LETTER TO EDITOR / CARTA AL EDITOR

Long survival of *Neisseria meningitidis* in freeze-dried cultures maintained in potentially unsuitable conditions

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Currently, diagnosis of *Neisseria meningitidis* in nasopharyngeal samples can be made by culture and nucleic acid amplification techniques[1]. In Cuba, molecular diagnosis of meningococcal meningitis was introduced at the National Reference Laboratory for Neisseria (NRLN), in 2010, through a PCR that amplifies a fragment of the ctrA gene using the protocol described by Taha, 2000[2]. This gene codes for a capsule protein that regulates the adhesion of *N. meningitidis* to the host, and 16 to 28% of meningococci isolates, especially in the nasopharynx, lack this gene[2]. In contrast, the sodC gene, related to the production of superoxide-dismutase of this organism, is less sensitive to antigenic variation, hence its importance for molecular diagnosis in patients and asymptomatic carriers[3]. Information on the meningococcal carriage is essential for public health policy[4]. Still, the high number of invasive meningococci disease (IMD) affecting Cuba during the 1980s and the absence of molecular tools prevented its accurate microbiological diagnosis in carriers[5]. This study aimed to identify

In Ciego de Avila province, *N. meningitidis*, by conventional and molecular methods in freeze-dried nasopharyngeal cultures of 50 *Neisseria spp.*, recovered from carriers during 1987-1988, one of the most affected by a large epidemic of IMD in Cuba[5]. Lyophilized material, which was preserved for 30 years, was reconstituted in 2 mL of nuclease-free sterile distilled water (Promega, USA). One milliliter was subcultured onto chocolate agar and incubated at 36.5-37°C for 18-24 hours, with 5-10% CO_2, and the other milliliter was used to perform DNA extraction. Conventional methods used as sugar utilization and Vitek®2 automated system (bioMérieux, France) identified Neisseria species. QIAamp®DNA Mini and Blood Mini method, QIAGEN, Germany made 1 DNA extraction. Two PCR systems were used for molecular identification of meningococci, a simple PCR test that amplifies a 523 bp fragment of the *ctrA* gene[2], and a real-time PCR (rt-PCR) that amplifies a 127 bp fragment of the *sodC* gene[3]. Serogroup identification of *Neisseria meningitidis* isolates was developed by slide agglutination using Remel™ Agglutinating Sera (Lenexa, USA). In addition, the main serogroups (A, B, C, W135, Y, X) of meningococci were investigated by rt-PCR[6], in positive samples identified by both simple and rt-PCR systems.

Pharyngeal carriage of *N. meningitidis* has been considered a prerequisite for the development of IMD and is known to be essential for transmission[4]. In this study, ten isolates (20%) recovered from lyophilized material of nasopharyngeal carriers were identified as meningococci by culture, the standard gold method for detecting bacterial carriage[7]. Nine of ten isolates were serogroup B, which was predominant during the epidemic of IMD in Cuba[5], and the other isolate was non-groupable.

Freeze-drying is more practical for the long-term preservation of

N. meningitidis cultures and optimal conditions for its conservation are refrigeration

(2-8°C) or freezing (-30 -70°C) temperatures, ampules are protected from the action of light and placed in an environment without humidity[8,9]. These conditions produce good genetic stability of the product, with a longevity of up to 20 years[8]. Moreira *et al.*, in Cuba, obtained a 46.3% of survival of N. meningitidis lyophilized cultures after one year of storage at 4°C[10]. Lyophilization is a very complex physical process affected by many parameters and variables such as growth medium, cell concentration, freezing rate, lyoprotectant, reconstitution medium, and time[11]. In the current investigation, freeze-dried ampules were stored at room temperature and unprotected from light. However, its more prolonged survival of 30 years is noteworthy under these unsuitable conditions[8,9]. Recently, Swain et al. demonstrated the relatively prolonged survival of the Cuban, New Zealand and Norwegian epidemic *N. meningitidis* serogroup B strains on inanimate surfaces for up to 8 days, depending on temperature and humidity, in comparison to other meningococci strains belonging to serogroup W135. In addition, carriage isolates appeared to survive better than invasive isolates, with a statistically significant difference ($P = 0.002$)[12].

Some authors recommend molecular tests for the identification and serogrouping of

N. meningitidis in cultures from carriers and lyophilized material[4,11]. The end point-PCR results that amplify a fragment of *ctrA* gene detected *N. meningitidis* in 76% of cell suspensions of *Neisseria spp*. Investigated (Figure 1). Meanwhile, the rt-PCR that amplified a 127 bp fragment of the *sodC* gene identified meningococci in 100% of cell suspensions (Figure 2), which highlights the capacity of this rt-PCR system for the definitive identification of *N. meningitidis*. There are few studies on using *sodC* gene in nasopharyngeal cultures from carriers[3,4]. Dolan *et al.* reported that sodC rt-PCR effectively identified 99.7% (624/626) of invasive and carriage *N. meningitidis* strains and was superior to the *ctrA* rt-PCR assay (71.6% 448/626)[3]. Jones *et al.* recently investigated three target genes (*ctrA, sodC* and *porA*) by whole-genome sequencing and rt-PCR. The *ctrA*

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gene was absent in a large percentage (58%, 54/93) of carriage isolates. However, both *porA* and *sodC* genes were well represented in the carriage collection (99% and 97%)[13]. In addition, Jordens & Heckels reported the development of a *porA* rt-PCR assay that identified several *N. meningitidis* isolates from carriers that were missed by using only the *ctrA* gene[7].

In the current study, 68.4% (26/38) of cell suspension positive to *N. meningitidis* by both PCR belonged to serogroup B, 5.3% was serogroup C, and the remainder (26.3%) were non-groupable. Martínez *et al.*, in a longitudinal study carried out on meningococci strains corresponding to nasopharyngeal carriers of the epidemic (1982-1992) and post-epidemic (1993-2002) stage in Cuba, detected a predominance of serogroup B (67.3%) in the epidemic phase and the non-agglutinable strains during the post-epidemic stage (70.7%)[14]. In Colombia, Moreno *et al.* also report a predominance of *N. meningitidis* serogroup B in carriers[15].

The absence of comprehensive information for carriage in developing countries limits clarification of the epidemiology of IMD16. In the case of the Caribbean region, in particular, there is no previous report about the use of molecular methods for identifying or seron-grouping *N. meningitidis* in patients or carriers. The current study supports the usefulness of molecular tools in future studies of nasopharyngeal carriers in the Cuban population. In addition, this genetic material is helpful for further genomic characterization of Cuban meningococci strains by multi-locus sequence typing and/or other sequence methods. Genomic surveillance for *N. meningitidis* is fundamental for understanding pathogen evolution and disease epidemiology and can be significantly improved using culture-free methods[17].

Conclusions

This study represents the first international report about a more prolonged survival of *N. meningitidis* in freeze-dried cultures from nasopharyngeal carriers that were kept under potentially unsuitable temperature, humidity and light

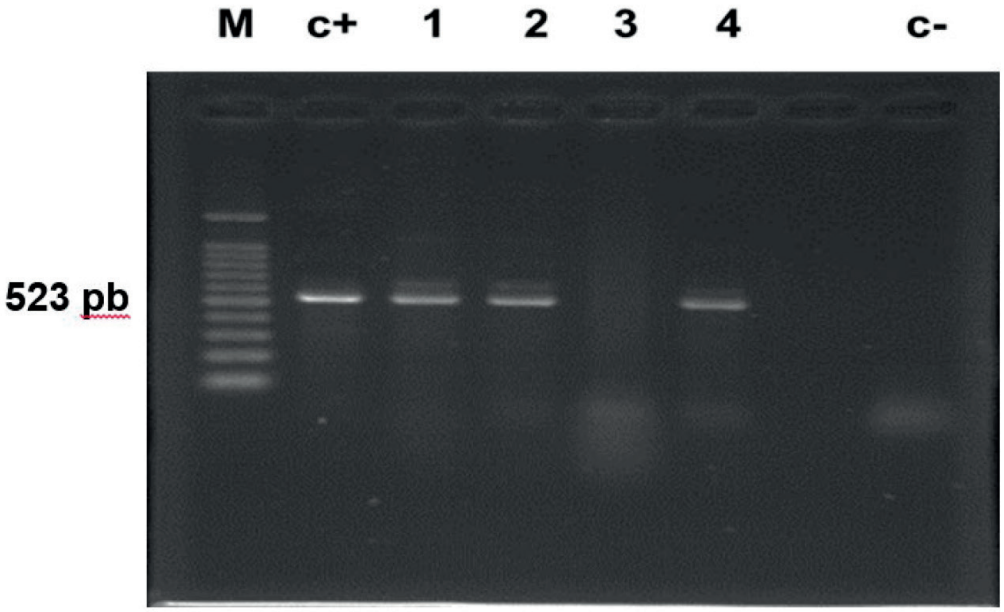

Figure 1. Amplifying *Neisseria meningitidis ctrA* gene in freeze-dried cultures of *Neisseria spp*. from carriers using a PCR with DNA extraction shown in 2% agarose gel stained with ethidium bromide, giving a 523 bp amplicon. Lanes: M: 100 bp DNA ladder; c+: positive control DNA of *N. meningitidis* ATCC 26195; lanes 1-4: DNA from carriers; c-: negative internal control (distilled water).

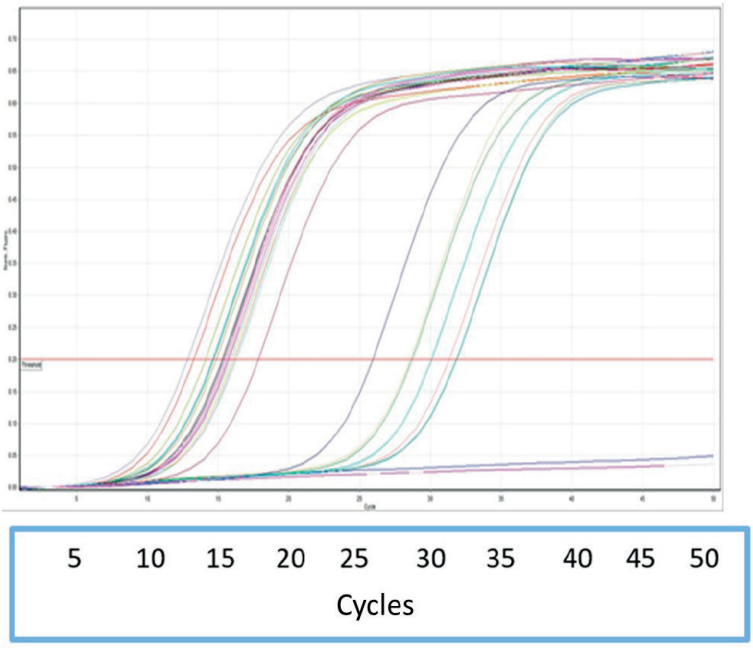

Figure 2. Specific amplification curves for real-time PCR with TaqMan that amplifies a 127 bp fragment of *Neisseria meningitides sodC* gene obtained from freeze-dried cultures of *Neisseria spp* in carriers. The horizontal line is the threshold.

exposure conditions. The sodC-based rt-PCR assay had an advantage over *ctrA* gene for detecting meningococci in cell suspensions of *Neisseria spp.* from lyophilized material obtained during the extensive epidemic of IMD in Cuba in the 1980s.

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Conflicts of Interest

None.

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