Diagnostic accuracy of loop-mediated isothermal amplification as a near-patient test for meningococcal disease in children: An observational cohort study

Diagnostic accuracy of 'loop mediated isothermal amplification' (LAMP) as a near-patient test for meningococcal disease in children

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Abstract

Background: Diagnosis of meningococcal disease relies on recognition of clinical signs and symptoms that are notoriously non-specific, variable, and often absent in the early stages of disease. Laboratory testing serves primarily to confirm the clinical diagnosis, as results may not be available for many hours or days. A method called 'loop mediated isothermal amplification' (LAMP) has previously been shown to be fast and effective for molecular detection of meningococcal DNA in clinical specimens.

Methods: We evaluated the diagnostic accuracy (sensitivity and specificity) of meningococcal LAMP as a near-patient test in the emergency department of a large children's hospital. Respiratory (nasopharyngeal swab) and blood specimens from children with suspected meningococcal infection were tested using a near-patient LAMP protocol and compared to reference laboratory testing.

Findings: Combined testing of respiratory and blood specimens using LAMP was accurate (sensitivity 89%; specificity 100%; PPV 100%; NPV 98%) and diagnostically useful (positive and negative likelihood ratios of ∞ and 0.11 respectively). The median time required for near-patient testing was 1 hour 26 minutes from sample to result.

Interpretation: Meningococcal LAMP is simple enough for use in any hospital with basic laboratory facilities, and near-patient testing using this method is both feasible and effective. In contrast to current UK NICE guidance, we found molecular testing of non-invasive respiratory specimens from children to be diagnostically accurate and clinically useful.

Funding: Health & Social Care Research and Development, Public Health Agency, Northern Ireland.

Introduction

Clinical diagnosis is the current standard for identifying invasive *Neisseria meningitidis* infection (meningococcal disease, MD) despite the signs and symptoms being notoriously non-specific, especially in young children.[1,2] Although MD can progress very quickly, the typical signs and symptoms (if present) do not appear until a median of 13 to 22 hours after the first onset of symptoms, and half of children who present to their GP in the early stages of MD are not referred or admitted to hospital at the first presentation.[3] Conversely, fear of missing the diagnosis also leads to overtreatment.[4] Most children admitted and treated as suspected MD turn out to have a less serious infection, and a previous study in our hospital found two thirds of children treated as possible MD had an alternative diagnosis.[5] Although introduction of an effective vaccine against serogroup B meningococcus should reduce the prevalence of meningococcal infection,[6] there is considerable scope to improve diagnostic testing.

Laboratory tests serve only to confirm the clinical diagnosis, as definitive results are rarely available in time to influence clinical decision making.[7] We previously developed a rapid and effective molecular test based on 'loop-mediated isothermal amplification' (LAMP) to detect meningococcal DNA (ctrA gene) in clinical specimens.[8] This assay gives equivalent performance to the current UK reference molecular test (TaqMan® real-time PCR[9–12]) but is faster and less expensive. In the UK, the National Institute of Health and Care Excellence (NICE) recognises that molecular (PCR) testing of sterile site specimens for meningococcus is very effective, but also notes that PCR is not available in most hospitals due to resource limitations.[13]

Detection of meningococcal DNA in a sterile site (blood or CSF) specimen confirms a diagnosis of invasive MD,[13] but this testing assumes that a level of clinical suspicion is already present. Collecting blood or CSF specimens from every patient with pyrexia but without the classical features of meningococcal infection is neither feasible nor desirable. Nevertheless, this group will include the small number of patients who have early-stage MD and who are at risk of being falsely reassured. Obtaining a nasopharyngeal specimen is relatively non-invasive, although current advice in the UK is not to test these for meningococcus due to the risk of detecting asymptomatic carriage.[13] We have previously shown, using sensitive and specific PCR methods, that carriage rates of pathogenic (capsular) meningococci in young children are very low.[14] Our previous study found molecular testing of throat swabs had a sensitivity of 81% and a specificity of 100% for diagnosis of MD. If a suitable molecular assay to detect capsular meningococci is used, we propose that near-patient testing of non-invasive respiratory specimens can provide valuable information to clinicians.

We report here a study to evaluate the diagnostic accuracy of meningococcal LAMP as a near-patient test on respiratory (combined nasal and throat swab) and blood specimens in patients with suspected MD. Respiratory, blood and CSF specimens were tested in the laboratory using a reference PCR assay. Conventional blood and CSF culture methods were used, as per normal clinical practice, and the diagnostic performance of standard 'non specific' laboratory tests was evaluated for comparison.

Materials and methods

Study design, inclusion criteria and specimens

The study was approved by the Office for Research Ethics Committee Northern Ireland [reference 09/NIR02/43]. Clinical specimens were collected as per normal Royal Belfast Hospital for Sick Children (RBHSC) practice, with near-patient testing of aliquots as described below. Written informed consent was obtained from the parents of all study participants before their near-patient test results were included in the study.

The study was designed as a prospective cohort study of diagnostic accuracy. Children (aged 0 to 13 years) presenting to the emergency department between November 2009 and January 2012 were eligible for inclusion. Patients with suspected meningitis or septicaemia entered a clinical care pathway and had a standard 'meningococcal pack' of investigations (Table 1). This group included those whom the admitting doctor suspected might have MD: children with fever, unwell appearance, non-blanching rash, signs of meningitis or signs of septicaemia.

Near-patient meningococcal ctrA LAMP

Near-patient testing of clinical specimens (nasopharyngeal swabs and EDTA blood) was done in a room adjacent to the pediatric emergency department. Further details of the near-patient LAMP protocol are given in the appendix. In brief, DNA was extracted from blood and respiratory (combined nasopharyngeal swab) specimens using a simple commercially available DNA extraction system. DNA extracts were analysed immediately using LAMP

reagents that were prepared in advance and stored frozen in the emergency department. Unlike molecular amplification methods such as PCR, positive LAMP reactions can be identified by visual inspection of the reaction tubes after incubation at an appropriate temperature. In this study, four near-patient LAMP tests were run for each patient (EDTA blood; combined nasopharyngeal swab; positive control; negative control) and test results were read after incubation at 63°C for 60 minutes.

Reference laboratory tests

Details of the gold standard reference laboratory tests are given in the appendix.

Statistical methods

Laboratory confirmed MD was defined as: "A clinically compatible case plus isolation of *N. meningitidis* or detection of *N. meningitidis* DNA from a normally sterile site (blood or CSF)".[15] Likelihood ratios (LR), sensitivity, specificity and positive and negative predictive values (PPV, NPV) were calculated for near-patient LAMP testing of nasopharyngeal specimens, blood specimens and both tests combined for diagnosis of MD. 95% confidence intervals were calculated for estimates of diagnostic accuracy. Staff in both the MRU and BHSCT laboratories were blinded to the results of near-patient testing.[16]

Role of the funding source

The funding sponsor had no role in the study design, data interpretation or decision to submit for publication.

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Results

Laboratory confirmed meningococcal disease

In total, 161 patients had a 'meningococcal pack' of investigations and were tested. Subsequently, one declined consent, and a further 12 were not approached for consent (two died; one child protection case; two discharged early; seven transferred to other units). The remaining 148 patients were consented into the study. Most were under five years old (median 11 months; range 17 days – 12.5 years) and 57% were male gender. 27/148 (18%) were found to have laboratory confirmed MD, and 121/148 (82%) had other conditions (Table 2). Only 7/27 (26%) of the children with confirmed MD had meningococcus isolated in blood culture, and only one of these was positive by blood culture alone. 26/27 (96%) were positive for meningococcal DNA in their blood by PCR. 8/27 (30%) of the children with confirmed MD had a lumbar puncture to obtain CSF (Table 3) and all were culture negative. Seven had CSF analysed for meningococcal DNA in the laboratory and 6/7 (86%) were positive. Antibiotics had been given prior to lumbar puncture to all patients in our study, giving CSF culture a sensitivity of zero. In contrast, molecular testing of CSF was clearly useful, although none of the patients here were positive by molecular testing of CSF alone.

11 children with laboratory confirmed MD had viral co-infections diagnosed by routine molecular virology testing, and three were infected with more than one virus. One child was positive in blood for enterovirus, seven were positive for respiratory viruses (picornavirus, rhinovirus, enterovirus, influenza A, bocavirus) and three were positive in stool specimens (astrovirus, rotavirus or picornavirus). Four of the children who did not have MD had other

invasive bacterial infections confirmed by routine bacteriology testing. Two had *S. pneumoniae* in blood culture; one had *E. coli* in blood, urine and CSF culture; one had *S. agalacticae* in blood culture.

Near-patient testing using meningococcal LAMP

The median time taken to complete the near-patient meningococcal LAMP tests (from starting extraction to reading results) was 1 hour 26 minutes. Results of near-patient testing using meningococcal LAMP are shown in Table 4. 141/148 children had a combined nasal and throat swab taken and tested using the near-patient LAMP protocol. The performance of near-patient nasopharyngeal testing (Table 4A) was as follows: sensitivity 84% (95% CI 65-94); specificity 100% (95% CI 98-100); PPV 100% (95% CI 81-100); NPV 97% (95% CI 91-99); positive LR ∞; negative LR 0.16 (95% CI 0.07-0.39). 144/148 children had sufficient blood taken for testing using the near-patient LAMP protocol. The performance of near-patient blood testing (Table 4B) was: sensitivity 84% (95% CI 72-99); specificity 100% (95% CI 93-100); PPV 100% (95% CI 75-100); NPV 97% (95% CI 90-99); positive LR ∞; negative LR 0.16 (95% CI 0.01-0.42). All 148 children had at least one specimen (nasopharyngeal swab and/or blood) analysed in the near patient setting. If patients who were positive by either of the near-patient tests were considered to be positive, the combined performance of near-patient LAMP testing of blood and/or nasopharyngeal specimens (Table 4C) was: sensitivity 89% (95% CI 72-96); specificity 100% (95% CI 97-100); PPV 100% (95% CI 83-100); NPV 98% (95% CI 93-99); positive LR ∞; negative LR 0.11 (95% CI 0.04-0.32).

Three patients with laboratory confirmed MD were negative by both near-patient LAMP tests. Two had classical signs and symptoms of MD, and the third was an atypical presentation; a two year-old girl recalled to the hospital when a blood culture taken the previous day grew *N. meningitidis.* At first presentation, her WCC and CRP were normal, and she was discharged on oral amoxicillin after a period of observation. When recalled 20 hours later, her parents reported that she had remained well since discharge. Physical examination revealed no meningism, normal perfusion and a few non-blanching spots on her abdomen. Her WCC had risen to 28.2 x 10^9/l and CRP to 211 mg/l. Her nasopharyngeal and blood specimens were both PCR positive for meningococcal DNA.

'Non-specific' laboratory tests

The results of 'non-specific' laboratory tests are shown in Table 5. Near-patient LAMP testing was substantially more accurate than any of the routinely used non-specific tests for diagnosis of MD.

Discussion

Compared to the gold standard laboratory diagnosis of MD, the near-patient LAMP assays gave impressive results. Comparison to WCC and CRP indicates that LAMP tests are more useful than conventional non-specific tests for both ruling in and ruling out meningococcal disease. In addition to being accurate, this study demonstrates that molecular testing in the emergency department is feasible. The LAMP assay performed similarly well in the near-patient setting and the laboratory setting, using different extraction protocols and different operators, with results typically available in less than two hours. We describe the accuracy of meningococcal LAMP in terms of likelihood ratios, which are not affected by disease prevalence.[17] Likelihood ratios greater than 10 and less than one are considered strong evidence for the value of a diagnostic test.[18] By this metric, the near-patient LAMP assay used here is clearly useful for diagnosis of MD.

There is likely to be resistance to testing of nasopharyngeal specimens for meningococcus because of the potential to detect asymptomatic carriage. No carriage was detected during this study. Although a larger study may have identified some carriage and reduced the specificity from 100%, the data supports our previous conclusion that molecular testing of nasopharyngeal specimens in very young children is diagnostically useful, and generally not confounded by carriage.[14] A relatively non-invasive nasopharyngeal meningococcal LAMP test on children presenting with 'fever without source' where the prevalence of meningococcal disease is very low should perform with reasonable accuracy. In this context a positive result is strongly suggestive of disease and could prompt further investigation and treatment. Patients with a negative result could be discharged safely after a period of observation, subject to the universal advice to re-attend if deterioration occurs. Most children

with fever still present to their general practitioner where the prevalence of meningococcal disease is much lower still.[19,20] A non-invasive test with a strong negative likelihood ratio used in combination with careful clinical assessment is likely to be even more valuable in this context.

Our data support the cost minimisation analysis conducted by NICE which suggests that rapid (<24 hours) access to molecular (PCR) test results can reduce costs through earlier discharge of the relatively well patients. NICE conclude that "the infrastructure does not currently exist to support such a strategy and is unlikely to exist within the next few years".[13] Meningococcal LAMP could be used immediately in any small to medium sized hospital with access to a basic laboratory to give results within a few hours. The meningococcal LAMP assay described here, used in combination with careful clinical assessment, could have led to earlier discharge of a significant number of 'not unwell' patients in this study who did not have MD.

Many of the MD cases in this study also had viral co-infections. As reported previously[21] we note that in the absence of rapid molecular testing for bacterial pathogens, positive molecular virology test results for viruses such as enterovirus or picornavirus should not reassure clinicians that an unwell patient does not have MD.

One limitation of our study was that the proportion of positive cases (19%) was lower than a previous study in our unit (33%) although this confirms there was no bias towards more severe case. The blood culture positive rate of ~25% is similar to previous studies[22,23] and the 'PCR only' case ascertainment (77%) is slightly higher than reports from the national reference laboratory[23] indicating a representative case mix of patients. We experienced

historically low levels of meningococcal disease during this study, with only 36 confirmed cases in Northern Ireland in 2011 compared with 94 cases in 2004. This has resulted in wider confidence intervals for our estimates of diagnostic accuracy. A small number of discordant test results were seen, which illustrates the problems that are common with definitive diagnosis of MD, even using the best available reference laboratory tests. This study was not designed to investigate whether early availability of definitive test results could influence patient management and improve clinical outcomes. A larger multi-centre trial of near-patient LAMP for children presenting with 'fever without source' could address this important question. Since this work was completed, the meningococcal LAMP test has been developed further, using freeze-dried reagents, real-time detection and improved amplification chemistry to improve sensitivity and reduce the assay time to below 20 minutes.

This study is the first to evaluate a rapid near-patient molecular diagnostic test for MD, and also the first demonstration of near-patient testing using LAMP in a UK hospital. The data support our initial hypothesis that testing of nasal and throat swabs using meningococcal LAMP in a near-patient setting is useful as a rapid diagnostic test for *N. meningitidis*. LAMP testing of nasopharyngeal or blood specimens in a near-patient setting was both feasible and accurate, and combining the results for both specimen types further increased the diagnostic accuracy. These data suggest the ability to rapidly detect a pathogenic (capsular) strain of *N. meningitidis* in the nasopharynx of a febrile child whose parents are sufficiently concerned to seek medical advice can give clinically useful information. Current advice from Public Health England (PHE) and NICE on testing of respiratory specimens is inconsistent. PHE recommends culture of nasopharyngeal swabs from suspected cases.[24] In contrast, NICE explicitly recommend that nasopharyngeal specimens are not tested, by any method.[13] The

potential to diagnose and treat life-threatening invasive disease early outweighs the small risk of detecting asymptomatic carriage in young children, and this should be considered in reviewed NICE guidance.

Research in context panel

Systematic review

A PubMed search on Dec 5th, 2014, with the terms ((near patient AND meningococc*) OR (near patient AND meningitidis)) found 16 papers, none related to near-patient testing. Searching with the terms ((point of care AND meningococc*) OR (point of care AND meningitidis)) found 7 papers, two reporting methods suitable for near-patient testing[8,25]. One of these[25] describes a LAMP-based testing system that "has potential" for point of care testing, but presents no clinical validation data in either laboratory or near-patient settings. The other[8] is the assay used here, and is the first published and clinically validated LAMP assay for rapid detection of meningococcal DNA. This study is the first to evaluate such an assay for near-patient testing.

Interpretation

There is currently no effective diagnostic test for meningococcal disease[2]. Cases with clear signs and symptoms are (or should be) diagnosed clinically, and laboratory testing serves mainly to confirm the clinical diagnosis. The decision to obtain and test sterile site specimens (blood or CSF) presumes that a clinical suspicion of serious invasive disease

already exists. No clinician who suspects meningococcal disease will wait for laboratory results before starting antibiotic treatment, so testing simply confirms an existing diagnosis.

Unfortunately, in the absence of definitive diagnostic testing, children with non-specific symptoms who are actually in the early stages of meningococcal disease are frequently overlooked. Half of children with confirmed meningococcal infection have been seen by a healthcare professional and either falsely reassured or discharged in the hours leading up to their clinical diagnosis and treatment.[3] It seems self-evident that identifying and treating these children earlier could improve clinical outcomes, although there is no data to confirm this. Without an accurate diagnostic test for meningococcal disease, ideally delivered at point-of-care, there is little prospect of identifying these cases earlier in a clinical trial.

This study confirms that molecular testing using LAMP to detect meningococcus is highly sensitive and specific, and demonstrates this for the first time in the context of near-patient testing in the emergency department. Molecular tests to detect this pathogen should not be confined to the reference laboratory.

Acknowledgements

We thank the staff of the Regional Virus Laboratory and the Pediatric Emergency Department for their support and assistance. This study was funded by a research fellowship from Health & Social Care Research and Development, Public Health Agency, Northern Ireland.

Declaration of interests

TWB received a grant from the Health & Social Care Research and Development, Public Health Agency, Northern Ireland. JPM, PVC and DJF hold share options in Hibergene Diagnostics Ltd. JPM, PVC and DJF hold patent US 8465927 B2 related to the meningococcal LAMP assays used in this study which is licensed to Hibergene Diagnostics Ltd. MDS has nothing to disclose.

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TABLE 1 'Meningococcal pack' investigations

Specimen	Volume	Test
Whole blood	At least 1ml	Blood culture, collected prior to antibiotic therapy if possible
EDTA blood	1ml	Meningococcal ctrA TaqMan® qPCR
EDTA blood	1ml[a]	Enterovirus / picornavirus RT-qPCR
EDTA blood	0.5ml	Full blood count
Clotted blood	0.5ml	Routine biochemistry: renal function; electrolytes; calcium; magnesium; C-reactive protein (CRP)
Heparinised blood	1.4ml	Coagulation screen
EDTA blood	2ml	Routine haematology / blood group
Combined nasal & throat swab	1ml[a]	Swabs combined in eNAT transport medium; meningococcal ctrA TaqMan® qPCR / LAMP and viral screen
CSF (optional)	1ml[b]	Routine biochemistry, WCC, culture and viral screen

[a]0.2ml aliquots of these specimens were processed using the near-patient LAMP protocol.

[b]Lumbar puncture to obtain CSF is done only if clinically indicated.

TABLE 2 Reference laboratory results for confirmed MD cases (n=27)

	Reference tests to confirm MD			Laboratory LAMP tests	
Patient no.	Blood culture	Blood PCR[a]	Serogroup[a]	Swab LAMP[b]	Blood LAMP
3	+	+	B	+	+
4	-	+	B	+	+
10	-	+	B	+	+
13	-	+	B	+	+
21	-	+	B	+	NA
29	+	+	B	-	+
30	-	+	B	+	+
36	-	+	B	+	+
73	-	+	B	+	+
85	+	+	Y	+	+
87	-	+	B	+	+
91	+	+	B	+	+
93	-	+	B	+	+
94	-	+	B	+	+
106	-	+	B	+	+
110	-	+	B	+	+
114	+	+	B	+	+
116	-	+	B	+	+
132	-	+	B	+	+
133	-	+	B	+	+
134	-	+	B	+	+
135	-	+	B	NA	+
145	-	+	B	+	+
146	+	+	B	+	+
157	-	+	B	+	+
158	-	+	B	+	+
160	+	+	B	NA	+

[a] PCR testing (ctrA TaqMan® assay and specific meningococcal serogrouping qPCR assays) were performed in Manchester (MRU), Belfast (BHSCT) or both.

[b] Combined nasopharyngeal swab; NA = specimen not available

TABLE 3 Results for patients with confirmed meningococcal disease where CSF was examined

Patient no.	Time from treatment (hrs)	CSF culture	CSF LAMP	CSF TaqMan
10	22	-	+	+
13	126	-	-	-
36	46	-	+	+
73	16	-	+	ND
94	42	-	+	+
114	2	-	+	+
145	48	-	+	+
158	44	-	ND	ND

ND, not done

TABLE 4 Results of near-patient meningococcal LAMP testing

A: Nasopharyngeal specimens (n=141)

		Meningococcal disease	
		Positive	Negative
Nasopharyngeal LAMP	Positive	21	0
	Negative	4	116

B: EDTA blood specimens (n=144)

		Meningococcal disease	
		Positive	Negative
Blood LAMP	Positive	22	0
	Negative	4	118

C: Combined results for both specimen types (n=148)

		Meningococcal disease	
Nasopharyngeal &/or blood LAMP		Positive	Negative
	Positive	24	0
	Negative	3	121

TABLE 5 Results of 'non-specific' laboratory tests

Test[a]	Sensitivity (95% CI)	Specificity (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)
CRP >10 mg/l	93% (77 to 98)	64% (55 to 72)	2.60 (1.99 - 3.39)	0.11 (0.03 - 0.44)
CRP >60 mg/l	63% (44 to 78)	91% (85 to 95)	7.43 (3.84 - 14.37)	0.41 (0.25 - 0.66)
Abnormal WCC	78% (59 to 89)	65% (56 to 73)	2.22 (1.61 - 3.05)	0.34 (0.17 - 0.70)
Abnormal neutrophils	85% (67 to 94)	52% (43 to 61)	1.78 (1.39 - 2.28)	0.28 (0.11 - 0.71)

[a]CRP cutoffs used in the literature range from 8 to 100 (reviewed by NICE[13]). Cutoffs used here were selected to optimise sensitivity (10 mg/l) or specificity (60 mg/l) based on ROC analysis (data not shown). The reference normal range for WCC in our laboratory is 5 to 13 x 10^9/l.

Supplementary material

Near-patient meningococcal LAMP protocol

DNA was extracted from EDTA blood (200µl) and eNat medium (200µl, containing combined nasal and throat swabs) using a QuickGene Mini80 system and DB-S DNA kit (Fuji Corporation, Tokyo, Japan). DNA extracts (100µl) were denatured (95°C, 5 minutes) cooled on ice and tested immediately. Each LAMP reaction (25µl) comprised 20µl of LAMP mastermix and 5µl of DNA extract in a 100µl PCR tube (Abgene Ltd., Epsom, UK). A base LAMP mastermix containing all reagents except DNA polymerase (Bst2.0, 8U/µl; New England Biolabs, Ipswich, USA) and Fluorescence Detection Reagent (FDR; Eiken Chemical Company, Japan) was stored at -20°C in 90µl aliquots prior to use. Components of the base mastermix (buffer, LAMP primers, betaine, $MgSO_4$ and dNTPs) were as described previously.[1] For each patient, a 90µl aliquot of mastermix was thawed, mixed with DNA polymerase (5µl) and FDR (5µl) and used immediately. Reactions were incubated at 63°C using a standard thermal cycler (GeneAmp 9700, Life Technologies, Paisley, UK). After 60 minutes, tubes were visually inspected for a colour change from pale orange to bright yellow/green to identify positive reactions, as illustrated in Figure S1.

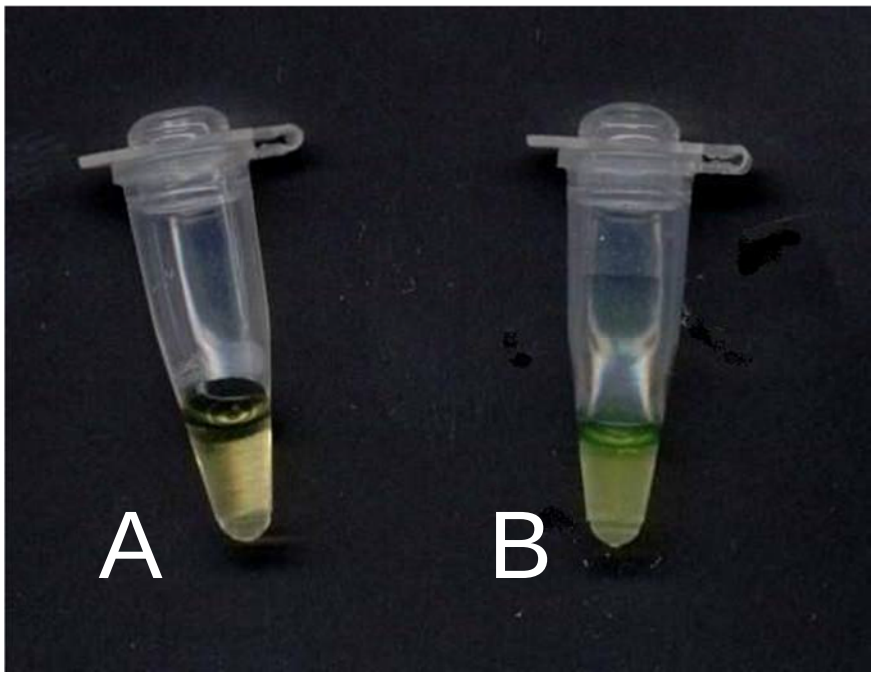

Figure S1. Negative (A) and positive (B) meningococcal LAMP reaction tubes.

Reference laboratory testing

Blood and CSF specimens were cultured according to UK standard methods[2,3] using an automated BacT/ALERT® 3D Microbial Detection System with BacT/ALERT® FA culture medium (bioMérieux, Marcy l'Etoile, France). Isolates were identified using the VITEK® 2 system (bioMérieux) and latex bead agglutination tests (Pastorex Meningitis Kit, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). PCR testing of EDTA blood was performed by the MRU or the Belfast laboratory or both. In Belfast, EDTA blood specimens (200µl) were extracted using the QIAsymphony SP automated extraction system and DSP Virus/Pathogen Mini Kit / Complex 200 protocol (Qiagen Ltd., Crawley, UK). PCR assays (10µl) to detect the meningococcal ctrA gene used Invitrogen Platinum® Quantitative PCR SuperMix-UDG (Life Technologies Ltd., Paisley, UK) with primers and a TaqMan® probe as described

previously.[4] An additional reverse primer (5'-TTGCCGCGGATTGGCCACCA-3') was used[5] to ensure that strains with known mutations in the ctrA gene could be reliably detected.[6,7] PCR assays were run on a LightCycler® 480II real-time PCR system (Roche Diagnostics Ltd., Burgess Hill, UK) using the following thermal cycling protocol: 50°C (10 minutes); 95°C (2 minutes); 45 cycles of 95°C (15 seconds) and 60°C (30 seconds). CSF specimens (200µl) were tested in the Belfast laboratory using either PCR or LAMP, as per the near-patient testing protocol, except that DNA extracts were prepared using a QIAamp DNA Blood Mini Kit (Qiagen Ltd.).

Serogrouping PCR

A commercially available real-time PCR kit (Diagenode Diagnostics, Léige, Belgium) was used to determine the genetic capsule type for confirmed cases, with specific primers and TaqMan® probes for capsule biosynthesis genes of the five serogroups (A, B, C, W135, Y) most frequently associated with MD. Reactions (10 µl) contained 1X Platinum® Quantitative PCR SuperMix-UDG (Invitrogen Ltd, Paisley, UK), 4 mM $MgCl_2$, 1 µl of primer and probe mix, 0.2 mg/ml bovine serum albumin (Sigma-Aldrich Ltd., Dorset, UK), nuclease free water and 2 µl of template DNA. Assays were run on a LightCycler® 480II real-time PCR system using the manufacturer's recommended cycling protocol: 50°C (2 minutes); 95°C for (10 minutes); 45 cycles of 95°C (10 seconds), 60°C (40 seconds) and 72°C (1 second).

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