Assessment of a two-step nucleic acid amplification assay for detection of *Neisseria meningitidis* followed by capsular genogrouping

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Immediate prevention of meningococcal disease relies in part on the prompt treatment with antibiotics of household and other close contacts of cases; however intervention with effective vaccination relies on identification of serogroup-causing strains. Parenteral antibiotic for patient with suspected meningococcal disease before hospital admission is currently recommended. Laboratory standard methods are hindered by failure to detect bacteria by this medical approach to improve patient prognosis. We assessed two polymerase chain reaction (PCR) assays to detect (crgA) *and define the serogroups* (siaD, orf-2*, and* ctrA) *of* Neisseria meningitidis *in 120 cerebrospinal fluid (CSF) samples from positive cases (culture or antigen detection or direct smear). The PCR sensitivity for the identification of* N. meningitidis *was 100% (95% confidence interval, CI, 96-100%) compared to a sensitivity of 46% for culture (95% CI 37-55%), 61% for latex agglutination test (95% CI 52-70%), and 68% for Gram stain (95% CI 59-76%); PCR specificity was 97% (95% CI 82-100%). PCR correctly identified the serogroups A, B, C, W_{135}, Y, and X in CSF samples with a sensitivity of 88% (95% CI 80-93%); the primer sets were 100% specific. The introduction of PCR-based assays shall increase laboratory confirmed cases, consequently enhancing surveillance of meningococcal disease.*

Key words: *Neisseria meningitidis* - meningococcal disease - polymerase chain reaction - genogroup

Thus far, meningococcal disease has been the main activity of local epidemiological surveillance, as well as other communicable diseases that require quick action to cases and their contacts. The Brazilian Ministry of Health demands that meningococcal disease cases, including suspected cases, are apprized within 24 h of diagnosis to ensure that chemoprophylaxis is given to all close contacts, what is fundamental for the prevention of secondary cases (Funasa 2005). Nevertheless, the reduction of secondary cases has almost no impact in the incidence of the disease in the community, as it accounts only 1-3% of all reported cases (Barroso et al. 1998). The changing epidemiology of meningococcal disease is a fact observed naturally or through the implementation of vaccination (Barroso et al. 1998, Carrol et al. 2000, Harrison 2006). Vaccination is the most effective measure to control the disease caused by *Neisseria meningitidis*, a human pathogen spread through direct contact with respiratory secretions from the asymptomatic nasopharyngeal carriers (Barroso et al. 1998, Harrison 2006). *N. meningitidis* capsular serogroups A, B, C, W_{135}, Y, and X are responsible for virtually all cases diagnosed world-wide, with group B and C predominating (van Deuren et al. 2000, Djibo et al. 2003, Lemos et al. 2006). Eventually cases due to sero-groups 29E and, to lesser extent, Z are diagnosed and reported (Bennett et al. 2004).

N. meningitidis is the major cause of community-acquired bacterial meningitis and septicaemia in Rio de Janeiro (Rio de Janeiro State Health Department). This bacterium has caused several epidemics into the Rio de Janeiro metropolitan area since the disease was recognized as autochthonous in 1916 (Vieira 1916, Renzo 1921, Barroso et al. 1996). In 2001 to 2005, the Rio de Janeiro State Health Department registered 2111 cases of meningococcal disease, with approximately 70% from the metropolitan area; 22% of these cases died. Microbiological confirmation deficiency (due to different problems) is a recurrent issue, most commonly because antibiotics were administered before the diagnosis is established. Of the 2111 registered cases, 83% (1533) were suspected cases since they have not been confirmed by culture or antigen detection (Fig. 1). These suspected cases are based on the clinical criteria alone (clinically diagnosed bacterial meningitis with a suggestive rash or purpura fulminans) or identification of Gram-negative diplococci in a Gram stain cerebrospinal fluid (CSF) smear (Funasa 2005).

Meningococcal disease normally begins suddenly and patient may progress to death within hours (van Deuren et al. 2000). Thus, any delays in diagnosis and treatment can therefore decrease the chances of survival. The case fatality rate of meningococcal disease is thought to be reduced by earlier treatment with parenteral antibiotics

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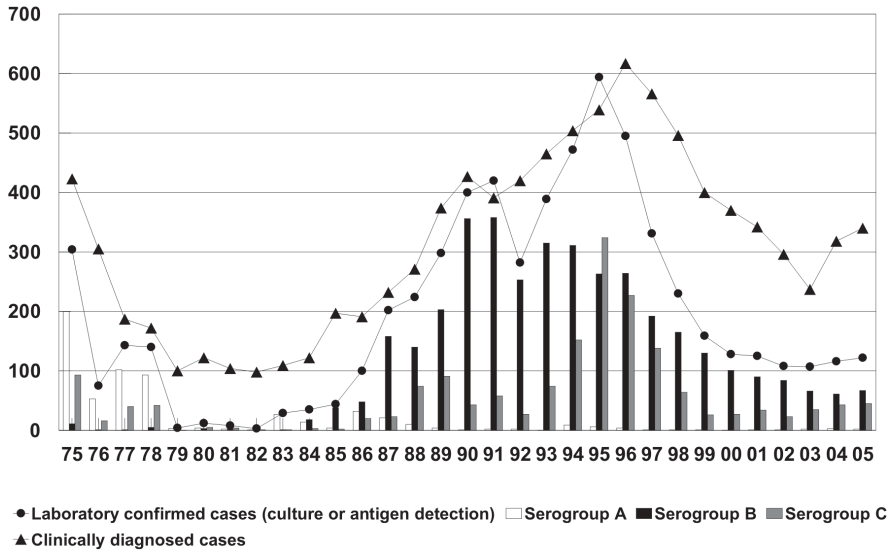

Fig. 1: meningococcal disease and the most common *Neisseria meningitidis* serogroups of cases diagnosed in the state of Rio de Janeiro between 1975 and 2005.

(Cartwright & Kroll 1997). Preadmission parenteral antibiotic is currently recommended and less lumbar punctures are done in patients with suspected meningococcal disease at presentation (Carrol et al. 2000). Neither early antibiotic administration nor a delayed lumbar puncture hinders microbiological diagnosis when nucleic acid amplification assay is used (Cartwright & Kroll 1997, Carrol et al. 2000, Ragunathan et al. 2000, Clarke & Edwards 2003). We conducted an assessment of a two-step polymerase chain reaction (PCR) assay to detect *N. meningitidis* DNA in CSF samples from positive cases (culture or antigen detection or direct smear) followed by genogrouping, as an adjunct for field-based surveillance of meningococcal disease.

MATERIALS AND METHODS

Setting, study design, and case definition - We conducted this study prospectively, between 2004 and 2005, in the city of Rio de Janeiro, the Capital of the state of Rio de Janeiro. The PCR assays were evaluated to detect *N. meningitidis* DNA and determine the serogroup in CSF samples for diagnostic purposes. Clinically diagnosed meningitis with *N. meningitidis* identified in CSF by conventional bacteriology criteria eligible cases were included: (i) culture, (ii) latex agglutination test or (iii) Gram stain smear.

Samples - Visual inspection, culture (heated blood agar), latex agglutination test (BD Directgen Meningitis Combo Test), Gram stain, and biochemical identification were performed on fresh CSF samples in the Meningitis Reference Laboratory, Instituto Estadual de Infectologia São Sebastião (IEISS), in the city of Rio de Janeiro. Outside this hospital, CSF samples were collected in sterile tubes and, frequently, a few drops inoculated in chocolate agar slant, then transported to that laboratory usually at room temperature. An aliquot (not less than 200 µl) of CSF specimens were placed in sterile vials, stored at –20ºC, before being transported frozen (dry ice) to the Instituto Oswaldo Cruz for molecular diagnosis. An appropriate sheet containing information regarding clinical and laboratory data accompanied each biological sample.

Of the 120 CSF samples received, 55 were culture-positive for *N. meningitidis*; a positive result corresponded to the criteria ii were also present in 34 and to the criteria iii in 36 of these samples. The remaining 65 CSF samples were culture-negative included by the following criteria: antigen detection-positive and direct smear-positive (n = 20), or antigen detection-positive (n = 19) or direct smear-positive (n = 26). CSF samples from patients with clinically diagnosed bacterial meningitis caused by other bacteria, identified as *Streptococcus agalactiae* (n = 6), *Mycobacterium tuberculosis* (n = 4), *Listeria monocytogenes* (n = 2), *Staphylococcus aureus* (n = 1), *Acinetobacter baumanii* (n = 2), *Streptococcus pneumoniae* (n = 4), *Escherichia coli* (n = 3), and *Haemophilus influenzae* type b (n = 2) were used as controls. The bacterial DNA from the CSF sample was isolated with the use of QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's protocol for DNA purification from Gram-negative bacteria. The protocol for DNA purification from Gram-positive bacteria was used whenever it was suitable.

PCR-based assays - PCR identification of *N. meningitidis* was based on the amplification of the *crgA* gene with specific oligonucleotide primers designed by Taha (2000), for which the amplicon length was 230 bp. For genogrouping, a multiplex PCR assay was performed with oligonucleotide primers in the *siaD* (B, C, Y, and W_{135}) and *orf-2* (A) genes (Taha 2000). Another PCR assay is necessary to discriminate serogroups Y and W_{135}, for which amplicon length was 120 bp. A PCR amplification

of specific regions of the *ctrA* gene for individual detection of serogroups 29E, X, and Z was also carried out (Bennett et al. 2004). PCR reaction mixture (50 µl) contained 0.6 µM of each oligonucleotide primer (Invitrogen), 200 µM dNTPs, 1X PCR buffer, 3 mM $MgCl_2$, 1U Platinun *Taq* DNA polymerase (Invitrogen), and 10 µl of DNA target. PCR parameters were 94°C for 3 min, 55°C for 30 s, 72°C for 20 s (initial cycle); followed by subsequent 35 cycles of 92°C for 40 s (denaturation), 55°C for 30 s (annealing), 72°C for 20 s (polymerization); and 72°C for 10 min (final cycle of polymerization), then holding at 4°C in a GeneAmp PCR System 9700 (PE Applied Biosystems). Amplicons were analyzed under UV fluorescence following electrophoresis on a standard 2% (wt/vl) agarose gel and staining with ethidium bromide. Negative control consisted of reaction mixture without DNA target. The DNA was included in each assay from standard strain (*N. meningitidis* ATCC 13090) or clinical isolates (*N. meningitidis* sero-groups A, B, C, W_{135}, Y, 29E, X, and Z) as positive controls.

Calculation of efficiency values - The sensitivity of the PCR screen and genogroup was estimated using CSF from positive cases corresponded to criteria i (n = 55), ii (n = 73), and iii (n = 82). To determine the specificity, amplification reactions were performed using genomic DNA extracted (DNeasy Tissue Kit, Quiagen) from *S. pneumoniae* (ATCC 49619), *H. influenzae* (ATCC 49766), *H. influenzaae* type b (ATCC 10211), *S. aureus* (ATCC 29213), *E. coli* K1 (clinical isolate), *S. agalactiae* (clinical isolate), *Enterococcus faecalis* (clinical isolate), and *Neisseria lactamica* (carrier isolate). It was also examined CSF samples from patients corresponded to bacterial meningitis other than meningococcal (n = 24). Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using a two-way table (Epi Info ^{TM}, Version 3.3.2, CDC). Exact binomial 95% confidence intervals (CI) were estimated.

RESULTS

In 2004 to 2005, 120 patients with meningococcal disease identified by one of the criteria defined in this study were investigated by means of PCR. Of these patients, 39% were up to 5 years old and 9% were infants. The median age was 16 years (range, 4 month to 63 years). Sixty-four (53%) patients were male and 56 (47%) were female. Visually inspected CSF samples from these cases were distinguished in four categories: limpid (12%), cloudy (73%), purulent (9%), and haemorrhagic (6%). The mean of CSF white blood cell count was 7557/mm^3 (range, 0 to 32,426/mm^3); 13% had less than 100/mm^3, and 17% without record of WBC count.

All 120 CSF samples from positive cases were correctly identified by means of PCR screen for the presence of the *crgA* gene, whilst direct smear revealed organisms in 82, antigen detection was positive in 73, and 55 isolates cultured from CSF. PCR sensitivity (100, 95 CI 96-100%), specificity (97, 95 CI 82-100%), positive predictive value (99, 95 CI 95-100%), and negative predictive value (100, 95 CI 86-100%) were calculated and compared with results obtained with conventional bacteriological methods (Table). The *crgA* gene PCR product amplified from standard strain (*N. meningitidis* ATCC 13090) and results obtained when the PCR genogroup assays were applied to clinical samples are

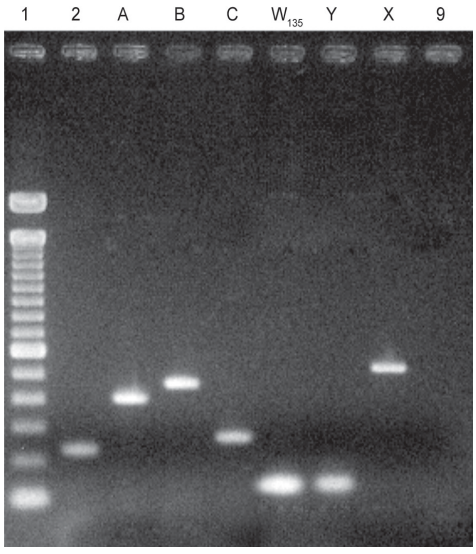

Fig. 2: polymerase chain reaction (PCR) amplification of the *crgA* target and the genes for the biosynthesis and transport of *Neisseria meingitidis* capsular polysaccharides. 1: 100 bp DNA Ladder (Invitrogen); 2: PCR amplification of the *crgA* gene (230 bp) from a reference *N. meningitidis* strain; 3-8: results obtained when the genogroup PCR was applied to biological samples, showing detection of serogroups A (400 bp), B (450 bp), C (250 bp), W_{135} (120 bp), Y (120 bp), and X (525 bp); 9: negative control.

TABLE I

Polymerase chain reaction, culture, antigen detection, and Gram stain results in 120 cerebrospinal fluid samples from infected patients with *Neisseria meningitidis*

	No. of samples		Sensitivity	Specificity	PPV	NPV
Procedure	+	–	(%)	(%)	(%)	(%)
crgA gene PCR	120	0	100	97	99	100
Genogrouping	105	15	88	100	100	67
Culture	55	65	46	100	100	33
Antigen detection	73	47	61	100	100	41
Gram stain	82	38	68	100	100	46

PPV: positive predictive value; NPV: negative predictive value.

shown in Fig. 2.

Genogrouping was performed with a sensitivity of 88% (95% CI 80-93%) for CSF (Table). Serogroup determined by PCR found out exactly that obtained by use of direct slide agglutination of each cultured bacteria (B = 28; C = 13; W_{135} = 2; Y = 1) or latex agglutination test (B = 11; C = 20). Also it could discriminate the provided latex agglutination test capability to detect capsule C/W_{135} and A/Y antigens (n = 8). The PCR assay determined the serogroup of 80% (95% CI 67-90%) of the culture proven cases, whilst latex agglutination test was positive in 62% (95% CI 48-75%). PCR failed to predict 10 serogroup B and 1 serogroup C in culture positive CSF specimens; whereas latex agglutination test did not detect 19 meningococcal capsule B and 2 capsule C antigens. All the samples that were positive by antigen detection (n = 73) were also positive by PCR. Of the samples included by the criteria iii alone (direct smear) the assay resolved the serogroup in 85% (22/26): 1 serogroup A, 13 serogroup B, 2 serogroup C, 4 serogroup W_{135}, and 2 serogroup X.

The specificity of the assay was tested with DNA purified from standard strains and clinical or carrier isolates (n = 8). There was one false-positive result obtained with PCR strategy that target *crgA* gene. A PCR product of 230 bp the same as for *N. meningitidis* was amplified from the *N. lactamica* carrier strain (data not shown). The oligo-nucletides used for genogrouping were specific for the selected capsular serogroups and did not amplify products from bacteria belonging to other species; PCR specificity was 100% (95% CI 86-100%). However the *ctrA* X set primers amplified a product from serogroup A strain of approximately 650 bp as it was reported before by Bennett et al. (2004), which can be easily distinguished from the 525 bp serogroup X product (data not shown). All CSF samples from bacterial meningitis other than meningococcal (n = 24) were negative by the two-step approach (*crgA* assay followed by genogrouping) for the presence of *N. meningitidis* DNA.

DISCUSSION

The rapid confirmation and serogroup information of *N. meningitidis* are fundamental to immediate management of cases and implementation of prophylactic measures among contacts (van Deuren et al. 2000, Taha et al. 2005). Estimation of the proportion of serogroup-causing strains during endemic periods or epidemic waves is essential for planning and assessment of vaccination strategies (Bash et al. 1996, Feavers 2000, Lemos et al. 2006, Harrison 2006). A low number of confirmed cases of meningococcal disease hamper the public health interventions in the community, once implementation of available meningococcal vaccines are based on the prevalence of capsular serogroups of cases diagnosed (Bash et al. 1996, Carrol et al. 2000, Feavers 2000, Barroso et al. 2002, Harrison 2006). This has been a critical issue for the control of meningococcal disease in Rio de Janeiro since the second half of the 1990s, when was noticed a decrease in the number of confirmed cases (Fig. 1).

Traditional non-culture laboratory methods, such as the latex agglutination test has been used to diagnosis and serogroup determination of *N. meningitidis*. It is useful when patient has received antibiotics before the diagnosis is established. Although a decrease in the sensitivity of this test is influenced by the time relationship between commencement of antibiotics and when the lumbar puncture is performed (Nigrovic et al. 2004). Also latex agglutination test is restricted for individual detection of serogroups A and C. *N. meningitidis* serogroup B latex reagent does not differentiate the *E. coli* K1 polysaccharide antigen; again it does not provide individual detection of serogroups W_{135} and Y, but jointly the serogroups A/Y or C/ W_{135} (BD Directgen Meningitis Combo Test). Identification of Gram-negative diplococci in a Gram stain CSF smear might suggest the diagnosis of *N. meningitidis*, although it does not give information on serogroup. In non-culture proven meningitis, PCR-based assay has proven to be a valuable approach for the demonstration of meningococcal aetiology in urban and remote areas (Carrol et al. 2000, Clarke & Edwards 2003, Taha et al. 2005). Furthermore, interlaboratory comparison has demonstrated this diagnostic technique is reliable to support the diagnosis and for epidemiological surveillance with similar results between the participants (Taha et al. 2005).

The *crgA* gene PCR has provided a rapid confirmation of *N. meningitidis* in CSF samples, with high sensitivity and specificity comparable with data presented elsewhere (Ragunathan et al. 2000, Clarke & Edwards 2003, Taha et al. 2005). False-positive results with some *N. lactamica* carriers isolates have been documented in studies validating molecular assays strategies that target *N. menin-gitidis*-specific genes (Guiver et al. 2000, Kesanopoulos et al. 2005). The inclusion of this non-pathogenic species decreased the specificity of the assay presented here. Nevertheless it is unlikely to be a problem when applied to solve clinical cases of suspected meningococcal disease. *N. lactamica* is an exceptional cause of human disease with four cases of meningitis reported in the medical literature (Denning & Gill 1991). Besides, the second step of the approach may confirm the presence of *N. meningitidis* DNA. The PCR genogroup is less sensitive (mean of 73%) according to data reported previously (Taha et al. 2005). However we could demonstrate a better performance compared to the results obtained with conventional bacteriology for diagnosis and serogrouping. The use of oligonucleotides to detect capsular serogroups 29E, X, and Z had not been applied before to CSF samples for diagnostic purposes (Bennett et al. 2004). These primer sets are useful to complement the investigation of meningococcal disease with the extent of capsular antigenic diversity among disease-causing strains.

The performance of the conventional PCR amplification protocols assessed was higher than in previous studies and compared with the real time PCR results to confirm the aetiology of meningococcal disease (Guiver et al. 2000, Taha 2000, Kesanopoulos et al. 2005, Taha et al. 2005). The use of improved DNA extraction procedure (Quiagen) might have increased the sensitivity of the

assays. In spite of the recommendation to send frozen samples, bacterial DNA detection was not affected with transport of fresh CSF specimens at room temperature to the Reference Laboratory. Some advantage of real time PCR is a high throughput and rapid turnaround time of samples (Guiver et al. 2000, Kesanopoulos et al. 2005). These aspects make the results available faster even with an increasing demand in terms of numbers of samples. Although the implementation of real time PCR protocol at a central level (Lacen) in each state of Brazil seems to be not plausible at present. It is reasonable to point out the routine use of molecular diagnosis shall increase the number of confirmed cases with a defined serogroup. It is important to notice the primer sets used for genogrouping cover all clinical and epidemiological significant serogroups associated with disease into this country (Lemos et al. 2006). Thus the benefits are clear to epidemiological surveillance and management of meningococcal disease in our community, by the reduction of the discrepancy between totals of meningococcal disease cases (clinical diagnosis alone and confirmed cases) notified and reported in the official statistics.

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