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Serratia marcescens in a neonatal intensive care unit: two long-term multiclone outbreaks in a 10-year observational study

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SUMMARY

We investigated two consecutive *Serratia marcescens* (*S. marcescens*) outbreaks which occurred in a neonatal intensive care unit (NICU) of a tertiary level hospital in North Italy in a period of 10 years (January 2003-December 2012). Risk factors associated with *S. marcescens* acquisition were evaluated by a retrospective case-control study. A total of 21,011 clinical samples was examined: *S. marcescens* occurred in 127 neonates: 43 developed infection and 3 died. Seven clusters were recorded due to 12 unrelated clones which persisted for years in the ward, although no environmental source was found. The main epidemic clone A sustaining the first cluster in 2003 reappeared in 2010 as an extended-spectrum β-lactamase (ESBL)-producing strain and supporting the second epidemic. Birth weight, gestational age, use of invasive devices and length of stay in the ward were significantly related to *S. marcescens* acquisition. The opening of a new ward for non-intensive care-requiring neonates, strict adherence to alcoholic hand disinfection, the timely identification and isolation of infected and colonized neonates assisted in containing the epidemics. Genotyping was effective in tracing the evolution and dynamics of the clones demonstrating their long-term persistence in the ward.

KEY WORDS: Serratia marcescens, Outbreak, Neonatal intensive care unit, Molecular epidemiology.

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INTRODUCTION

S. marcescens, a ubiquitous environmental gram-negative organism, is a recognized cause of outbreaks in NICUs (Hejazi *et al.*, 1997). It is an important opportunistic pathogen and it may cause serious infections in newborns including sepsis, pneumonia, brain abscess and meningitis (Polilli *et al.*, 2011). Reports of *S. marcescens* outbreaks in NICUs have increased in the last decade (Miranda-Novales *et al.*, 2003; Giles *et al.*, 2006) identifying low birth weight and prematurity as major risk factors for invasive infections with a significant impact on both infant morbidity and mortality (Arslan *et al.*, 2010).

Several possible environmental sources of *S. marcescens* in hospitals have been implicated (Madani *et al.*, 2011; Cullen *et al.*, 2005; Civen *et al.*, 2006; Sartor *et al.*, 2000), but no point of source has been identified (Prasad *et al.*, 2001; Maragakis *et al.*, 2008). Colonized symptom-free patients have been recorded as the most important reservoir of the bacterium (Fleisch *et al.*, 2002; Miranda *et al.*, 1996; Voelz *et al.*, 2010). High patient density and low nurse-to-patient ratio have also been reported as potential sources for the spread of the pathogen via contaminated hands of healthcare workers (Milisavljevic *et al.*, 2004; Gillespie *et al.*, 2007). Typically the management of such outbreaks includes patient isolation, cohort nursing of the infected and colonized infants, strict hygienic measures, and ward

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closure (Assadian *et al*., 2002; Buffet-Bataillon *et al*., 2009; Perotti *et al*., 2007).

Once entrenched in an NICU, *S. marcescens* is difficult to eradicate and this condition causes persistent problems and occasionally recurrent outbreaks (Bosi *et al.*, 1996; Miranda *et al*., 1996; Sarvikivi *et al*., 2004). Thus, a satisfactory typing method is important in determining the source of infection, the dynamics and the evolution of *S. marcescens* strains during outbreaks (Hejazi *et al*., 1997; Steppberger *et al*., 2002; David *et al*., 2006; Enciso-Moreno *et al*., 2004; Ligozzi *et al*., 2010). In recent times ESBLs have had a large spread in the hospital environment and outbreaks in NICU settings due to ESBL-producing *S. marcescens* have been reported in Italy (Villari *et al*., 2001; Crivaro *et al*., 2007).

Herein, we detail the investigation of two long-term multiphase outbreaks, both caused by several genetically unrelated *S. marcescens* strains, that occurred in a NICU of a tertiary care hospital in Northern Italy, within a 10-year prospective observational period from January 2003 to December 2012.

Aims of the present study were:

1) to analyze the molecular epidemiology of *S. marcescens* isolates in outbreaks;
2) to evaluate risk factors for *S. marcescens* acquisition in NICU patients through a case-control study;
3) to implement appropriate control measures.

MATERIALS AND METHODS

This study is consistent with the Declaration of Helsinki and was subject to an assessment of the local ethics committee.

Setting

The NICU of Modena University Hospital has 20 beds for the admission of critically ill newborn infants: 2 isolation rooms, each with a single bed, 2 intensive care rooms, each with 3 intensive care beds and 12 intermediate care beds shared amongst 3 rooms. Nurse-to-patient ratio ranges from 1:3 to 1:4 for intensive care beds and 1:6 for intermediate care beds.

Approximately 400 newborns are admitted to the NICU annually, born at the same centre or in provincial hospitals.

S. marcescens had never been isolated in the NICU before January 2003. From January to March 2003, 2 cases of *S. marcescens* invasive infection and 4 cases of colonization marked the onset of an outbreak and prompted an investigation. Over a 55 month period, increases in clinical isolates demonstrated 5 epidemic clusters and 1 sporadic case, lasting for varying periods and separated by varying time intervals. The outbreak persisted until July 2007 involving 67 newborns and was considered to be over in August 2007, after the discharge of the last patient infected by *S. marcescens*. No other case was recorded in the NICU until January 2010 when a resumption in clinical *S. marcescens* isolates identified the onset of a new outbreak which lasted 20 months and involved 58 patients. The outbreak was declared to be over after the last colonized infant was discharged in August 2011. As of this date to December 2012, only a few sporadic cases of *S. marcescens* colonization were recorded in the ward.

Information regarding staffing and crowding of the NICU was reviewed for the period of the outbreaks, starting from 2 weeks prior to the first one, up to 2 weeks after the last *S. marcescens* positive culture in each cluster.

Microbiology

During outbreak 1, weekly microbiological cultures from throat and bowel of all NICU patients were instituted. The bacteriological screenings were stopped after 2 negative records following the discharge of the last *S. marcescens* contaminated patient. The carriage testing on all new hospitalized neonates continued even after the last case in July 2007. At the onset of outbreak 2, microbiological screenings were taken weekly on admitted patients. At a later stage, during both outbreaks, several samples from nurses' and physicians' hands were examined to identify possible carriers.

Large environmental investigations were performed during clusters III and IV (outbreak 1) and cluster VI (outbreak 2). Surveillance samples including air, water, baby incubators, antiseptics, soap, distilled water, humidifiers, inhalation therapy equipment, sinks, taps, monitors, stethoscopes and room surfaces were collected and culturally analyzed to identify possible sources and reservoir of infection.

Clinical specimens included patient blood samples, cerebrospinal fluid (CSF), tracheal and bronchial aspirate, urine, surgical wound matter, gastric juices, stool, conjunctival swabs and throat swabs. Clinical and surveillance samples were processed by standard methods (Caroll *et al.*, 2007). All isolates were studied for phenotypic identification and antibiotic susceptibility by means of an automated system (VITEK 2, BioMérieux, France). The tested antimicrobial agents included: ampicillin, ampicillin+clavulanate, aztreonam, cefazolin, ceftazidime, cefotaxime, ceftriaxone, piperacillin, piperacillin/tazobactam, gentamicin, amikacin and imipenem. ESBL activity and overproduction of Ampicillinase C (AmpC) were tested by standard methods (Crivaro *et al.*, 2007). The identified strains were stored at -70°C for further studies.
Molecular typing of isolated strains was performed. Only the first patient isolates were included in the genetic analysis. The genomic relatedness was analyzed by RFLP-PCR, using the flagellin gene as the target for the PCR amplification and RFLP analysis, as described by Parvaz *et al.*, 2002. Aliquots of amplification products were digested with *Alu*I endonuclease (Roche Diagnostic, Penzberg, Germany). Restriction fragment were analyzed by agarose (4%) gel electrophoresis. The analysis of restriction patterns was elaborated using Diversity Database Software (Bio-Rad Laboratories, Milan, Italy).

Case-control study
A retrospective case-control study was conducted among newborns hospitalized in the NICU during the outbreak. Cases were defined as neonates infected or colonized by *S. marcescens*. Only the first positive culture was used to define a case. A positive rectal and/or pharyngeal culture in the absence of clinical signs and/or symptoms of infection defined the carriage. The presence of clinical signs and/or symptoms compatible with infection and *S. marcescens* isolated from normally sterile sites defined the infection. Controls were defined as neonates admitted to the NICU during the outbreak, in whom *S. marcescens* was not isolated in any clinical specimens. Medical records were reviewed. Data included sex, gestational age, birth weight, underlying disorders, administration of critical care and NICU length of stay (LOS). The associations were calculated using contingency table methods and tested for significance using Pearson's chi-square test.

RESULTS

Outbreaks
Seven epidemic clusters followed in the NICU during the study period, spanning 1-16 months. Of 127 neonates involved, 84 (66%) were symptom-free colonized while 43 (34%) developed clinical infections. The temporal trend of *S. marcescens* clinical cases detected in the ward is shown in Figure 1. On total cases the median gestational age was 29.5 weeks (27-33.7) with a median birth weight of 1,192 grams (842.5-2042.5). Vaginal delivery was recorded in 24% of patients. Eighty-five percent of newborns were delivered preterm, and 13% were twins. Babies were born at the hospital in 85% of cases. The median LOS in the NICU was 64 days (26-94).
The median LOS in NICU before *S. marcescens* acquisition was 15.5 days (7.75-34.25). Central venous catheterism (CVC) in 39.3% of cases and mechanical ventilation (MV) in almost 30.7% of cases were administered. Relative to the infected patients, their median gestational age was 28 weeks (26.5-34) with a median birth weight of 900 grams (737-1988). Clinical infections had different severity and localization. The most severe pictures were sepsis (21%) and pneumonia (19%), related to 3 fatal cases. The overall mortality was 7%. Individual data for 18 patients with systemic infections are presented in Table 1. Other minor infections included conjunctivitis (37%), urinary infections (25%) and lymphadenitis (2%).

Infection control measures
During the first cluster, hospital infection control strategies and hand-washing protocols were employed, including the strict observance of the use of new gloves for each patient, and the adoption of alcohol-based antiseptic gel in the intensive care rooms. The NICU was closed to new admissions for 1 month. By cluster III, a strict isolation policy was instituted; the unit was divided into infected and non-infected areas and the nurses were cohorted. In addition to this, intensive staff educational training regarding hand-washing was carried out and the operational protocol was modified, introducing the routine use of alcohol-

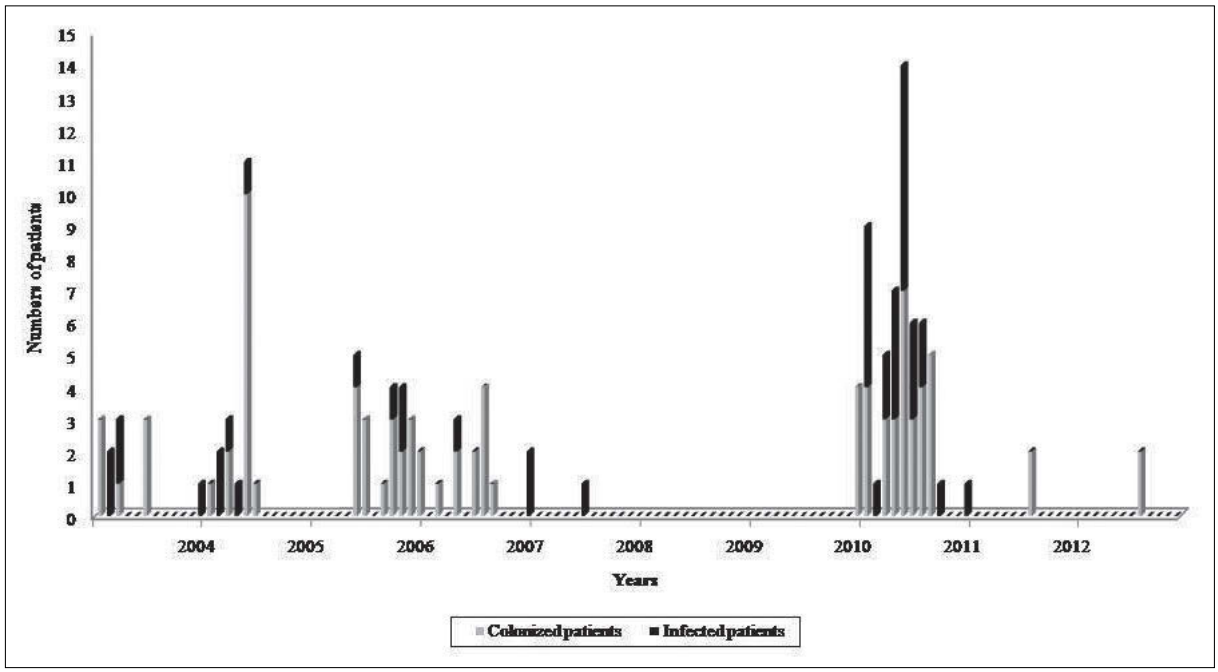


FIGURE 1 - *Patients infected or colonized by S. marcescens distributed by date of the first positive culture from January 2003 to December 2012.*

based antiseptic gel. This practice was retained and is still in use today. During cluster IV, a new ward reserved for newborns who did not need intensive care was created. The NICU was closed again to new admissions for two weeks in June 2006, during cluster VI.

The restriction of hospitalizations was then maintained until the termination of the epidemic to ensure the proper application of the cohorting and of the other rules of containment which had been instituted.

During outbreak 1, the NICU was found to be repeatedly overcrowded. The mean NICU occupation rates were consistently high and frequently exceeded standard levels, particularly from January 2003 to December 2005 before the opening of a new ward. During this period the occupation rates reached values of 106%, 113% and 116% respectively in the three first clusters. Subsequently, the average has not exceeded 93%. Nursing and professional medical team services however remained standard during the overcrowding periods, resulting in a reduced nurse-to-patient ratio. Overcrowding related to a high rate of bed occupancy was not found in connection with the resumption of the outbreak in January 2010. However, from August 2009 to May 2010 renovations in the NICU required the transfer of neonates to a new open space ward. This temporary arrangement proved inadequate for the proper management of patients and led to a situation of high patient density coinciding with the onset and peak (highest number of cases) of cluster VI.

Microbiology

A total of 84 symptom-free newborns resulted *S. marcescens* colonized, 61% in pharynx, 39% in bowel, 24% in both sites. The rate of carriers referring to all patients detected as *S. marcescens* positive in the NICU during each cluster epidemic was 55% in cluster I, 100% in cluster II, 68% in cluster III, 85% in cluster IV, 0% in cluster V, 57% in cluster VI, 100% in cluster VII. Cultures of all samples taken from the hands of healthcare workers did not show positivity for *S. marcescens*. With regard to the environmental monitoring, the investigation failed to reveal a point source for the outbreaks. During outbreak 1, despite the numerous environmental samples examined, only one strain was obtained from the nozzle of a soap dispenser. In outbreak 2, two *S. marcescens*

TABLE 1 - *Characteristics of 18 neonates infected with S. marcescens in the NICU from 2003 to 2010.*

Cluster/case number	*Date m/y*	*RFLP-PCR (pattern)*	*Birth location*	*Gestational age (wks)*	*Birth weight (gr)*	*Risk factors or underlying disorders*	*Type of infection*	*Isolate - body site*	*LOS in NICU prior to infection (days)*	*Total LOS in NICU (days)*
I/1	03/2003	A	Inborn	28	840	CVC, MV	Pneumonia	Respiratory	16	94
I/2	03/2003	A	Outborn	33	980	CVC	Sepsis, meningitis, brain abscess	Blood, gastrointestinal	6	66
I/3*	03/2003	A	Outborn	27	865	CVC	Sepsis	Blood, gastrointestinal, respiratory	15	19*
III/4	01/2004	C	Inborn	25	900	CVC, VPS	Sepsis	Blood	54	68
III/5	03/2004	C	Inborn	27	800	-	Pneumonia	Respiratory	61	92
III/6	03/2004	D	Outborn	35	1,876	CVC	Sepsis	Blood, pharynx, gastrointestinal	0	26
III/7	04/2004	D	Inborn	24	749	MV	Sepsis	Blood, gastrointestinal	3	109
III/8	06/2004	B	Inborn	24	632	CVC, MV	Pneumonia	Pharynx, respiratory	9	14*
IV/9	10/2005	B	Inborn	23	660	Cardiopathy, NEC, retinopathy	Pneumonia	Respiratory	12	186
IV/10	05/2006	G	Inborn	26	680	CVC	Clinical sepsis	Pharynx, gastrointestinal	64	82
V/11	01/2007	F	Inborn	27	664	CVC, MV	Pneumonia	Respiratory	39	73
V/12	01/2007	C	Inborn	35	2,593	CVC, MV, surgery	Sepsis	Blood, surgical wound	12	38
V/13	07/2007	A	Inborn	38	2,720	CVC, MV, surgery	Sepsis	Blood	51	67
VI/14	05/2010	E	Inborn	25	735	CVC, MV, surgery	Sepsis	Blood, gastrointestinal	41	97
VI/15	05/2010	H	Inborn	25	857	CVC, MV, surgery	Pneumonia	Respiratory	21	140
VI/16	06/2010	A	Inborn	26	950	CVC, MV	Sepsis	Blood, gastrointestinal	3	10*
VI/17	06/2010	A	Inborn	26	690	CVC, MV	Pneumonia	Respiratory, fharynx	85	142
VI/18	07/2010	A	Inborn	33	1970	Anatomics alterations VM, CVC	Pneumonia	Respiratory, conjunctiva, gastrointestinal	12	173

RFLP-PCR: Restriction Fragment Length Polymorphism - Polymerase Chain Reaction; LOS: length of stay; CVC: central venous catheterism; MV: mechanical ventilation; NEC: necrotizing enterocolitis; Clinical sepsis: presence of clinical signs and symptoms of sepsis infection with at least two abnormal laboratory results when blood culture is negative. *Patient death.

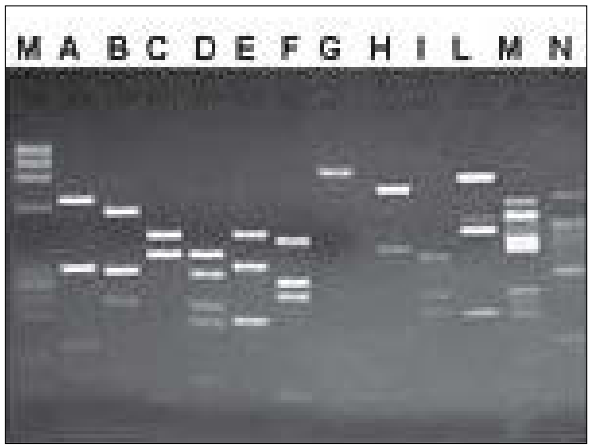
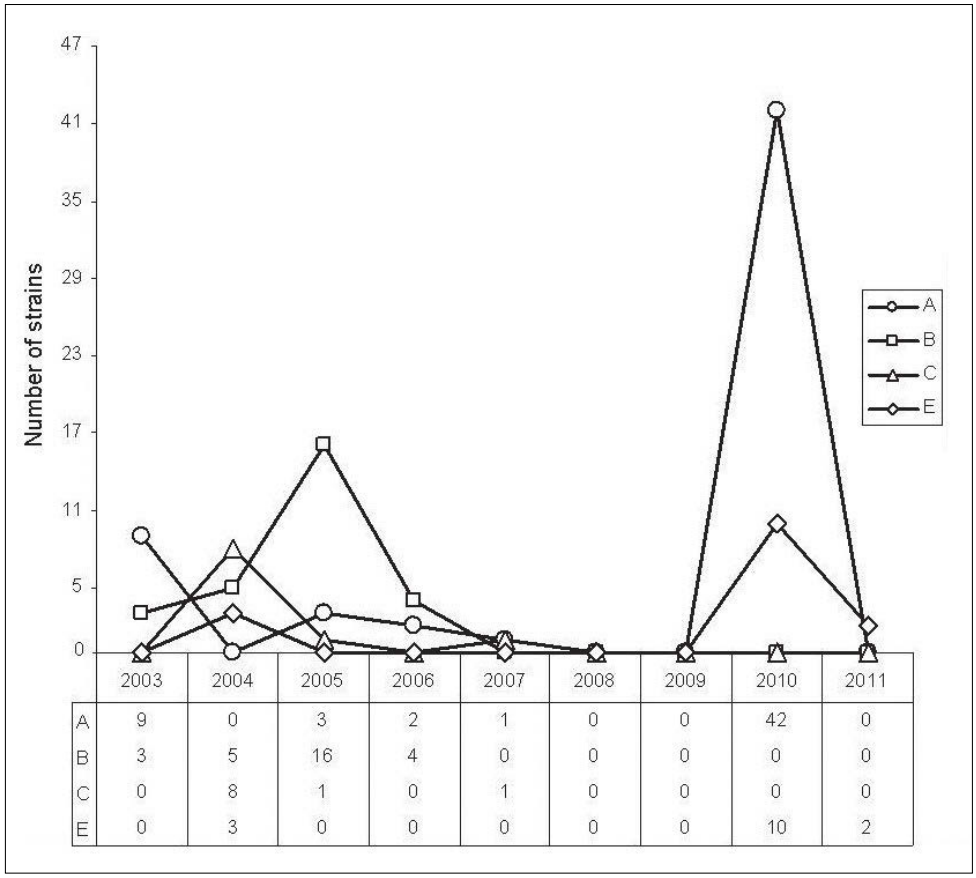


FIGURE 2 - *Representative RFLP-PCR banding patterns of S. marcescens isolates recovered during 2 outbreaks in NICU. Lane 1: DNA molecular standard; lane 2: pattern Ax57 strains; lane 3: pattern Bx28 strains; lane 4: pattern Cx10 strains; lane 5: pattern Dx2 strains; lane 6: pattern Ex15 strains; lane 7: pattern Fx4 strains; lane 8: pattern Gx5 strains; lane 9: pattern Hx1 strain; lane 10: pattern Ix1 strain; lane 11: pattern Lx1 strain; lane12: pattern Mx1 strain; lane 13: pattern Nx2 strains.*

strains were found, one from a surface swab and one from respiratory equipment, probably contaminated by the hands of operators.

During the study period, a total of 21,011 samples were analyzed. A total of 467 strains were identified from: blood (30), tracheal aspirate (34), urine (36), surgical wound matter (2), gastric juice (4), conjunctival swabs (34), throat swabs (216) and stool (121), with the highest value of positivity found in urine and throat swabs cultures (7% in both) followed by tracheal aspirate and stool cultures (5% and 6% respectively). Phylogenetic analysis classified the 127 examined strains into 12 patterns (A, B, C, D, E, F, G, H, I, L, M, N) of which four more frequently found: 57xA (45%), 28xB (22%),15xE (12%) and 10xC (8%) Figure 2. The trend of the different clones during the observation period is shown in Figure 3. In outbreak 1, genotype A was related to clusters I and IV, and to the last sporadic case. Genotype B was found in clusters II, III and IV. Genotype C appeared in clusters III and persist-



	2003	2004	2005	2006	2007	2008	2009	2010	2011
A	9	0	3	2	1	0	0	42	0
B	3	5	16	4	0	0	0	0	0
C	0	8	1	0	1	0	0	0	0
E	0	3	0	0	0	0	0	10	2

FIGURE 3 - *Temporally trend of four main epidemic clones of S. marcescens during 2 outbreaks in NICU.*

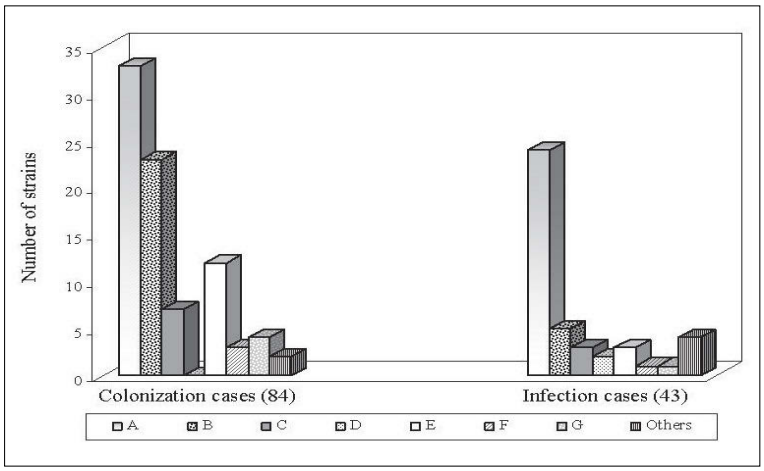


FIGURE 4 - *Distribution of unrelated S. marcescens genotypes in infection and colonization cases in NICU.*

TABLE 2 - *Statistical analysis of risk factors associated with S. marcescens infection and colonization*

Characteristics	*S. marcescens case patients n (%)*				*p value*
	Infections	*Colonizations*	*Total infections/colonizations*	*Total control patients*	
	43 (33.8)	84 (66.1)	127	160	
Sex n (%)					0.867
Males	21 (48.8)	49 (58.3)	72 (56.7)	88 (55.0)	
Females	22 (51.2)	35 41.7)	55 (43.3)	72 (45.0)	
Birth weight					0.001
<1000	22 (51.2)	21 (25.0)	43 (33.8)	18 (11.2)	
>1000 <1500	7 (16.2)	25 (29.7)	32 (25.1)	29 (18.1)	
>1500 <2500	10 (23.2)	20 (23.8)	30 (23.6)	52 (32.5)	
>2500	4 (9.3)	18 (21.4)	22 (17.3)	61 (38.1)	
Gestational age					0.001
≤26 weeks	13 (30.2)	11 (13.0)	24 (18.9)	6 (3.7)	
27 to 32 weeks	19 (44.1)	42 (50.0)	61 (48.0)	50 (32.2)	
33 to 37 weeks	8 (18.6)	15 (17.8)	23 (18.1)	56 (35.1)	
>37 weeks	3 (6.9)	16 (19.0)	19 (14.9)	48 (30.0)	
Critical care					0.01
CVC	6 (13.9)	9 (10.7)	15 (11.8)	28 (17.5)	
MV	2 (4.6)	7 (8.3)	9 (7.0)	24 (15.0)	
CVC + MV	13 (30.2)	7 (8.3)	20 (15.7)	36 (22.5)	
CVC + MV + surgery	6 (13.9)	3 (3.5)	9 (7.0)	11 (6.9)	
None	16 (37.2)	58 (69.0)	74 (58.2)	61 (38.1)	
Underlying disorders					0.295
Hydrocephalus	0	2 (2.3)	2 (1.5)	4 (2.5)	
Intrauterine growth retard	5 (11.6)	3 (3.5)	8 (6.3)	15 (9.3)	
Anatomic alterations	4 (9.3)	9 (10.7)	13 (10.2)	7 (4.3)	
Respiratory distress	18 (41.8)	14 (16.6)	32 (25.1)	37 (23.1)	
None	16 (37.2)	56 (66.6)	72 (56.7)	97 (60.6)	
Mean LOS, days, SD, (range)			57.8±42.61 (1-192)	27.6±26.66 (2-120)	

CVC: central venous catheterism; MV: mechanical ventilation; LOS: length of stay.

ed in cluster IV and V. Other minor clones (D, E, F, G) have emerged in clusters III, IV and V. In outbreak 2, the genetic strain A recurred as dominant clone with strain E and other new minor strains (H, I, L, M, N). The relation of the different genotypes to cases of infection and colonization is shown in Figure 4.

The prevalent clones (A, B, C, E) resulted in both infection and colonization groups. Sepsis was associated with 4xA, 2xC, 2xD, 1xE, 1xG, pneumonia with 3xA, 2xB, 1xC, 1xF, 1xH and cases of mortality with 2xA and 1xB. Genotype A proved to be the main epidemic.

Regardless of the different genetic pattern, all *S. marcescens* isolates except one (type E) in outbreak 1 displayed identical antibiograms. All strains belonging to patterns A, B, C, D, F and G were susceptible to ceftriaxone, ceftazidime, cefotaxime, cefepime, imipenem, meropenem, aztreonam, ciprofloxacin, levofloxacin, gentamicin, piperacillin, amikacin and trimethoprim-sulphamethoxazole. Pattern E strains were resistant to gentamicin, piperacillin and amikacin. No reduced susceptibility to extended-spectrum cephalosporins was found in isolated strains (ceftazidime and cefotaxime minimum inhibitory concentration ≤1 μ/mL).

During outbreak 2, instead, ESBL activity emerged in strains of type A and was found in 40 of 42 of isolates. The same character of antibiotic resistance was also highlighted in all the isolates of types I and L. All ESBL positive strains showed a similar pattern with limited sensitivity to imipenem, meropenem, levofloxacin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole. The search for beta-lactamase AmpC was constantly negative. The other strains belonging to pattern E, H, M and N showed no ESBL activity with a pattern of resistance limited to ampicillin and cefazolin.

Case-control study

A total of 287 patients were considered in the case-control study, including 127 cases and 160 controls. Statistical analysis identified birth weight, gestational age, required critical care and LOS as significant risk factors (RFs) associated with *S. marcescens* clinical illness or carriage (Table 2). Sex and underlying disorders were not statistically significantly associated with *S. marcescens* acquisition.

DISCUSSION

A program of "alert" organisms surveillance including *S. marcescens* in the mainly high-risk settings was operating in Modena University Hospital at the onset of the first epidemic cluster. This program provides preventive strategies such as: daily monitoring of "alert" pathogens, storage of isolates, filing and computer analysis of data by a software package. This allowed us to recognize the epidemic emergency in a timely fashion and to start appropriate control measures.

The results of these activities implemented in the NICU seem to have affected the severity and containment of the epidemics, as found in other studies (Polilli *et al.*, 2011; Maltezu *et al.*, 2012; Rabier *et al.*, 2008; Bayramoglu *et al.*, 2011; Jang TN *et al.*, 2001). In particular, the associated mortality rate of 7% that we recorded is lower than those reported in literature, ranging between 10 and 20% (Fleish *et al.*, 2002; Arslan *et al.*, 2010; Bizzarro *et al.*, 2007).

Periodic cultural screening on all newborns and the subsequent genotyping of the isolates, allowed the quick identification of both colonized and symptomatic patients, and the timely cohorting and strategic clinical response. Moreover, the screening facilitated precise tracking of the spread of the bacterial strains in the NICU. The diffusion of the molecular typing results among staff members was deemed to have played an important educative role regarding the cross-transmission mechanisms of the epidemic strains, assisting in underlining the appropriateness of proposed infection control measures.

High patient density and under-staffing, which have been identified as important reasons for breaches in standard hygiene procedures, are suspected to contribute to the outbreaks reported in this study.

Overcrowding was addressed during cluster IV with the opening of a new ward for neonates not requiring intensive care. Since then, only 3 infection cases were recorded until the conclusion of the first epidemic and no other clinical cases occurred during the subsequent 29 months.

The case control studies identified significant risk factors including reduced gestational age and birth weight, intensive care support requirement and longer NICU stay which have also been highlighted in other investigations (Voelz *et al.*, 2010).

Colonizations or less serious infections were more often associated with healthier, older newborn infants. The high percentage of carriers in pharynx and bowel found in the studied patients supports the suggestion that colonized babies are the most important reservoir of the microorganism (Miranda *et al.,* 1996; Voelz *et al*., 2010; Sarvikivi *et al*., 2004).

During the outbreaks, different *S. marcescens* genotypes were identified by RFLP-PCR. The identification of *Serratia* heterogeneous strains in long-lasting outbreaks seems mainly due to the genetic instability of this microorganism as well as entry of new strains in the ward, in accordance with other published findings (Parvaz *et al*., 2002; Manning *et al.,* 2001; Friedman *et al.,* 2008). A more virulent strain is usually responsible for clinical cases of infection, while other minor strains are usually associated with asymptomatic colonization (Maltezu *et al*., 2012; Rabier *et al*., 2008; Bayramoglu *et al*., 2011; Cimolai *et al*., 1997).

A relationship between genetic patterns and intrinsic virulence factors cannot be concluded in this report, as the different clones were related to both infection and colonization cases. Most of the clones were involved in more than one cluster and remained for a long time in the ward. Of special note, strain A involved in the first clinical cases was related again to the last case of outbreak 1 and to 42 cases of outbreak 2, therefore it persisted in the NICU for 8 years. In addition, genotype E, which appeared in May 2004, resurfaced in April 2010 during the second epidemic. These observations support the hypothesis that *S. marcescens* strains may determine environmental colonization persisting for a long time in the ward, even though investigations fail to find any point of source.

The index case of outbreak 1 was identified in an extremely low-weight preterm baby, born at our hospital by surgical delivery.

The newborn infant was immediately placed in the NICU and colonization occurred 36 days following admission. The source of the first infection was never identified. The onset of outbreak 2 in January 2010 is related to the admission to the NICU of a patient from another hospital, who had sepsis due to an ESBL-producing *Klebsiella pneumoniae* strain. A few days after her admission in the ward the baby was a carrier of ESBL-producing *S. marcescens* strain of type A. ESBL activity in *S. marcescens* might be likely acquired through horizontal gene transfer from the *K. pneumoniae* strain.

This ESBL-producing clone A spread rapidly in the ward claiming the second epidemic as the dominant strain. During both epidemics cross-transmission through staff hand or temporarily contaminated instruments was suggested by three isolates: one from the nozzle of a soap dispenser, one from a surface swab and one from a respiratory equipment. ESBL-producing *S. marcescens* strains had never been isolated before in the ward though ESBL-producing Gram-negative bacteria (mainly *K. pneumoniae* and *Escherichia coli*) have constantly increased in recent years in our NICU.

In fact, the ESBL isolation rate has risen from 10% in 2008 to 43% in 2010. During the peak of outbreak 2 in July 2010, 93% of patients had Gram-negative ESBL-producing strains. In particular, 23 neonates who gained *S. marcescens* had simultaneous colonization by other ESBL-producing bacteria, resulting in 4 cases of invasive infections.

Besides conjugal plasmid transfer, empiric antimicrobial therapy with ampicillin and gentamicin practised in this NICU might have contributed to selection and spread of ESBLs in the ward. The acquisition of new resistance traits can alter the microbial ecology of the department exacerbating the difficulty of treatment and control of micro-organisms involved in the outbreak. In particular, cause for concern is the emergence and persistence in the ward of the highly diffusive genetically mutated strain A.

The authors believe that *S. marcescens* may be endemic in this NICU requiring constant adhesion to the protocols introduced to avoid further epidemic spreads. Molecular typing of isolates proved to be a very useful tool for identification and tracing of consecutive epidemic clusters involving genetically unrelated clones. It should be considered that strain heterogeneity itself does not exclude an outbreak.

The results of this observation period confirm the ability of *S. marcescens* to implant in the wards, to remain even if not recognized for long periods and to determine new episodes in critical moments. Thus, it is essential to remain vigilant while maintaining strictly control strategies. In this centre's NICU the overcrowding limitation,

the cohorting and the isolation practices along with intensive staff training, performed to guarantee adherence to adopted surveillance protocols, have contributed to the control of *S.marcescens* spread in the ward. In addition to this, the measures applied for *S.marcescens* limitation seem to have had a positive impact on the cross-transmission of all multidrug-resistant Gram-negative bacteria in the NICU. In fact, the monitoring of the last two years of observation had shown a downward trend in ESBLs detection, recording rates of 10% in 2011 and 7% in 2012. Since the selection, the emergence and circulation of new multidrug-resistant strains are favoured by the same risk factors highlighted for *S. marcescens* acquisition, the same strategies can be validly applied in NICUs for the control of other horizontally transmitted pathogens.

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