines<sup>[16]</sup>: ampicillin (30 µg), amoxicillin-clavulanic acid (30 µg), carbenicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg),

imipenem (30 µg), aztreonam (30 µg), gentamicin (10 µg). For the purpose of the study, a Gram-negative organism resistant to at least 3 different groups of antimicrobial agents (penicillins, cephalosporins, aminoglycosides, carbapenems) was defined as multidrug-resistant Gram-negative (MDRGN).

Metallo-β-lactamase production by imipenem-resistant strains of *P aeruginosa* was detected by the EDTA-imipenem disk synergy test<sup>[17]</sup>. PCR amplification was carried out by using oligonucleotide primers targeting conserved regions of *blaVIM* and *blaIMP*<sup>[18]</sup>. Sequencing of VIM genes was carried out as previously described<sup>[18]</sup>.

Pulsed field gel electrophoresis (PFGE) was carried out on all available isolates of *P aeruginosa*. Chromosomal DNA was digested using *XbaI* endonuclease and resolved by PFGE in a CHEF-Mapper apparatus (Bio-Rad, Hercules, CA, USA). Strains were interpreted as indistinguishable, closely related, possibly related or unrelated strains according to the criteria of Tenover *et al*<sup>[19]</sup>.

**Statistical analysis** The association between potential risk factors and colonization by imipenem-resistant, imipenem-susceptible *P aeruginosa* strains and other MDRGN organisms was analyzed for variables present at admission and during the NICU stay. Time dependent variables, (*ie* length of stay and of antimicrobial use), were measured as between admission and the first isolation of a MDRGN organism.

One-way ANOVA and Kruskal-Wallis statistic test were used for parametric and non parametric analysis, respectively, to evaluate differences between the variables considered. The relative risk (RR) and the 95% confidence intervals (CI) for the risk factors were also calculated. The association was tested by Pearson's  $\chi^2$  test and Fisher exact test for frequency analysis. A Cox proportional hazards regression model was used to identify risk factors associated with colonization by imipenem-resistant *P aeruginosa*. Confounder selection included variables significant at  $P \leq 0.10$  in the preliminary analysis. For the purpose of this analysis, birthweight was stratified in 3 categories:  $\leq 1500$  g, 1501–2500 g, and  $> 2500$  g. Length of stay was also stratified in three categories:  $\leq 1$ , 2, and  $> 2$  weeks, respectively. All *P*-values were two-sided and *P*-values less than 0.05 were considered statistically significant. Data were analyzed by the Epi Info software (version 6.0, Centers for Disease Control and Prevention, Atlanta, GA, USA) and SPSS Software (version 14.0, SPSS Inc, Chicago, IL, USA)

## Results

During the study period, a total of 221 neonates were

admitted to the NICU for at least 48 h. Among these, 11 could not be submitted to rectal swab cultures and were excluded from further analysis.

A total of 1021 rectal swab cultures were sequentially obtained from 210 neonates. The characteristics of the study population are summarized in Table 1. The average length of stay was 22.3 d (median 13 d, range 3–140). Ninety-two (43.8%) neonates received antibiotics during the NICU stay. Criteria for infection were met by 25 (11.9%) neonates, of whom 2 died.

**Table 1.** Baseline characteristics of patients at the time of admission to the NICU, Palermo, Italy, 2003 January to 2004 January. Information about delivery type was available for 203 of the 210 infants. Information about APGAR score was available for 189 of the 210 infants.

Population	No infants (%)
Gender (male)	106 (50.5)
Birthweight (g)	
$\leq 500$	2 (0.9)
501–1000	11 (5.2)
1001–1500	17 (8.1)
1501–2000	45 (21.4)
2001–2500	36 (17.1)
$> 2500$	99 (47.3)
Gestational age (weeks)	
24–29	13 (6.2)
30–36	99 (47.1)
$> 36$	98 (46.7)
Inborn	109 (51.9)
Age at admission $> 24$ h	16 (7.6)
Twin birth	25 (11.9)
Cesarean delivery	153 (75.4)
APGAR score at 5 min $< 5$	2 (1.1)
Malformation	20 (9.5)

One-hundred and sixteen (55.2%) of 210 subjects were colonized with MDRGN at some point over the study period. The most frequent MDRGN species identified were *Enterobacter cloacae* (35.3%), *Pseudomonas aeruginosa* (31.0%), *Klebsiella oxytoca* (21.6%), *Escherichia coli* (19%), *K pneumoniae* (18.1%) and *Serratia marcescens* (7.8%). In particular, 22 neonates were colonized with an imipenem-resistant, MBL-producing *P aeruginosa* strain and 14 by an imipenem-susceptible *P aeruginosa* strain. No infection was detected in neonates colonized by these organisms.

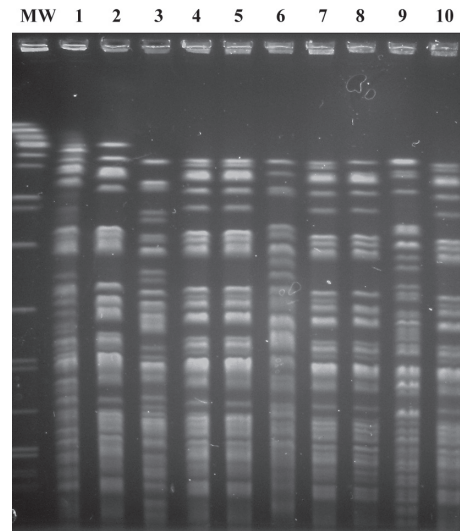
Screening test of the MBL-producing strain of *P aeruginosa* gave positive PCR results with the primers for *blaVIM* genes. By sequence analysis, the strain was found to carry a novel variant of the MBL *blaVIM* family,

designated *bla*VIM-11 (GenBank accession number AY635904).

The monthly incidence of acquisition of MDRGN, imipenem-resistant and imipenem-susceptible *P aeruginosa*, respectively, over the 12-month study period is illustrated in Figure 1. Strain relatedness of the *P aeruginosa* isolates was determined by PFGE analysis (Figure 2). A single pulsotype, named A, was shared by all imipenem-resistant *P aeruginosa* isolates, suggesting cross-transmission throughout the study period. On the contrary, the 14 imipenem-susceptible isolates were attributed to 11 different pulsotypes, named B to L. Only two were shared by 2 and 3 isolates from different neonates, respectively, whereas the remaining ones were unique.

Figure 3 shows the pattern of transmission of *P aeruginosa* strains of pulsotype A among neonates during the 52 weeks of the period under investigation. A stepwise pattern was evident, but in some weeks the strain was not isolated, suggesting the possible contribution of an environmental reservoir to the persistence of the strain in the NICU.

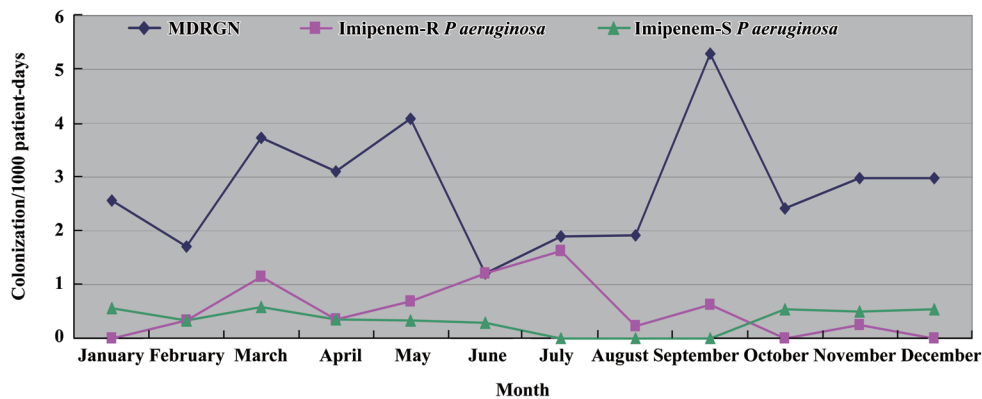
We looked at the influence of selected clinical parameters at admission and during the NICU stay for their association with colonization with at least one strain of MDRGN, with imipenem-resistant, MBL-producing and imipenem-susceptible *P aeruginosa* strains. The association was initially tested by univariate analysis (Table 2). The only characteristic at admission significantly associated with colonization by *P aeruginosa* was a higher birthweight (Table 2). Furthermore, the NICU procedures most strongly associated with the acquisition of a colonization by MBL-producing *P aeruginosa* proved to be infant feeding, length of stay and administration of ampicillin-sulbactam (Table 2). Of interest, all of these



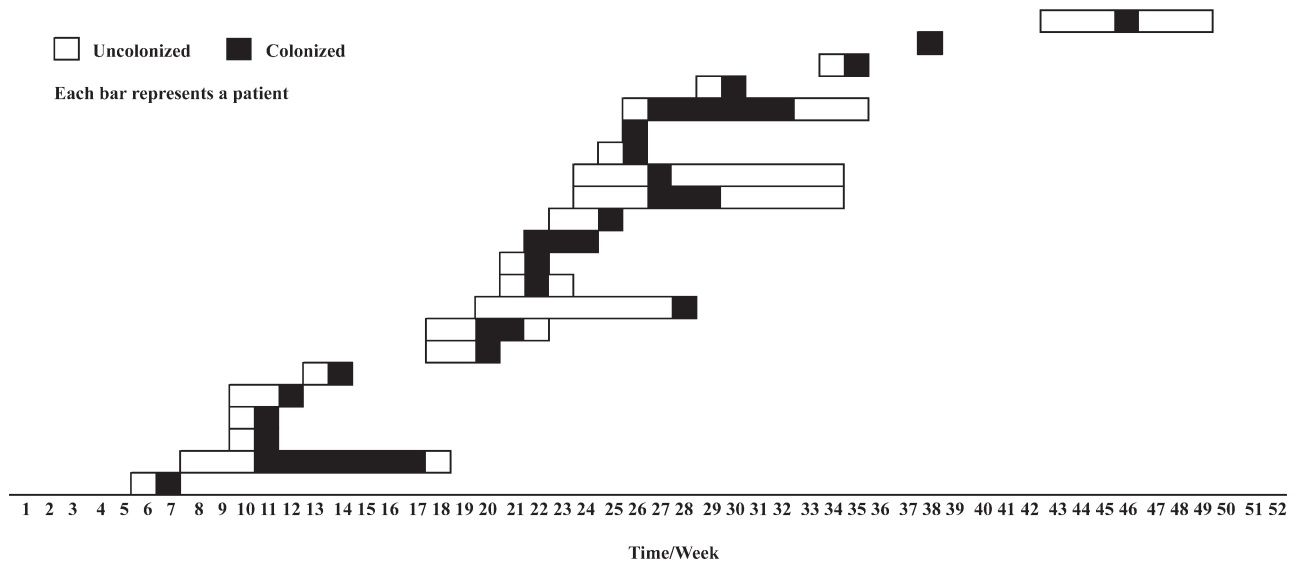
**Figure 2.** PFGE profiles (pulsotypes) of genomic DNA of representative *Pseudomonas aeruginosa* isolates, digested by *Xba*I. Lanes: MW, Braenderup H9812; 1 and 2, pulsotype C; 3, pulsotype D; 4, 5, 7, 8 and 10, pulsotype A (imipenem-resistant clone); 6, pulsotype E and 9, pulsotype F.

characteristics, except for birthweight and length of stay, significantly differed between neonates colonized by imipenem-resistant and imipenem-susceptible isolates of *P aeruginosa* (Table 2).

The risk of colonization in neonates positive for *P aeruginosa* of pulsotype A compared with those culturing positive for other multiresistant Gram-negatives, including imipenem-susceptible *P aeruginosa*, was positively correlated with breast milk feeding (RR=1.6, 95% CI, 1.3–1.9;  $P<0.001$ ) and administration of ampicillin-sulbactam (RR=1.2, 95% CI, 1.1–1.5;  $P=0.01$ ), and inversely correlated with exclusive feeding by formula



**Figure 1.** Incidence density of cases of imipenem-resistant, MBL-producing *Pseudomonas aeruginosa*, imipenem susceptible *P aeruginosa* and other MDRGNs colonization in a NICU, Palermo, Italy. 2003 January – 2004 January.



**Figure 3.** Pattern of transmission of *Pseudomonas aeruginosa* pulsotype A during the 12-month period of study in a NICU, Palermo, Italy. 2003 January to 2004 January.

**Table 2.** Risk factors associated with MDRGN colonization during the study period in a NICU, Palermo, Italy, 2003 January to 2004 January. Birthweight and length of stay are expressed as mean $\pm$ SD.

Characteristic	Colonized with other species of MDRGN <i>n</i> =80 (%)	Colonized with imipenem-resistant, MBL-producing <i>Pseudomonas aeruginosa</i> <i>n</i> =22 (%)	Colonized with imipenem-susceptible <i>Pseudomonas aeruginosa</i> <i>n</i> =14 (%)	<i>P</i>
Birth weight (g)	1993.5 (740.6)	2352.3 (1130.7)	2603.57 (757.2)	0.019
Length of stay (d)	16.8 (19.0)	9.6 (5.9)	7.6 (8.7)	0.053
Breast milk feeding	35 (43.8)	21 (95.5)	6 (42.9)	<0.0001
Formula feeding	45 (56.3)	1 (0.5)	8 (57.1)	<0.0001
Ampicillin-sulbactam	35 (43.8)	15 (68.2)	3 (21.4)	0.019

(RR=0.7, 95% CI, 0.6–0.8;  $P$ <0.001). The administration of gentamicin (RR=1.1, 95% CI, 0.9–1.4;  $P$ =0.06) was not associated.

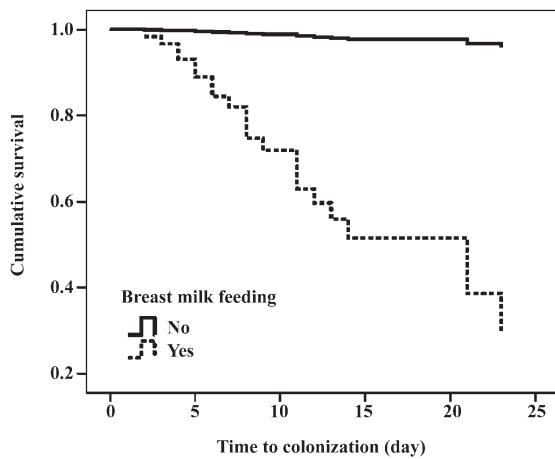
The significant variables at  $P$  0.10 were further analyzed in the Cox regression model. A summary of results is provided in Table 3. In this model, birthweight of more than 2500 g (HR, 3.66; 95% CI, 1.21–11.13) and breast milk feeding (HR, 29.16; 95% CI, 3.91–217.69) were independently associated with an increased risk of colonization by MBL-producing *P. aeruginosa*, whereas length of stay of more than 2 weeks (HR, 0.10; 95% CI, 0.00–0.11) and exclusive feeding by formula (HR, 0.18; 95% CI, 0.05–0.61) were inversely associated.

A survival curve comparing breast milk feeding status and number of days to first imipenem-resistant, MBL-

**Table 3.** Hazard ratios estimating the risk of colonization by imipenem-resistant, MBL producing *Pseudomonas aeruginosa* during the study period in a NICU, Palermo, Italy, 2003 January to 2004 January.

Variable	Hazard ratio (95% CI)	<i>P</i>
Birthweight (g)		
≤1500	1.00	
1501–2500	1.31 (0.41–4.23)	0.648
>2500	3.66 (1.21–11.13)	0.022
Length of stay (weeks)		
≤1	1.00	
2	0.004 (0.00–2.84)	0.099
>2	0.10 (0.00–0.106)	0.011
Breast milk feeding	29.16 (3.91–217.69)	0.001
Formula feeding	0.18 (0.05–0.61)	0.006
Ampicillin-sulbactam	1.50 (0.61–3.72)	0.379

producing *P aeruginosa* isolation is shown in Figure 4.



**Figure 4.** Survival estimates of 116 neonates, showing pulsotype A *Pseudomonas aeruginosa* colonization according to breast milk feeding.

### Discussion

The present study describes the transmission among 22 neonates admitted to the NICU throughout 12 months of a strain of imipenem-resistant *P aeruginosa* producing a novel *blaVIM* sequence. Colonization and infection by *P aeruginosa* in hospitalized neonates has been previously described<sup>[1,2,10]</sup>. However, the drug resistance pattern of the strain detected in our investigation is of special concern, because of the threat to the clinical utility of carbapenems, important therapeutic agents for the treatment of severe infections with MDRGN, particularly those carrying genes for extended-spectrum  $\beta$ -lactamases<sup>[7]</sup>. Although this organism was detected by surveillance cultures, we detected no infections in colonized neonates. Thus, its presence in the NICU would normally have been undetected.

As in previous studies, molecular typing by PFGE proved to be an invaluable tool to discriminate unique from shared strains and trace transmission routes. In particular, *P aeruginosa* isolates identified in the NICU were clearly differentiated in two groups: indistinguishable imipenem-resistant and MBL-producing isolates, an epidemiological synonym of clonality and cross-transmission, and highly heterogeneous imipenem-susceptible isolates, with only 2 micro-clusters of colonization cases.

In this study we found an association between colonization by a well-defined imipenem-resistant, MBL producing *P aeruginosa* strain and breast milk feeding.

Contamination of human breast milk by *P aeruginosa* in NICU has been described in published reports<sup>[13,14]</sup>. Of particular interest, an outbreak in France in 2001 was caused by contamination of a milk bank pasteuriser and bottle warmer<sup>[13]</sup>. On the other hand, appropriate preparation and handling of formula for neonates in health-care facilities has received great attention since some years, whereas safe handling of human milk has been focused on only more recently<sup>[20]</sup>. High risk neonates are often unable to directly breastfeed. In these cases, mechanical expression, use of collection kits, cold storage, transport, and warming need to be carried out with techniques adequate to prevent contamination of milk<sup>[21]</sup>. Accordingly, guidelines of the American Dietetic Association have recently been updated, also including new chapters on handling of expressed human milk<sup>[20]</sup>.

The pattern of spread documents some periods of cross-transmission, when *P aeruginosa* pulsotype A was recovered from neonates hospitalized during overlapping intervals, alternating with apparent eclipsing phases of the strain in the NICU. In many outbreak and non-outbreak contexts, the principal reservoir of MDRGN in NICU has proved to be the intestinal tract of the neonates and contaminated hands of healthcare workers the major vehicle of the continued dissemination of cross-colonizing strains<sup>[10,11]</sup>. Analytical limits of the detection method and timing of surveillance cultures might account for the holes in the transmission chain during the 12 months of observation. However, the apparent interruptions in the transmission route of the *P aeruginosa* strain could have been likely overcome by some environmental reservoir responsible for survival and perpetuation of the strain in NICU. Recovery of Gram-negative from inanimate surfaces, patient care equipment, contaminated fluids, water sources and water reservoirs has been repeatedly reported, with special reference to *P aeruginosa*<sup>[1,5]</sup>. Examination of formula, milk handling room and environmental flora was beyond the purposes of the study and was not carried out during the period under study. Therefore, association between the endemic imipenem-resistant *P aeruginosa* strain and the breastfeeding handling chain was based on epidemiological, but not microbiological, evidence. The results of the study were presented to the NICU staff and resulted in more emphasis on infection control measures; mainly hand washing, strict separation of clean and dirty procedures, attention to the general hygiene of rooms and equipment and safer management of all feeding-related aspects. Moreover, possible environmental reservoirs were sanitized or eliminated. Follow-up surveillance periods

have been repeatedly conducted, and have documented the interruption of the transmission chain.

This surveillance study identified a specific practice likely responsible for the sustained circulation of imipenem-resistant, MBL producing *P aeruginosa* in the NICU under investigation. The ability to specifically detect circulation of clinically relevant strains through surveillance, timely hygienic interventions and cooperation by NICU personnel are crucial to minimize or control health care-associated infections.

As colonization by a multidrug-resistant organism may lead to serious consequences in NICU, such a study may highlight the need for development and application of strategies to prevent an invaluable nutrition support from becoming an insidious risk factor for acquisition of health care-associated infections.

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### Author contribution

Caterina MAMMINA, Paola DI CARLO, and Domenico CIPOLLA designed research; Matilde TANTILLO, Maria Rosa Anna PLANO, and Angela MAZZOLA performed research; Caterina MAMMINA, Paola DI CARLO, Alessandra CASUCCIO, and Giovanni CORSELLOAC analyzed data and wrote the paper.

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