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Epidemiology and molecular characterization of *Neisseria lactamica* carried in 11–19 years old students in Salvador, Brazil

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ABSTRACT

Neisseria lactamica is a nonpathogenic commensal bacterium that is potentially associated with the development of natural immunity against *N. meningitidis*. However, the genetic variation present in natural populations of *N. lactamica* has not been fully investigated. To better understand its epidemiology and genetic variation, we studied *N. lactamica* carriage in 1200 students aged 11–19 years old in Salvador, Brazil. The carriage prevalence was 4.5% (54/1200), with no statistical difference among sex and age, although we observed a trend towards higher carriage prevalence among 11-year-old individuals. Whole genome sequence analysis revealed a high genetic diversity among the isolates, with the presence of 32 different STs, 28 (87.5%) of which were new. A total of 21/50 (42%) isolates belonged to three different clonal complexes. While none of the isolates contained *nadA* or *fHpb* alleles, we detected 21 FetA variants, 20 NhbA variants and two variants of PorB. The data provide detailed information on circulating *N. lactamica* isolates in adolescents in Brazil and are complementary to studies in other countries.

1. Introduction

Neisseria lactamica is a lactose fermenting diplococcus closely related to *N. meningitidis*, which lives in a commensal relationship with humans. This bacterium is frequently isolated from the nasopharynx of children, and is rarely associated with invasive disease as it lacks several virulence factors usually found in *Neisseria meningitidis* (Changal et al., 2016; Everts et al., 2010).

N. lactamica is of interest as it has been implicated in the age-related development of natural immunity against *N. meningitidis* (Gold et al., 1978). Although poorly understood, the prevalence of *N. lactamica* carriage in young children (< 5 years of age) is significantly higher compared to *N. meningitidis*. Furthermore, these children developed significant IgG responses that were cross-reactive with serogroup A, B, and C meningococci soon after colonization with *N. lactamica* (Gold et al., 1978).

N. lactamica does not express the meningococcus protective capsule (Kim et al., 1989) and the outer-membrane protein PorA (Ward et al., 1992). However, there is similar relatedness among some outer membrane proteins, including porin B (PorB) (Bennett et al., 2008), iron-regulated enterobactin (FetA) (Bennett et al., 2009) and neisserial heparin-binding antigen (NhbA), although the variants are mainly not overlapping in the two species (Lucidarme et al., 2013).

The evidence of cross-reactivity responses against common antigens (Cann and Rogers, 1989; Troncoso et al., 2002) encouraged the development of anti-meningococcal vaccines based on *N. lactamica* (Griffiss et al., 1991; Finney et al., 2008; Gorringe et al., 2009).

Some of the subcapsular antigens common to *N. lactamica* and *N. meningitidis* are included in the multiple component serogroup B meningococcal vaccine, 4CMenB (Serruto et al., 2012). The use of vaccines containing surface proteins shared with *N. lactamica* could interfere in the colonization of the nasopharynx by *N. lactamica*, potentially hampering the acquisition of natural immunity (Lucidarme et al., 2013; Troncoso et al., 2002). The impact of meningococcal vaccines on

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neisserial species with similar surface proteins warrants further investigation (Toneatto et al., 2017).

Although there is a degree of relatedness between some of the surface antigens, the commensal *N. lactamica* has not been prioritized to the same degree as *N. meningitidis*, especially with regard to studies on epidemiology and genetic variation (Alber et al., 2001; Bennett et al., 2005; Kristiansen et al., 2012; Lucidarme et al., 2013). Furthermore, there is no information regarding the circulation and genetic diversity of *N. lactamica* isolates in Brazil.

Previously, we conducted a cross-sectional study to assess the meningococcal carriage status of 11–19-year-old student's resident in Salvador (Nunes et al., 2016; Moura et al., 2017). Although the laboratory methodology was primarily designed for meningococcus isolation, all lactose-fermenting Gram-negative diplococci were registered and stored for further investigation. In the present study, we describe the epidemiology and the genetic profiles of *N. lactamica* isolates recovered from 11 to 19-year-old carriers in Salvador, Brazil.

2. Material and methods

2.1. Ethical considerations

This study was approved by the Ethics Committee of the Instituto Gonçalo Moniz, FIOCRUZ-BA (CAAE #16099713.1.0000.0040). Written informed consent from all study participants (or guardians) was obtained before sample and data collection.

2.2. Isolation and identification of N. lactamica

N. lactamica isolates ($n = 54$) were recovered from the oropharyngeal swabs collected from 1200 students, aged 11–19 years old, attending a total of 134 different public schools in Salvador, Brazil, during September to December 2014 (Nunes et al., 2016). The swabs were immediately used to inoculate selective agar medium (modified Thayer-Martin agar containing vancomycin, colistin, nystatin, and trimethoprim) and transferred to a polystyrene tube containing 1 mL of skim milk-tryptone-glucose-glycerin (STGG) transport medium (O'Brien et al., 2001). After 24–48 h of incubation, the plates were inspected and colonies with the characteristic morphology of *Neisseria* spp. were subcultured on blood agar medium for species identification by Gram staining, oxidase reaction, and carbohydrate utilization tests. The results were confirmed using API-NH1 strips (bioMérieux, Hazelwood, MO, USA), as described previously (Nunes et al., 2016). *N. lactamica* isolates were stored in brain heart infusion (BHI) broth containing 20% (v/v) glycerol at −80 °C.

2.3. Molecular characterization

Of the 54 *N. lactamica* isolates, 50 were characterized by whole genome sequencing (WGS). Genomic DNA was extracted as previously described (Kretz et al., 2016), and sequenced using MiSeq v2 chemistry (Illumina, San Diego, CA, USA). Genome assembly was carried out using CLC Genomics Workbench, ver. 9.0.0 (CLC bio, Aarhus, Denmark) with read trimming and mapping of reads back to contigs. The multilocus sequence typing (MLST) alleles, sequence types (STs) and clonal complexes (cc) were identified by comparison of the assembled genomes with *Neisseria* PubMLST database (http://pubmlst.org/neisseria/), using a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The presence and diversity of *porB*, *fetA* and *nhba* were investigated using sequence analysis as previously described (Kretz et al., 2016). Partial protein sequences were used for PorB, FetA typing, and full-length protein sequences were used for NHBA typing.

2.4. Phylogenetic analysis

Single nucleotide polymorphisms (SNPs) were identified using kSNP version 3 software (Gardner and Hall, 2013) with a kmer length of 25. A maximum likelihood phylogenetic tree was constructed from the core SNPs and the Tamura-Nei model, using MEGA7 (Tamura et al., 2013) and 500 bootstraps interactions.

2.5. Data analysis

Statistical analysis was done using STATA statistical software version 12 (College Station, TX, USA). The prevalence of *N. lactamica* carriage was calculated for the total sample and for subgroups (sex and age). Univariate analysis to identify exposure associated with *N. lactamica* carriage was performed; the chi-square test was used to determine statistical significance.

3. Results

3.1. N. lactamica carriage

Among the 1200 students screened, *N. lactamica* was isolated from 54 (4.5%) individuals (Nunes et al., 2016). There was no significant difference in carriage prevalence based on gender: 31 (57.4%) females and 23 (42.6%) males. Although the *N. lactamica* carriage rate was slightly higher among 11-year-old students (9.7%), it was not statistically significant (Fig. 1). The prevalence of *N. lactamica* carriage across the various age groups was similar to that of *N. meningitidis* in the same population (Fig. 1). Only one participant was co-colonized by both *N. meningitidis* and *N. lactamica*.

3.2. Molecular characterization

A total of 50 *N. lactamica* isolates identified by conventional methods were characterized by WGS. Thirty-two different STs were identified, 28 (87.5%) of which were new. The majority of the isolates (29/50, 58%) lacked association within any known cc in the PubMLST database. A total of 21 (42%) isolates belonged to three different cc: cc613 (13/50; 26%); cc1494 (5/50; 10%); cc624 (3/50; 6%). We were unable to determine the ST of one isolate (M37159) due to a deletion of the *pdhC* housekeeping gene (Table 1). The phylogenetic analysis showed a high level of genetic variability with many different ST identified; and isolates belonging to the same ST and/or cc type to cluster together (Fig. 2). A total of 11329 core SNPs were identified with a difference of 80–5615 SNPs between all isolates analyzed.

Among the outer membrane proteins, two PorB variants were identified: 3–599 (10/50; 20%) and 3–596 (40/50; 80%), the latter being novel and most prevalent among the isolates (Table 1). All but nine of the isolates contained the FetA VR (variable region), with 21 FetA VRs in total. The most prevalent was F1-29 (12/41; 29.3%), and we identified four new variants: F1-143 (2/41; 4.9%), F1-204 (1/41; 2.4%), F4-68 (1/41; 2.4%), and F5-120 (1/41; 2.4%) (Table 1).

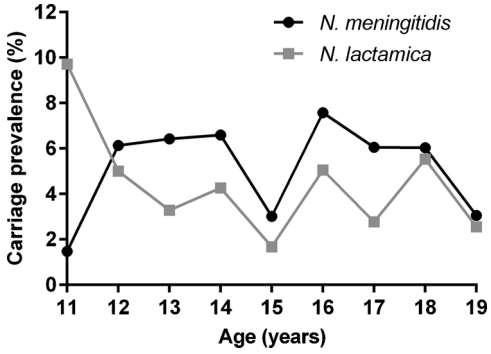

Fig. 1. Carriage prevalence for *N. lactamica* and *N. meningitidis* among adolescents resident in Salvador, Brazil, 2014 by age group. Note, the data for *N. meningitidis* was offset by 0.5% on the y-axis for clarity.

Table 1
Genetic profile of 50 *Neisseria lactamica* isolates.

ID N°	ST	Clonal Complex	PorB	FetA	NhbA
M37058	11636	1494	3–599	F1-105	291
M37063	**11637**	NA	3–596	F5-88	88
M37071	**11637**	NA	3–596	F4-17	88
M37073	**11639**	613	3–599	F4 – 68[a]	305[b]
M37078	**11640**	624	3–596	–[¶]	268[b]
M37079	**11641**	NA	3–596	F5 – 120[a]	533[b]
M37083	**11637**	NA	3–596	F5-88	88
M37084	11457	613	3–596	F1-33	305[b]
M37091	**11643**	NA	3–596	–[¶]	526
M37094	**11644**	NA	3–596	F1 – 143[a]	928
M37101	**11645**	NA	3–596	F1-68	88
M37105	**11644**	NA	3–596	F1 – 143[a]	928
M37107	**11647**	NA	3–596	F1-120	92
M37110	**11643**	NA	3–596	–[¶]	526
M37119	**11649**	NA	3–596	F2-7	88
M37123	**11650**	NA	3–599	F1 – 204[a]	268[b]
M37130	**11651**	NA	3–596	F4-19	526
M37131	**11636**	1494	3–599	F1-105	262
M37132	**11653**	613	3–596	F1-29	932
M37136	**11654**	NA	3–596	F1-29	88
M37137	**11655**	NA	3–596	–[¶]	88
M37140	613	613	3–599	F2-7	305[b]
M37142	**11637**	NA	3–596	F4-17	88
M37143	**11654**	NA	3–599	F1-29	88
M37146	**11658**	NA	3–596	F5-13	268[b]
M37147	**11654**	NA	3–596	F2-1	88
M37155	**11660**	NA	3–596	F1-29	88
M37157	613	613	3–596	F1-29	175[b]
M37158	624	624	3–596	–[¶]	929
M37159[*]			3–596	–[¶]	462[b]
M37160	613	613	3–596	F1-29	545
M37161	613	613	3–596	F1-29	175[b]
M37162	**11661**	NA	3–596	–[¶]	207[b]
M37163	613	613	3–596	F1-112	523[b]
M37164	**11662**	624	3–596	–[¶]	268[b]
M37166	613	613	3–596	F1-29	633
M37167	**11663**	NA	3–596	F2-7	548[b]
M37168	**11664**	NA	3–596	F1-22	470
M37169	**11665**	1494	3–599	F1-105	262
M37170	613	613	3–596	F5-92	305[b]
M37171	613	613	3–596	F1-29	175[b]
M37172	**11667**	1494	3–596	F5-18	262
M37173	613	613	3–596	F1-29	175[b]
M37174	**11668**	NA	3–596	F2-4	545
M37175	**11669**	NA	3–596	–[¶]	462[b]
M37176	**11670**	NA	3–599	F1-105	262
M37177	**11671**	NA	3–599	F6-3	308[b]
M37178	613	613	3–596	F1-29	262
M37179	**11672**	1494	3–599	F1-105	262
M37957	**11654**	NA	3–596	F1-29	88

ST = sequence type; PorB = porin B; FetA = iron-regulated enterobactin; NhbA = neisserial heparin binding antigen. The new STs that were described for the first time in this study are indicated in bold.

[a] New FetA variant.

[b] New NhbA variant.

[¶] *fetA* gene deleted.

[*] Isolate with *pdhC* housekeep gene absent, no sequence type and no clonal complex assigned.

Although relatively rare, cc613 and cc1494 were predominantly associated with F1-29 (8/13; 61.5%) and F1-105 (4/5; 80%), respectively (Fig. 3). Furthermore, three isolates assigned to cc624 lacked the *fetA* gene (Table 1, Fig. 4).

The analysis of the molecular data revealed 20 different variants of intact *nhba,* including eight new variants. The most common was variant 88 (12/50; 24%) followed by 262 (6/50; 12%) (Table 1). When compared with the clonal complexes, the data showed that all the isolates of the variant 88 lacked association within the cc available in the PubMLST database, while most of the variant 262 (4/6; 66.7%) were associated with the cc1494 (Table 1, Fig. 4). None of the isolates were found to contain *nadA* or *fHbp* alleles.

4. Discussion

N. lactamica carriage is normally higher during the early childhood years, decreasing with the age (Cartwright et al., 1987). The participants screened in the present study were aged 11–19 years old, younger children were not included in the study and this may explain the lack of association between age and *N. lactamica* carriage prevalence. However, our data revealed a trend towards higher carriage prevalence among the youngest (11-year-old) individuals, although this was not statistically significant. Similar findings were observed with gender; a higher proportion of *N. lactamica* prevalence is usually observed among females, possibly due to prolonged or closer contact with children (Cartwright et al., 1987). However, we found no significant differences in isolation of *N. lactamica* from male or female participants. This may be due to the age of the females enrolled in this study, many of whom may have no regular or prolonged contact with young children. The overall carriage rate (4.5%) among study participants is consistent with previous reports involving participants with higher age (Kremastinou et al., 2003; Leimkugel et al., 2007) and lower than those where young children were included (Saez-Nieto et al., 1985; Kremastinou et al., 1999; Bennett et al., 2005; Kristiansen et al., 2012).

The hypothesis that *N. lactamica* carriage could protect against meningococcal infections, either by occupying a biological niche that would otherwise be available to meningococci or by inducing natural immunity, highlights the importance of studying the population structure of *N. lactamica* (Lucidarme et al., 2013; Troncoso et al., 2002). Furthermore, administration of the 4CMenB vaccine and the presence of *N. lactamica* could be synergistic, by priming or boosting the immune response. However, vaccination could result in the elimination or prevention of *N. lactamica* carriage resulting in loss of natural immunity (Lucidarme et al., 2013). Previous studies demonstrated that *N. lactamica* populations are highly diverse (Bennett et al., 2005), and these findings have implications for the design of vaccines based on this organism. In the present study, the molecular data obtained by WGS was similar to those of previous studies; there was a high level of genetic variability among the *N. lactamica* isolates (Alber et al., 2001; Bennett et al., 2005; Kristiansen et al., 2012). Despite this diversity, we observed a high prevalence of cc613, followed by cc1494 and cc624, results that are consistent with those reported in the United Kingdom (Bennett et al., 2005) and Burkina Faso (Kristiansen et al., 2012).

Few studies have evaluated the distribution of outer membrane proteins among *N. lactamica* isolates (Bennett et al., 2008; Bennett et al., 2009; Lucidarme et al., 2013). In respect to the components of the 4CMenB vaccine, we found that none of the *N. lactamica* isolates contained NadA or FHPB, which are used in the vaccine preparation, in agreement with a previous report (Lucidarme et al., 2013). Rather, the FetA and *nhba* variants identified in this study were highly diverse within the same group and different STs, and only two *porB* variants were detected. Accordingly, as seen with the United Kingdom cc624 *N. lactamica* isolates (Bennett et al., 2009; Lucidarme et al., 2013), we observed *fetA* deletions only in isolates of cc624 and several STs for which no cc is assigned (Figs. 3 and 4). Comprehensive molecular epidemiology and surveillance including a higher number of isolates is needed to evaluate the impact of 4CMenB on *N. lactamica* carriage.

The analysis of the genetic diversity of the *N. lactamica* isolates using WGS provided crucial information regarding the genetic diversity of this poorly investigated bacterium. Moreover, these isolates provided valuable unexplored genomic data for further analysis that will assist our understanding of the carriage dynamics of *Neisseria* species.

In summary, *N. lactamica* carriage in Salvador showed no variation across the 11–19 age groups, in comparison with that seen for carriage of *N. meningitidis*. The genetic distribution and diversity of corresponding antigen genes among the *N. lactamica* isolates were similar to those reported in other studies. Continuous study on the

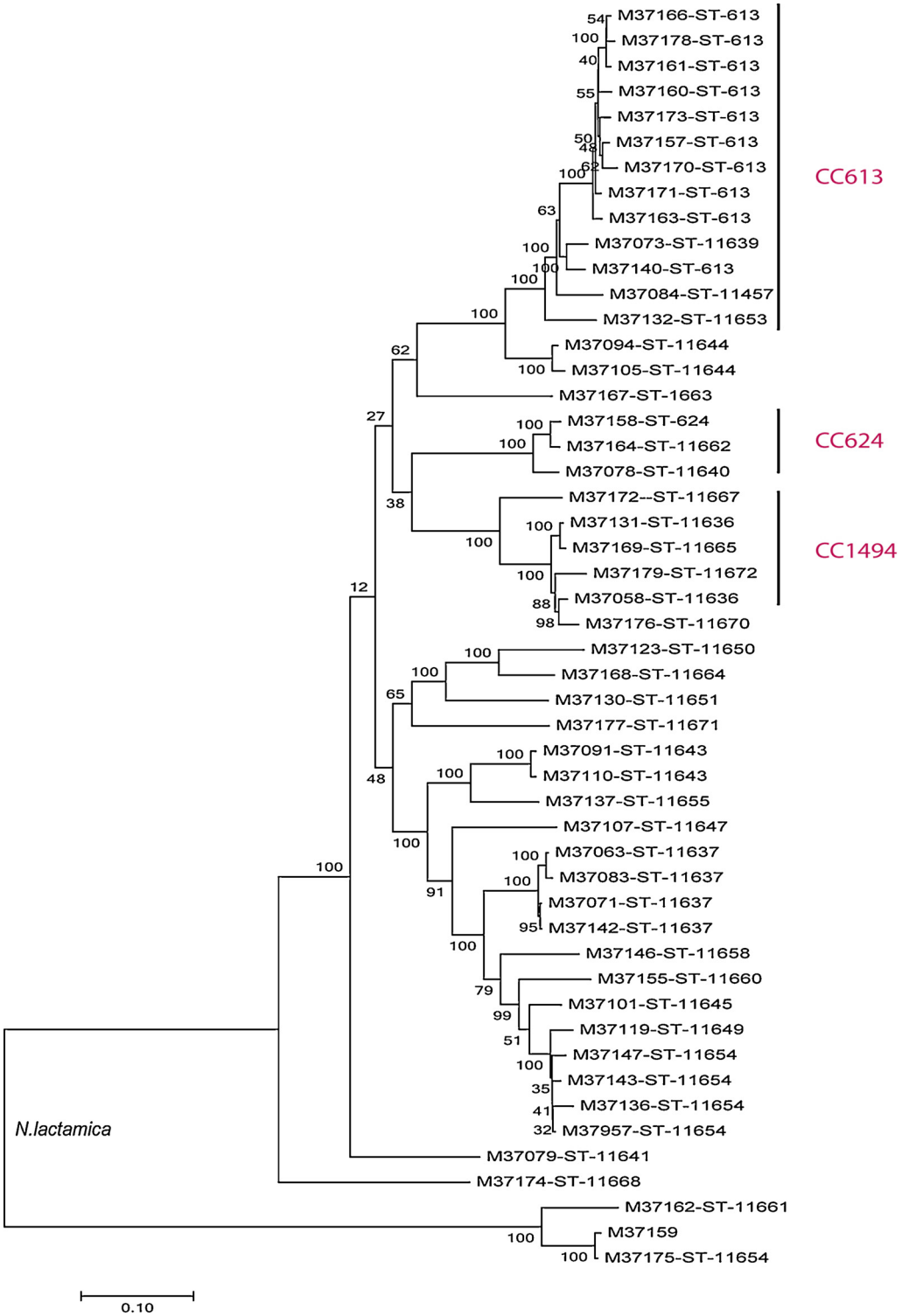

Fig. 2. Phylogenetic tree of the *N. lactamica* isolates based on the whole-genome sequence. The *N. lactamica* isolates are labelled with sample ID, sequence type (ST) and clonal complex (cc) (when assigned) they belong to. Internal nodes are labeled with bootstrap values. The scale bar is based on the 11329 positions in the core SNP matrix and indicated nucleotide substitutions per site.

characterization of circulating strains of *N. lactamica* may contribute to a better understanding of meningococcal colonization, virulence factors and vaccine responses.

Acknowledgements

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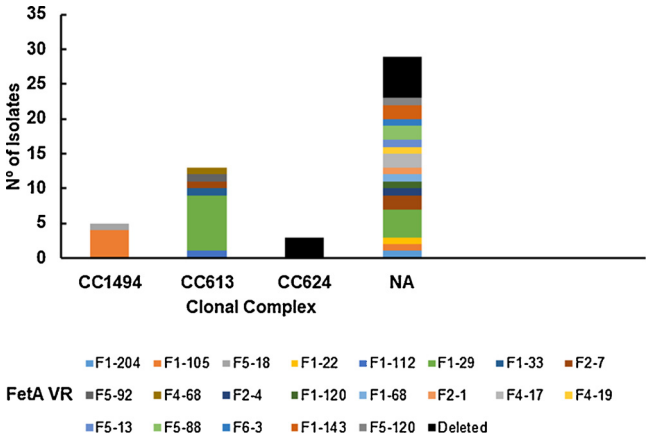

Fig. 3. FetA variants distribution among *N. lactamica* clonal complexes. VR-variants; cc- Clonal Complex; NA- clonal complex not assigned; Deleted – *fetA* gene deleted.

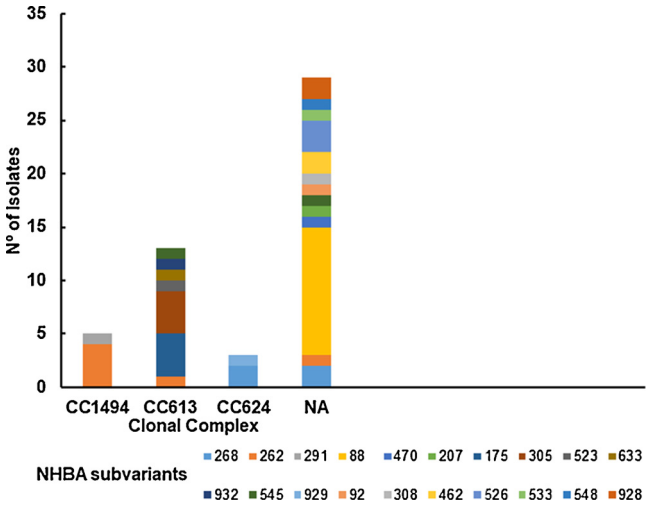

Fig. 4. NhbA variants distribution among *N. lactamica* clonal complexes. cc-clonal complex; NA- clonal complex not assigned.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijmm.2018.03.007.

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