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Molecular epidemiology of a *Malassezia pachydermatis* neonatal unit outbreak

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Abstract

The non–lipid-dependent yeast *Malassezia pachydermatis* is predominantly zoophilic but occasionally colonizes the human skin. This yeast caused an outbreak in a neonatal iIntensive care unit (NICU). This study aimed to describe the molecular epidemiology of this *M. pachydermatis* outbreak. All the *M. pachydermatis* isolates collected at a French University Hospital from January 2012 to April 2013 were included in the study. *M. pachydermatis* isolates, sampled from various biological samples sites in 25 patients, were identified via MALDI-TOF mass spectrometry and typed using intergenic-spacer 1 (IGS1) nucleotide sequence polymorphisms analysis. By analyzing 90 IGS1 sequences (including 43 deposited in GenBank), we found that of the 186 *M. pachydermatis* isolates, 47 were viable for typing and all of them clustered within type 3; 78.7% clustered within the 3D subtype; the remaining clustered within three newly described subtypes: 3E (4.3%), 3F (8.5%) and 3 G (8.5%). No particular subtype was associated with a collection site or a particular time period. This first molecular investigation of a *M. pachydermatis* outbreak in neonates showed that multiple genotypes can colonize the same neonate patient by. The source of this polyclonal outbreak could not be identified. It stopped after infection control measures, including the prohibition of a lipid-rich moisturizing hand cream used by the health care staff, had been implemented.

Key words: *Malassezia pachydermatis*, neonates, outbreak, typing, IGS1.

Introduction

The lipophilic yeast *M. pachydermatis* belongs to the resident skin flora of many animal species and is commonly isolated from the skin and mucus membranes of dogs. Yeasts overgrowth on the skin could be promoted by excessive sebum production and/or disruption of epidermal

barrier, which may occur in hypersensitivity diseases; keratinization disorders; infections, including ectoparasitic diseases; and endocrine disorders.[1,2] Whereas *M. pachydermatis* is the only primarily zoophilic species in the genus *Malassezia*, it occasionally colonizes the human skin and has also been isolated from various human biological samples (sputum, bronchial alveolar lavage, peritoneal fluid, etc.).[2,3] *M. pachydermatis* also causes blood-stream infection, especially in neonates, in whom this yeast has been involved in health-care associated outbreaks.[2,4,5] Several studies have stressed the importance of molecular typing to understand the clonal nature, the transmission routes and spreading mechanism of a fungus involved in an outbreak.[6] The genetic diversity of *M. pachydermatis* strains obtained from animals has been highlighted by analyzing the nucleotide sequences of the D2 variable region of the 26S rRNA gene and the chitin synthase 2 (CHS2) gene.[2,7,8] More recently, Sugita el al. identified three major types (1, 2, and 3) in *M. pachydermatis* isolated from dogs and cats, which were further separated into 10 subtypes (1A, 1B, 1C, 1D, 2A, 2B, 3A, 3B, 3C, and 3D, as detailed in Table 1) by using the 18S-26S rRNA intergenic spacer (IGS) 1 region.[9] The present study aimed to describe the molecular epidemiology of *M. pachydermatis* outbreak that occurred in a neonatal intensive care unit (NICU).

Methods

Patients and samples

From January 10 2012, date to diagnosis of the first *M. pachydermatis* fungemia, to February 2013, all infants with a birth weight lower than 3,100 gr hospitalized at the NICU of the University Hospital of Amiens (France) were systematically screened for the presence of *M. pachydermatis* colonization. A total of 390 samples were analyzed: 12 stools, 103 nasal, 108 axilla, 106 anal, 21 blood, 4 skin, 24 urine, and 12 respiratory samples. Additionally, for the purpose of outbreak investigation, two nasal swabs, two ear swabs, and one interdigital spaces swab were collected from 710 healthcare workers, including 120 electro-radiology manipulators and electro-physiologists, 110 physiotherapists, 330 nurses and nursery nurses, 45 assistants, and 105 medical doctors. We also collected 60 environmental samples, including: 37 electronic equipment (cardiac monitoring systems, mixers, phone, ultrasound probes, etc.) and 23 baby incubators. All samples were inoculated onto both Sabouraud dextrose (Bio Rad, Marne la Coquette, France)[10,11] and Chromagar *Candida* (Becton Dickinson, Le Pont de Claix, France) medium plates and incubated at 32°C for 7 days. A patient was considered colonized with *M. pachydermatis* if this yeast was cultured from at least one of his samples. Neither Dixon's nor Leeming-Notman medium were available at the hospital's laboratory. Therefore, only *M. pachydermatis* and no other *Malassezia* spp could be isolated. Yet this approach adequately addressed the investigation of the *M. pachydermatis* blood stream infections outbreak.

M. pachydermatis identification

Isolated yeast colonies were identified according to their macroscopic and microscopic features. The macroscopic features of the predominant colonies included their shape, size, color, consistency, and the characteristics of surrounding culture medium. Microscopic features of the yeast cells in culture were described after lactophenol staining and included the predominant morphology, size, and budding base of the yeasts at 1000X magnification. The strains were kept at −80°C. Both MALDI-TOF mass spectrometry identification and typing was performed at the Parasitology and Mycology laboratory of Marseille's University Hospital La Timone in October 2014.

MALDI-TOF mass spectrometry identification

The isolates were subcultured on Sabouraud's medium and incubated for 3–7 days at 32°C. MALDI-TOF mass spectrometry identification of the *M. pachydermatis* isolates was performed with a Microflex LT^{TM} (Bruker Daltonics, Germany) instrument, by comparing the isolate's spectra to an in-house reference spectra library that included *Malassezia* spp. reference spectra via the MALDI Biotyper v3.0 (Bruker Daltonics) software, as previously described.[12]

M. pachydermatis molecular typing

IGS1 typing was performed on the 47 *M. pachydermatis* isolates that had been collected from 24 children and one adult, as detailed in Table 2. DNA was extracted from a colony suspension in 800 μl of lysis buffer (bioMérieux, Craponne, France) using a NucliSENS^{TM} easyMAG^{TM} V2 (bioMérieux) following the manufacturer's instructions, and eluted in 50 μl H_2O. The IGS1 domain from the rRNA gene was amplified using the previously described PCR conditions and the primer set designed by Sugita et al.: 26S-F (5′-ATCCTTTGCAGACGACTTGA-3′) and Mala-R (5′-TGCTTAACTTCGCAGATCGG-3′).[13] Thirty-five cycles of PCR amplification were performed under the following conditions: denaturation for 30 s at 95°C, primer annealing for 30 s at 58°C and polymerization for 1 min at 72°C in a total reaction volume of 20 μl of an amplification mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin,

Table 1. IGS1 genotype-based Sugita groups and their accession numbers.

Accession no.	Source	Country	IGS1 Type	Base pair
AB118940	Dog, otitis externa	Hungary	2A	759
AB118596	Dog, otitis externa	Hungary	2A	759
AB118597	Dog, otitis externa	Hungary	3C	861
AB118598	Dog, otitis externa	Hungary	2A	759
AB118599	Dog, otitis externa	Hungary	2A	759
AB118600	Dog, otitis externa	Hungary	1C	591
AB118601	Dog, ear of healthy subject	Hungary	1D	601
AB118602	Dog, otitis externa	Hungary	1B	567
AB118603	Dog, ear	Hungary	1C	591
AB118604	Dog, otitis externa	Hungary	3D	898
AB118605	Dog, ear	Hungary	3D	898
AB118606	Dog, otitis externa	Hungary	2B	761
AB118607	Dog, ear	Hungary	1A	552
AB118608	Dog, ear	Hungary	A1	552
AB118609	Dog, chronic otitis externa	Hungary	2A	759
AB118610	Dog, chronic otitis externa	Hungary	2A	759
AB118611	Dog, dermatomycosis	Slovakia	3D	898
AB118612	Dog, otitis externa	Slovakia	3D	898
AB118613	Dog, otitis externa	Slovakia	3D	898
AB118614	Dog, dermatomycosis	Slovakia	3D	898
AB118615	Cat, dermatomycosis	Slovakia	3B	859
AB118616	Dog, dermatomycosis	Slovakia	3D	898
AB118617	Dog, vagina	Slovakia	3D	898
AB118618	Dog, dermatomycosis	Slovakia	2B	761
AB118619	Dog, dermatomycosis	Slovakia	3D	898
AB118620	Dog, otitis externa	Slovakia	3D	898
AB118621	Dog, otitis externa	Slovakia	3D	898
AB118622	Dog, seborrhoeic dermatis	Slovakia	3A	749
AB118775	Dog, femur of healthy subject	Japan	2B	761
AB118776	Dog, externa of healthy subject	Japan	1A	552
AB118777	Dog, externa of healthy subject	Japan	1A	552
AB118778	Dog, externa of healthy subject	Japan	1A	552
AB118779	Dog, umbilical area of healthy subject	Japan	1A	552
AB118780	Dog, femur of healthy subject	Japan	1A	552
AB118781	Dog, externa of healthy subject	Japan	1A	552
AB118782	Dog, externa of healthy subject	Japan	1A	552
AB118783	Dog, externa of healthy subject	Japan	2A	759
AB118784	Dog, dorsal area of healthy subject	Japan	2A	759
AB118785	Dog, externa of healthy subject	Japan	3D	898
AB118786	Dog, externa of healthy subject	Japan	1A	552
AB118787	Dog, externa of healthy subject	Japan	1A	552
AB118788	Dog, externa of healthy subject	Japan	1A	552
AB118941	Dog, otitis externa	Sweden	3D	898

2.5 mM of each deoxynucleotide triphosphate, 1 U Taq polymerase, and 0.5 μg of each primer. Amplicons were visualized after ethidium bromide staining of agarose gel electrophoresis and purified with the UltraClean GelSpin DNA Purification Kit (MO BIO Laboratories, Inc., CA, USA) according to the manufacturer's recommendations. Both strands were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems, France), the primers Mala_F (5′-TCTTCCTTGTCCTCTGACTT-3′), IGS1_R (5′-GTATGTTATGCTGTGCTGTG-3′), M.pach-IGS1F (5′-TCCAGCCACGCTCCACACGA-3′), and M.pach-IGS1R (5′-TCGTGTGGAGCGTGGCTGGA-3′). Sequence reaction products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems) and analyzed with an ABI 3130 Genetic Analyzer (Applied Biosystems).

Table 2. Origin and IGS1 typing results of the *Malassezia pachydermatis* strains involved in the neonatal intensive care unit.

Patient	Sex, Age (days)	Sample ID	Collection date	Site	IGS 1 Type	IGS 1 Length (base pairs)	GenBank accession no.
P1	M, 15	MP1	27/03/2013	nasal	3D	895	KP639639
		MP2	27/03/2013	urine	3F	894	KP639680
		MP3	27/03/2013	axilla	3D	903	KP639640
P2	M, 12	MP4	10/04/2013	anal	3D	894	KP639656
P3	F, 22	MP5	06/03/2013	skin	3D	895	KP639666
		MP6	06/03/2013	axilla	3D	894	KP639665
		MP7	06/03/2013	nasal	3D	896	KP639664
		MP8	06/03/2013	stools	3D	895	KP639663
		MP9	21/02/2013	axilla	3D	894	KP639662
		MP10	21/02/2013	nasal	3D	897	KP639661
P4	F, 60	MP11	13/03/2013	axilla	3D	897	KP639667
		MP12	13/03/2013	urine	3D	898	KP639668
		MP13	10/02/2013	nasal	3D	896	KP639669
P5	M, 32	MP14	25/03/2013	anal	3D	900	KP639660
P6	F, 59	MP15	03/01/2013	nasal	3D	895	KP639673
P7	M, 21	MP16	09/08/2012	axilla	3F	894	KP639682
P8	F, 22	MP17	31/07/2012	axilla	3F	894	KP639681
		MP18	31/07/2012	anal	3F	894	KP639683
		MP19	25/07/2012	urine	3D	894	KP639674
P9	F, 23	MP20	28/07/2012	axilla	3G	894	KP639678
P10	F, 37	MP21	12/05/2012	axilla	3D	898	KP639675
		MP22	12/05/2012	skin	3D	897	KP639641
P11	F, 19	MP23	12/05/2012	anal	3E	894	KP639684
		MP24	12/05/2012	stools	3D	894	KP639642
P12	F, 11	MP25	25/05/2012	urine	3D	902	KP639643
P13	M, 15	MP26	28/06/2012	axilla	3D	896	KP639644
P14	F, 37	MP 27	21/02/2012	stools	3D	894	KP639645
		MP28	21/02/2012	axilla	3G	895	KP639676
		MP29	21/02/2012	axilla	3D	898	KP639652
		MP30	21/02/2012	blood	3D	895	KP639653
		MP31	21/02/2012	anal	3D	902	KP639651
P15	F, 8	MP32	16/04/2012	axilla	3D	891	KP639646
P16	M, 3	MP33	13/04/2012	axilla	3D	899	KP639647
P17	M, 19	MP34	19/04/2012	nasal	3D	895	KP639670
		MP35	19/04/2012	urine	3D	895	KP639672
		MP36	19/04/2012	axilla	3E	894	KP639685
		MP37	19/04/2012	nasal	3D	895	KP639671
P18	M, 21	MP38	05/04/2012	skin	3D	895	KP639648
P19	M, 27	MP39	22/03/2012	axilla	3D	896	KP639649
		MP40	22/03/2012	nasal	3D	897	KP639650
P20	F, 19	MP41	21/02/2012	nasal	3D	891	KP639654
		MP42	21/02/2012	anal	3G	894	KP639677
P21	M, 49	MP43	13/04/2012	nasal	3D	899	KP639655
P22	M, 25	MP44	13/04/2012	nasal	3D	894	KP639657
P23*	M, 33 years	MP45	16/11/2011	skin	3D	897	KP639658
P24	M, 18	MP46	12/01/2012	nasal	3G	894	KP639679
P25	M, 63	MP47	21/02/2012	anal	3D	900	KP639659

ID, identification; NICU, neonatal intensive care unit.

*This strain, isolated from an adult kidney transplant recipient patient hospitalized in the same hospital, but in a ward distant to the NICU.

Sequences were assembled, edited, and manually corrected using Sequencher (v. 4.1.4). The IGS1 sequences types of our isolates was determined by comparing their sequences with those deposited in the NCBI GenBank nucleotide database, which were collected from healthy or skin diseased dogs and cats, as described in,[9] which are described in Table 1. These IGS1 *M. pachydermatis* nucleotide sequences were aligned using the multiple sequence alignment program ClustalW 1.8 included in the Molecular Evolutionary Genetics Analysis (MEGA) v. 4.1 software. An UPGMA dendrogram was generated with 1000 bootstrap replications using MEGA.[14] A phenogram using a Minimum Spanning Tree (MST) graphing algorithm was generated using SplitTree software.[15] Using as the priority rule for MST construction the classification of *M. pachydermatis* genotypes into different groups.

Statistical analyses

Statistical analyses were performed using IBM SPSS software (v. 20.0; IBM SPSS Inc., New York, USA). The Chi-square test was used. A P value $> .05$ was considered significant.

Results

Epidemiological survey

Sixty-four infants were admitted to the NICU during the *M. pachydermatis* outbreak period (January 2012 to April 2013). A blood-stream infection was diagnosed in two patients, on February 2 and September 28, 2012, respectively. From January 2012 to April 2013, 390 biological samples, including 12 stools, 103 nasal, 108 axilla, 106 anal, 21 blood, 24 urine, 4 unspecified cutaneous, and 12 respiratory samples were analyzed. *M. pachydermatis* was grown in 186 (47.7%) of the samples collected from 60 of 64 (93.8%) neonates. The prevalence of *M. pachydermatis* colonization, in infants was 93.8%. The infants colonized by *M. pachydermatis* were born at a mean of 29.1 (±3.1) weeks of amenorrhea; their mean birth weight was 1353.2 (±765.8) gr. *M. pachydermatis* was isolated in 54 (89%) of the 61 infants with a central venous catheter and in 49 (86%) of the 57 on parenteral nutrition. *M. pachydermatis* positive culture occurred throughout the outbreak; there was no notable time cluster.

No *M. pachydermatis* culture was positive in the 710 health-care worker samples and 60 samples from the NICU environment collected from January 2012 to April 2013.

Control interventions

To address this *M. pachydermatis* outbreak and to prevent further systemic infection cases the following interventions were implemented: (i) the colonized or infected neonates were treated with 3 mg or 6 mg /kg /d of fluconazole, respectively; (ii) hand hygiene and no jewelry rule for the medical and nursing staff was strengthened; (iii) local and environmental cleaning was strengthened; and (iv) the use of lipid-rich fatty creams or gels was strongly discouraged.

Molecular typing results

Seventy-six of the 186 *M. pachydermatis* isolates, collected from 24 children and one adult, had been kept. IGS1 region nucleotide sequencing was successful in 47 (62%) of these 76 isolates. Failure occurred because some isolates could not be cultured. The characteristics of the 47 *M. pachydermatis* species IGS1 sequences and their respective GenBank accession numbers are detailed in Table 2. They had been collected from various biological samples, including skin (4), nose (12), anal (7), axilla (15), urine (5), stool (3), and blood (1) of 24 children at the NICU; one isolate was collected from an adult patient hospitalized at the kidney transplant unit.

The joint analysis of the 47 IGS1 sequences from our study and the 43 sequences of Sugita et al.[9] (a total of 90 IGS1 sequences) highlighted that all of our 47 *M. pachydermatis* isolates clustered within Type 3 (Fig. 1). Figure 2 shows the characteristic IGS1 sequence features of each *M. pachydermatis* Type 3 subtypes. Noteworthy, our findings allowed describing than *M. pachydermatis* IGS1 subtype of isolates obtained from different samples of neonate patients were: 3D (78.7%), 3E (4.3%), 3F (8.5%), and 3 G (8.5%), with the appearance of three new subtypes in type 3 (Table 2): two isolates classified in subtype 3E, 4 isolated classified in subtype 3F and four isolated in subtype 3 G, as illustrated in Figure 3. No particular subtype was associated with any collection site or particular time period.

Subtype 3D was isolated from patients at the NICU and in one adult kidney transplant recipient (Table 2). The two newly described subtypes, 3F and 3E, were only isolated from patients at the NICU. Whereas six patients were colonized by multiple *M. pachydermatis* genotypes, the 19 other patients were colonized by a single genotype.

Discussion

The non–lipid-dependent *M. pachydermatis* species involved in the present outbreak has the particularity to preferentially colonize and cause diseases in animals, whereas lipid-dependent species, such as *M. globosa*, *M. sympodialis*, *M. furfur,* and *M. restricta*, colonize the skin and are common human superficial mycoses agents. [16–20] Hence, reports of human systemic infections due to *M. pachydermatis* are scarce.[10,21] Severe *M. pachydermatis* infections have infrequently been reported in adults,[2,21–23]

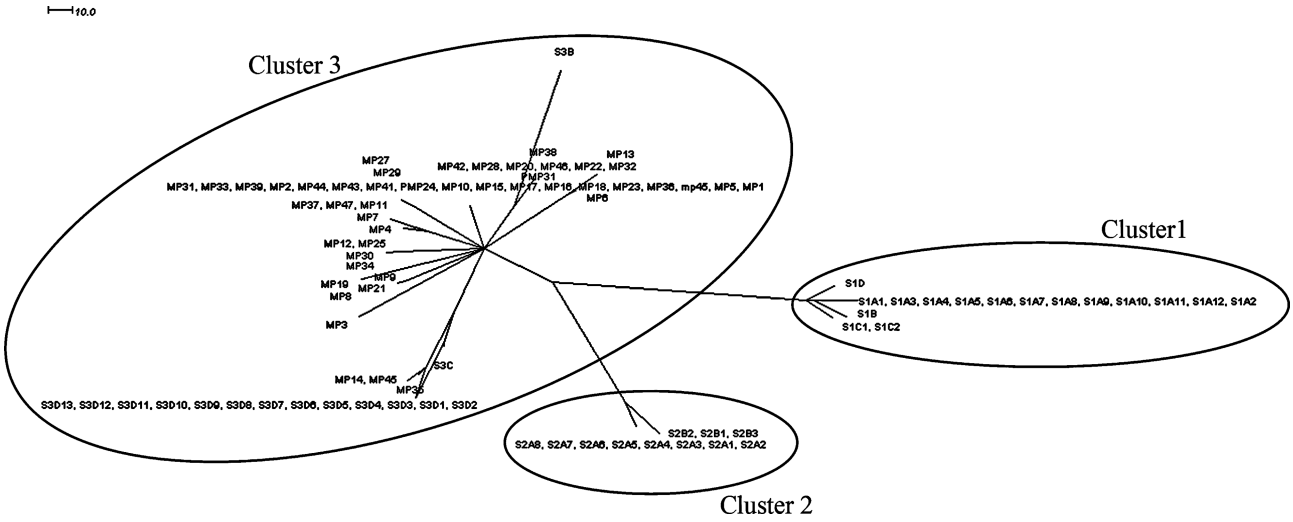

Figure 1. A Minimum Spanning Tree based on the IGS 1 sequences of 90 *M. pachydermatis* isolates: 47 were collected in this outbreak investigation and 43 had been deposited by Sugita et al. [9]. S, sequences from Sugita et al.; S1A, Group 1 subtype A1; S1B, Group 1 subtype B1; S1C, Group 1 subtype C1; S1D, Group 1 subtype D1; S2A, Group 2 subtype A2; S2B, Group 2 subtype B2; S3A, Group 3 subtype A3; S3B, Group 3 subtype B3; S3C, Group 3 subtype C3; S3D, Group 3 subtype D3; MP, sequences from this study's isolates.

and in most cases systemic *M. pachydermatis* infections occurred in hospitalized neonates. Four studies investigated the epidemiology of *M. pachydermatis* in neonates.[4,21,24,25] Welbel et al. investigated a hospital-acquired bloodstream infections outbreak due to *M. pachydermatis* in premature infants and highlighted that parenteral nutrition and/or intravenous lipid infusion were the major risk factor of *M. pachydermatis* infection and that person to person, probably via the caregiver's hands was the most likely transmission route.[2,4,26] These authors hypothesized that *M. pachydermatis* patient's colonization was acquired following contact with the parents or the nursing staff. Systemic infection route was traced to subsequent intravenous catheters colonization in neonates whose skin was colonized by *M. pachydermatis*. Several studies highlighted that the prolonged use of indwelling catheters and parenteral lipid emulsions have been as key risk factors for *M. pachydermatis* infection and additionally *Malassezia* species have also been found to survive for long periods on the surfaces of baby incubators.[25,27] Although this had not been quantified in our study, empirical observation suggested that *M. pachydermatis* colonization increased in the members of the medical staff who use hand creams. One limitation of our epidemiological investigation was that *M. pachydermatis* could not be cultured from the numerous health-care worker samples that had been collected in this study. One explanation of this finding might be that the dates of the sampling campaigns had been notified before to the staff, which might have induced systematic stringent hand disinfection or cleaning practices before.

Recently, Al-Sweih et al. described a case of *M. pachydermatis* fungemia in a preterm neonate, where the isolate was identified via rRNA nucleotide sequence analysis of the ITS and D1/D2 regions.[28] We identified via MALDI-TOF mass spectrometry and IGS1 nucleotide sequence analysis the *M. pachydermatis* isolates collected from different body sites in neonate infants. MALDI-TOF mass spectrometry is rapid, simple, and accurate, provided that a comprehensive reference spectra database is used.[2,29,30] The nucleotide sequence of the IGS1 region that we used for typing is a highly polymorphic region within *M. pachydermatis* genome; it is greater than 400 bp long and share approximately 40 to 50% similarity among *M. pachydermatis* isolates. In contrast, the IGS1 region is approximately 70 bp long and share more than 85% similarity within the other species in the genus.[31,32] It thus can be used for nucleotide sequence-based identification of *M. pachydermatis* but not of the other *Malassezia* species.

In the present study, following Sugita's IGS1 isotype classification,[9] we identified three new isotypes (3E, 3F, 3 G) within group 3. We demonstrated that isolates of subtype 3D were frequently (78.7%) isolated in this French NICU patients' population. In dogs, the most prevalent isolates from skin lesions of atopic dermatitis also belong to subtype 3D in Japan and Taiwan but more frequently to subtype 3C in Korea.[33] In line with Kobayashi et al., who reported no significant association between the IGS1 genotype and disease presentation or colonization site,[31] we found no association between any particular subtype with collection site or a particular time period. Noteworthy, our findings

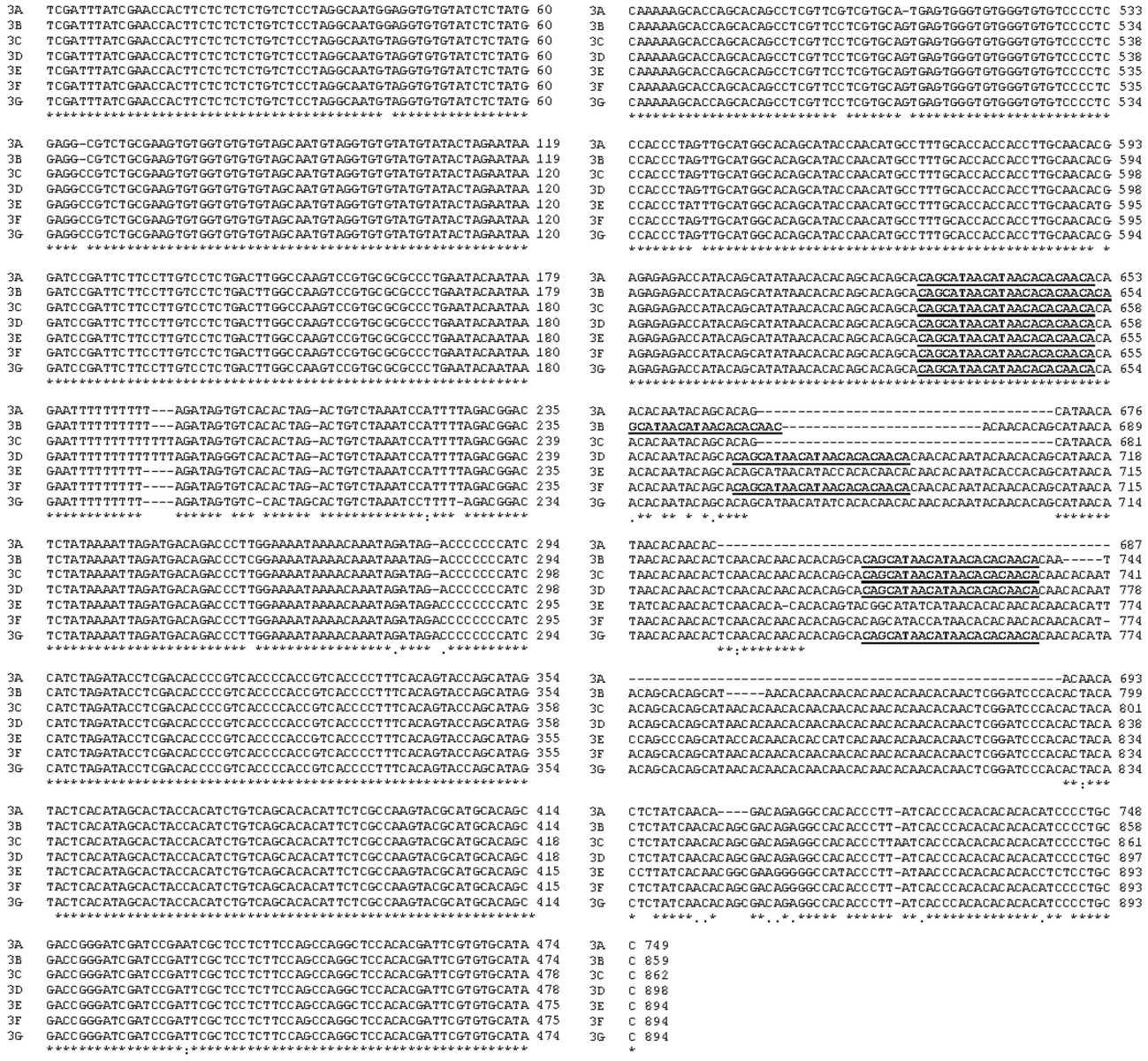

Figure 2. Alignment of the *Malassezia pachydermatis* IGS1 sequence of subtypes 3A to 3 G. Asterisks (*) are used when the nucleotide at a particular position is identical in all groups. Dashes (_) represent alignment gaps.

highlighted that neonates might be colonized by multiple genotypes.

The results of the present study indicate that patients were colonized by multiple *M. pachydermatis* genotypes. Probably, patient's colonization was acquired after diverse contact with the nursing staff. However, one limitation of our study was that only 25% (47/186) of the isolates collected from 40% (24/60) of colonized neonates could ultimately be typed.

In conclusion, the rRNA gene IGS1 region typing proved simple and effective in investigating the present NICU *M. pachydermatis* outbreak. Whereas we described three novel of *M. pachydermatis* subtypes, the subtype 3D was the most frequent in this French low-birth weight neonate population. Typing highlighted the polyclonal nature of this *M. pachydermatis* outbreak that ended after the implementation of several control measures including strengthening hygiene and restricting the use of lipid-rich fatty creams or gels by the medical and nursing staff.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

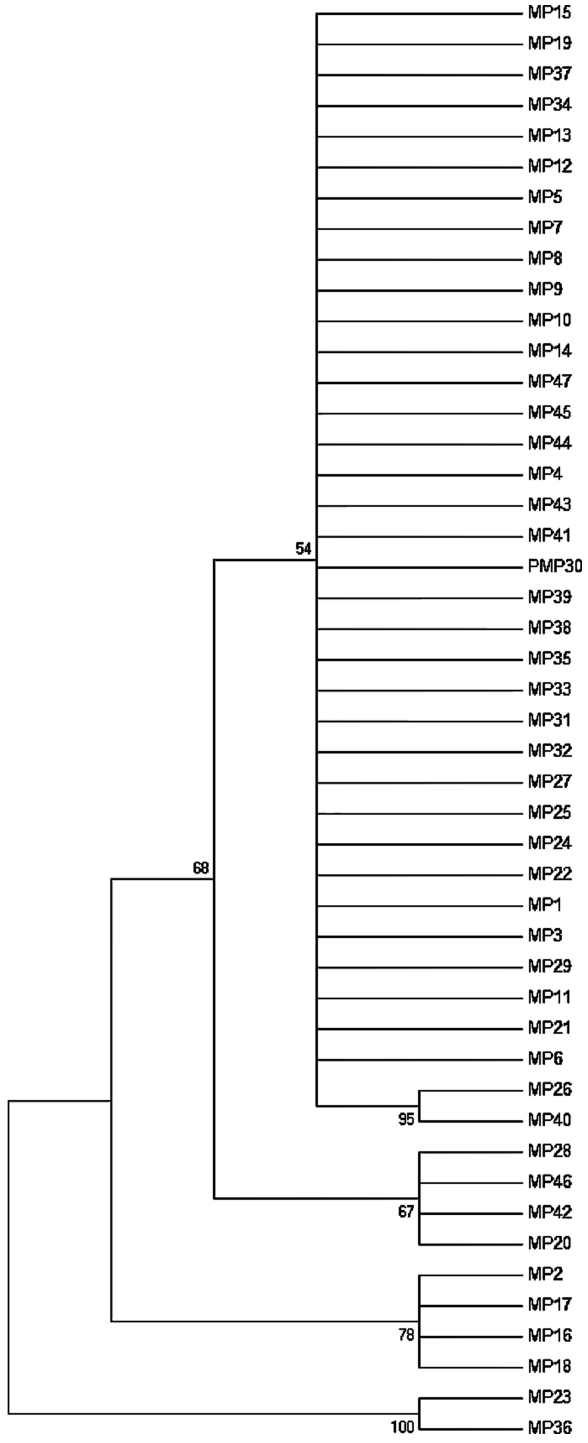

Figure 3. Phylogenetic tree of the 47 IGS1 sequences of the *Malassezia pachydermatis* strains isolated in this outbreak investigation.

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