ORIGINAL ARTICLE

Comparative genomics of *Neisseria meningitidis* strains: new targets for molecular diagnostics

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Abstract

In 2010, Jaton *et al*. (False-negative PCR result due to gene polymorphism: the example of *Neisseria meningitidis*. J Clin Microbiol 2010;48:4590–2) reported an isolate of *Neisseria meningitidis* serogroup B that was not detected by the *ctrA* quantitative real-time PCR (qRT-PCR) used in our diagnostic laboratory. Sequence analysis of *ctrA* revealed several single nucleotide polymorphisms responsible for the negative qRT-PCR. Therefore, we sequenced the genome of this isolate and performed comparative genomics to propose new gene targets for the specific detection of *N. meningitidis* from clinical specimens. We identified 11 genes as specific to *N. meningitidis* genomes and common to at least 177 (97%) of the 183 genomes available. Among them, three genes (*metA, tauE* and *shlA*) were selected to develop new qRT-PCRs for the detection of *N. meningitidis* DNA. The three qRT-PCRs were highly sensitive and specific, and they exhibited a good reproducibility when tested on plasmidic positive controls and genomic DNA extracted from strains of *N. meningitidis* and other relevant bacterial species. The clinical sensitivity and specificity of *metA* and *tauE* qRT-PCRs were both 100% based on a testing of cerebrospinal fluid samples positive for *N. meningitidis* or other clinically relevant bacteria. Despite a 100% specificity, the sensitivity of the *shlA* qRT-PCR was only 70%. We thus recommend using the *metA* and/or *tauE* qRT-PCRs developed here. To prevent PCR failure in the presence of new polymorphic strains, the detection of dual targets by duplex qRT-PCR would be more accurate and suitable for the diagnosis of *N. meningitidis* from clinical specimens.

Keywords: *ctrA*, Genome sequence, *metA*, molecular diagnostics, *Neisseria meningitidis*, quantitative real-time PCR, *shlA*, *tauE*
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Introduction

Bacterial meningitis, a severe infection affecting the central nervous system [1,2], is mainly caused by three bacteria: *Streptococcus pneumoniae, Haemophilus influenzae* type b and *N. meningitidis*. *N. meningitidis* causes invasive meningococcal infections including meningitis and septicaemia, which are life-threatening diseases requiring prompt treatment with an appropriate antibiotic [1]. *N. meningitidis* is frequently localized in the nasopharynx [3,4], and its carriage rate in the nasopharyngeal cavity varies from 5 to 10% of adolescents and young adults [5]. Mortality associated with *N. meningitidis* remains—it is about 10% in industrialized countries—despite modern healthcare management. Moreover, morbidity may include long-term sequelae such as hearing loss, cognitive dysfunction, developmental delay and motor nerve deficits [6]. Twelve serogroups of *N. meningitidis* have been identified on the basis of the capsular polysaccharide structures, and among them six serogroups (A, B, C, W, X and Y) are the major causes of severe invasive disease [4,7–9].

Before the availability of PCR-based testing, diagnosis of *N. meningitidis* infections was mainly performed by bacterial

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culture, which is relatively slow, as it requires approximately 24 to 48 hours for a confirmed positive result. Moreover, prior antibiotic therapy affects the results of conventional bacteriologic methods, including bacterial culture, decreasing the detection rate of the pathogenic bacteria by approximately 30% [10]. In addition to the low sensitivity of culture, errors in phenotypic identification may occur, even when matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technology [11] is used. Indeed, Cunningham *et al.* [12] recently reported the misidentification of *N. polysaccharea* strains as *N. meningitidis* using MALDI-TOF MS with high identification scores ranging from 2.068 to 2.241. Such misidentification is expected because these two species are closely related, as demonstrated by their high percentage of 16S rRNA identity (99.3%).

During the last 20 years, the diagnosis of bacterial meningitis has been improved by the development of PCR assays that specifically target the responsible pathogens. To this end, Corless *et al.* [2] described in 2001 quantitative real-time PCR (qRT-PCR) assays targeting the *ply, bexA* and *ctrA* genes for detection of *S. pneumoniae, H. influenzae* and *N. meningitidis*, respectively. Therefore, several diagnostic laboratories, including our own, used the *ctrA* gene as a target for the detection of *N. meningitidis* from clinical samples and especially from cerebrospinal fluid (CSF) specimens. However, some studies reported that the chromosomal locus containing the *ctrA* gene could be subject to rearrangement, and hence 16 to 28% of *N. meningitidis* isolates lack the *ctrA* gene [13–15]. Thus, targeting this gene can lead to false-negative detection of *N. meningitidis*. Importantly, we recently had a case of *N. meningitidis* meningitis in a 2-year-old child confirmed by culture, for which the PCR was negative due to the presence of several single nucleotide polymorphisms (SNPs) in the *ctrA* gene at the binding positions of the reverse primer and the probe [16]. A similar false-negative result of the *ctrA* qRT-PCR was also documented in Italy [17].

Given the need for accurate tools to diagnose this major pathogen, we describe here the genome sequence of the *N. meningitidis* isolate reported by Jaton *et al.* [16] and provide a detailed comparative genomic analysis to identify new target genes. We developed three new quantitative real-time PCRs for specific diagnosis of *N. meningitidis* species and evaluated their performance using genomic DNA from isolates and clinical samples.

Materials and Methods

Isolate

The *N. meningitidis* CHUV (Nm CHUV) isolate under investigation was cultured from a CSF sample taken from a 2-year-old child hospitalized at the University Hospital Center of Lausanne for meningitis with petechiae [16].

Genome sequencing

Genomic DNA was extracted and purified using the Wizard Genomic DNA purification kit (Promega, Duebendorf, Switzerland). Genomic libraries were constructed using the Nextera XT library kit (Illumina, San Diego, CA, USA) in order to combine different genomic projects in the same sequencing run. Constructed libraries were normalized on specific beads according to the Nextera XT protocol and pooled into a single library for paired-end sequencing of 2 × 150 bp using the MiSeq (Illumina).

Genome assembly and annotation

The reads were filtered based on their quality and size using the fastq-mcf program (Ea-utils: http://code.google.com/p/ea-utils). Genome assembly was done using the SPAdes assembler with different kmer values (from 81 to 127). The assembly with lowest number of contigs and highest N50 was selected for further analysis. To perform genomic comparison, we retrieved from the National Center for Biotechnology Information (NCBI) database all complete (n = 14) and incomplete (n = 169) genomes of *N. meningitidis*, as well as 14 non-*meningitidis Neisseria* genomes including *N. gonorrhoeae, N. cinerea, N. bacilliformis, N. elongata, N. flavescens, N. lactamica, N. macacae, N. mucosa, N. polysaccharea, N. sicca, N. subflava, N. wadsworthii, N. weaveri* and an unclassified *Neisseria* strain. The genome of the Nm CHUV isolate, as well as the 14 complete genomes of *N. meningitidis* and the 14 non-*meningitidis Neisseria* genomes, were reannotated using the RAST Server [18]. To identify the multilocus sequence typing (MLST) profile, the Nm CHUV genome was submitted to the pubMLST database (http://www.pubMLST.org).

Genomics comparison to identify new target genes for diagnostic qRT-PCR

Pairwise genome sequence identities were calculated by the JSpecies program (http://www.imedea.uib.es/jspecies). After annotation, the gene pool of Nm CHUV was compared by BLASTN with those of all non-*meningitidis* (cutoffs: gene coverage > 40%, E value $<10^{-3}$) to identify genes specific to Nm CHUV. The latter were subjected to a reciprocal BLASTN with all *N. meningitidis* gene pools (n = 35 628 genes) to identify genes common to the 14 *N. meningitidis* genomes. This subset of common *N. meningitidis* genes was subsequently subjected to a BLASTN similarity search against the National Center for Biotechnology Information (NCBI) nonredundant database 'nt.' Genes specific to *N. meningitidis* species and common to all *N. meningitidis* complete genomes were kept. Finally, the

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presence of these genes was also assessed in the 169 available whole genome shotgun sequences of *N. meningitidis*. From the specific and conserved genes within the 183 available genomes of this species, three genes were selected for the development of new specific qRT-PCRs because of their presence in all 183 genomes analysed and their predicted function based on the hypothesis that a gene with a key function is less likely to be lost throughout evolution.

Quantitative real-time PCR development

Primers and probes targeting the genes of interest were designed using Primer3 0.4.0 software [19]. The specificity of the primers and probes was checked by BLASTN against the nonredundant NCBI database and by *in silico* PCR from the online database (http://insilico.ehu.es/). Targeted gene sequences (about 500 bp) containing the qRT-PCR primer and probe sequences were amplified by PCR using primers described in this study. PCR products were purified and cloned into a pCR2.1-TOPO vector (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA) as previously described [20]. The recombinant control plasmids were produced in *Escherichia coli* TOP10 competent cells and quantified using a Nanodrop ND-1000 (Witech, Littau, Switzerland). Several tenfold dilutions were performed to prepare plasmid solutions from 10^5 copies/μL to 1 copy/μL. In addition, primers and probe solutions were tested at two concentrations (100 nM, 200 nM) to determine the optimal concentrations for the qRT-PCR assays that were always performed with 5 μL of template DNA in a final volume of 20 μL, as previously described [20]. The DNA amplification and quantification was performed on a StepOne instrument (Thermo Fisher Scientific Life Sciences) with the following conditions: a holding stage (50°C for 2 minutes and 95°C for 10 minutes) followed by a cycling step repeated 40 times (95°C for 1 second and 60°C for 20 seconds).

To evaluate the efficiency, analytical sensitivity and reproducibility of the qRT-PCRs, each primer concentration was tested with each probe concentration on a dilution series of the control plasmid. The analytical specificity was tested on 29 extracted genomic DNAs of reference and clinical isolates (collected from our clinical diagnostic laboratory) including *N. meningitidis* (n = 5), other *Neisseria* species (*N. gonorrhoeae* (n = 3), *N. elongata* (n = 2), *N. flavescens* (n = 4), *N. mucosa* (n = 3), *N. bacilliformis* (n = 1), *N. weaveri* (n = 1), *N. lactamica* (n = 1), *N. canis* (n = 1), *N. zoodegmatis* (n = 1), *Neisseria* sp. (n = 2)) and organisms not belonging to the *Neisseria* genus (*E. coli* (n = 1), *Staphylococcus aureus* (n = 1), *Pseudomonas aeruginosa* (n = 1), *H. influenzae* (n = 1) and *Streptococcus mitis* (n = 1)). All genomic DNA concentrations were standardized to 10^4 DNA copies/μL. The specificity of our three qRT-PCRs was also determined using clinical CSF samples positive for *Listeria monocytogenes* (n = 2), *S. pneumoniae* (n = 2), *H. influenzae* type b (n = 1) and c (n = 1) and *Enterococcus faecalis* (n = 5). The sensitivity was also tested on CSF samples positive for *N. meningitidis* (n = 10), by tenfold dilution, in duplicate, until negativity. These ten *N. meningitidis* strains belonged to serotypes W135/Y (n = 3), serotype C (n = 2), serotype B (n = 1) and undetermined serotypes (n = 4). Sterile DNA-free water was used as negative control.

Results

Genome analysis

Genome assembly of the 2 044 080 paired reads from isolate Nm CHUV produced 144 contigs with lengths ranging from 1008 to 95 448 bp and an average contig size of 14 940 bp. The genome size was estimated to be 2 151 415 bp with a GC content of 51.65% and coverage of 142 fold. No plasmids were identified. Table I summarizes the general features of the Nm CHUV genome and the published complete genomes of *N. meningitidis* strains, which belong to different serogroups including A, B and C as well as one that was nongroupable. Almost all were isolated from Europe, except one each from China, the United States and the Gambia in Africa. Genome comparison of this Nm CHUV isolate with the 14 available complete genomes of *N. meningitidis* (Table I) revealed that this isolate is most similar to the *N. meningitidis* M04-240196 isolate, with 99.72% average nucleotide identity. Pairwise nucleotide identity with the other 13 complete *N. meningitidis* genomes ranged between 97.10 and 97.83% (Table I).

As expected, sequence analysis of the *ctrA* gene obtained by whole genome sequencing confirmed the existence of SNPs in the Nm CHUV isolate. One SNP (G to A) was located on the probe sequence, and four SNPs (A, T, A, G to G, G, C, G, respectively) were located on the reverse primer sequence. Interestingly, the recently described Nm M04-240196 isolate exhibits exactly the same SNPs in the *ctrA* gene. MLST results (*abcZ* 4, *adk* 10, *aroE* 15, *gdh* 8, *pdhC* 11, *pgm* 9, *fumC* unknown) show that the isolate Nm CHUV belongs to clonal complex 269, which is represented by the type strain M04-240196. However, it cannot be classified to a known sequence type because it harbours an unknown *fumC* allele (no known allele type assigned). Moreover, strain M04-240196 and CHUV differ by 777 SNPs along the genome sequence.

Gene pool comparison of the Nm CHUV genome (n = 2602 genes) with those of 16 genomes of non-*meningitidis* species (n = 55 038 genes) by BLASTN identified 129 genes that are specific to the genome of the Nm CHUV isolate. Reciprocal BLASTN comparison of these specific genes with the 14 complete genomes of *N. meningitidis* revealed that 23 of these 129 unique genes are

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TABLE 1. General features of *Neisseria meningitidis* CHUV genome and the published complete genomes of 14 additional *N. meningitidis* isolates

Organism	BioProject	Size (Mb)	GC%	Genes	Proteins	Serogroup	Geographic origin	% of genome identity[a]
Neisseria meningitidis CHUV	—	2.15	51.65	2657	2602	B	Switzerland	—
Neisseria meningitidis MC58	PRJNA57817	2.27	51.5	2675	2602	B	United Kingdom	97.83
Neisseria meningitidis M01-240355	PRJNA162075	2.29	51.5	2667	2596	B	United Kingdom	97.32
Neisseria meningitidis 053442	PRJNA58587	2.15	51.7	2550	2479	C	China	97.22
Neisseria meningitidis 8013	PRJEA161967	2.28	51.4	2660	2589	C	France	97.19
Neisseria meningitidis FAM18	PRJNA57825	2.19	51.6	2557	2486	C	United States	97.40
Neisseria meningitidis G2136	PRJNA162085	2.18	51.7	2600	2529	B	United Kingdom	97.19
Neisseria meningitidis H44/76	PRJNA162083	2.24	51.4	2097	2538	B	Norway	97.19
Neisseria meningitidis M01-240149	PRJNA162079	2.22	51.4	2609	2545	B	United Kingdom	97.19
Neisseria meningitidis M04-240196[b]	PRJNA162081	2.25	51.4	2627	2555	B	United Kingdom	99.72
Neisseria meningitidis NZ-05/33	PRJNA162077	2.25	51.3	2634	2563	B	New Zealand	97.19
Neisseria meningitidis WUE 2594	PRJEA162093	2.23	51.8	2691	2624	A	Germany	97.10
Neisseria meningitidis Z2491	PRJNA57819	2.18	51.8	2578	2508	A	Gambia	97.05
Neisseria meningitidis alpha14	PRJNA61649	2.15	51.9	2512	2442	cnl	Germany	97.19
Neisseria meningitidis alpha710	PRJNA161971	2.24	51.7	2650	2580	B	Germany	97.36

[a]Percentage of identity of the genome of the *N. meningitidis* CHUV strain with the published genomes of other *N. meningitidis* isolates, calculated by the JSpecies program (using BLAST and Nucmer programs); cnl, capsule null locus.
[b]This single genome exhibited more than 99% average nucleotide identity.

conserved in all 15 genomes. The 23 genes were subjected to BLASTN analysis against the nonredundant database to remove those present in other bacteria. Finally, the presence of 11 genes unique to *N. meningitidis* was investigated in the 169 draft genomes of *N. meningitidis* (incomplete genomes) available. Taken together, these analyses allowed us to identify 11 genes that were specific to *N. meningitidis* and common to more than 97% of the 183 available genomes (Supplementary Table S1). These 11 genes encode proteins involved in bacterial membrane structure, the toxin/antitoxin system or export/import metabolism. Three of these 11 genes, present in all 183 *N. meningitidis* genomes, were selected as potential targets for the development of qRT-PCR assays for the diagnosis of *N. meningitidis*: *metA*, encoding a periplasmic protein associated with the phenol degradation pathway; *tauE*, encoding a sulphite exporter; and *shlA*, encoding a putative large exoprotein involved in haeme utilization or in adhesion.

Quantitative real-time PCR assays

The primer and probe sequences for the three quantitative and standard PCRs are listed in Table 2. The optimal concentrations of primers and probes for the qRT-PCRs were estimated at 200 and 100 nM, respectively, for the primer and probe solutions (data not shown).

The efficiency of the three qRT-PCRs was tested on control plasmids with different DNA copy numbers. We observed a good correlation between DNA copy number and cycle threshold (C_t) values for all three targets: *metA* (R^2 = 0.9946; slope, −3.88), *tauE* (R^2 = 0.9951; slope, −3.67) and *shlA* (R^2 = 0.9945; slope, −3.80). Control plasmids, at a concentration of 5 × 10^5 DNA copies/reaction, were detected for *metA*, *tauE* and *shlA* at C_t values of 17.45 ± 0.149, 17.17 ± 0.09 and 16.54 ± 0.05, respectively, whereas the concentration of 5 DNA copies/reaction was detected at C_t values of 36.49 ± 0.663, 35.01 ± 0.584 and 35.43 ± 0.919, respectively. Evaluation of the analytical sensitivity for the three targets showed 100% positive detection at a concentration of ten DNA copies per reaction and ≥80% positive detection at a concentration of five DNA copies per reaction (Fig. 1). In the presence of fewer than five DNA copies, detection was variable for the three targets. The reproducibility of the three qRT-PCRs was evaluated with ten replicates of each control plasmid (Fig. 1). The specificity testing revealed positive amplification with C_t values ≤23.63 for all 5 *N. meningitidis* isolates and negative results for the other bacteria tested.

The clinical specificity using the PCR advance kit of these qRT-PCRs tested on CSF samples revealed 100% specificity for *tauE*, *metA* and *shlA*. Indeed, when CSF positive for other bacteria were tested, no DNA was amplified. The clinical sensitivity of *metA* and *tauE* qRT-PCRs was excellent (100%), comparable to that of the *ctrA* qRT-PCR used here as the reference standard, whereas *shlA* was only detected in 70% of *N. meningitidis*–positive samples tested.

Discussion

Rapid and accurate identification of pathogenic bacteria responsible for severe infections is crucial for a quick, adequate and efficient treatment. This holds especially true for bacterial infections caused by *N. meningitidis* such as septicaemia and meningitis that are serious causes of morbidity and mortality. Here we report a comparative genomic approach to identify genes conserved and specific to *N. meningitidis* species, and we develop new qRT-PCRs for the specific detection of this pathogen.

New molecular diagnostic approaches such as qRT-PCR assays have revolutionized the hospital practices in the

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TABLE 2. Primers and probes designed and validated in this study

Gene name	Primer and probe name	Sequence	Product size (bp)
Primers and probes for quantitative PCR assay			
tauE	*tauE*-F	TTTCCGATGCTCGGTACAACC	81
	tauE-R	CAGGCTTGGTAATGCCACCA	
	tauE-Probe	6FAM-TCATGCCATTGTCTAAGGTTGTTGCCT	
metA	metA-F	GCGAATTTGCTAATCCTATTTATGTGC	94
	metA-R	AAATTTTGCGCCATTACAGGTG	
	metA-Probe	6FAM-AACCAGCGCAACGAAAATTGCAA	
shlA	shlA-F	CCATGGTTGCAGTAGCCGAAA	99
	shlA-R	CGCAAAGGTCGCCTGAAGTT	
	shlA-Probe	6FAM-TGCCAACAGCCAGGGCAAAGG	
Primers for standard PCR and cloning			
tauE	*tauE*-VF	TGCAATCTATCGTTTTTGTTGC	660
	tauE-VR	TCCTTAACCGAATTCCAACAT	
metA	metA-VF	CCAGCCAAATTGCTTATGCT	509
	metA-VR	GTGCATGAATACCCGGTCTC	
shlA	shlA-VF	TGAATAAAGGTTTACATCGCATT	508
	shlA-VR	ATAATCACGTCGGCCTTTTG	

metA, gene encoding a periplasmic protein named MetA; *shlA*, gene encoding a putative large exoprotein involved in haeme utilization or in adhesion (part of the ShlA/HecA/FhaA family); *tauE*, gene encoding the sulphite exporter protein TauE/SafE.

domain of clinical microbiology [21]. Compared to standard phenotypic methods including bacterial culture or Gram staining, these methods significantly reduce the time for bacterial identification from about 24 hours to 2.5 hours [12]. However, in the case of *N. meningitidis*, some concerns have emerged about the genes targeted by qRT-PCRs currently available for diagnostic laboratories. First, the *ctrA* gene has been reported to be absent in up to 60% of nongroupable strains, generally associated with nasopharyngeal carriage [22]. Moreover, the *ctrA* gene was reported to exhibit several SNPs in the regions targeted by the primers and probes for qRT-PCR [16]. Both the lack of *ctrA* and the presence of polymorphisms in the gene may lead to false-negative results. The *sodC* gene has been described as an alternative target for identification of *N. meningitidis* strains, especially for those lacking the capsule [13]. However, *sodC* was proposed to be acquired by *N. meningitidis* by horizontal gene transfer from *H. influenzae* [13,23], suggesting inadequate specificity with possible cross-detection of *H. influenzae*. Moreover, we showed by comparative genomics that this gene was not conserved in and not specific to all 183 *N. meningitidis* genomes analysed in this study.

The comparative genomics approach used in this study identified 11 genes specific and common to more than 97% of *N. meningitidis* genomes. Among these, three genes were selected to develop three diagnostic qRT-PCRs. These three new qRT-PCRs showed high analytical sensitivity and reproducibility when tested on *N. meningitidis* plasmid positive control and good specificity when tested on genomic DNA extracted from bacterial strains involved in meningitis (*S. pneumoniae* and *H. influenzae*) or important pathogens (*P. aeruginosa, E. coli* and *S. aureus*). Both qRT-PCRs for *metA* and *shlA* detection were specific for *N. meningitidis* because all amplifications were negative on clinical samples previously positive for other bacteria. Clinical sensitivity was excellent for the *metA* and *tauE* qRT-PCRs, whereas the *shlA* qRT-PCR exhibited a 70% clinical sensitivity. However, we were not able to test these qRT-PCRs using representative isolates of all *N. meningitidis* serogroups, and thus our results may not be generalizable for all serogroups at this time.

ShlA, ctrA and *sodC* appear to be suboptimal targets for diagnostic PCR, whereas our *metA* and *tauE* qRT-PCR assays represent a good alternative and sensitive targets for the detection of *N. meningitidis* from clinical samples. The occurrence of SNPs or gene loss of the targeted genes in some *N. meningitidis* isolates cannot be excluded. Thus, we believe that combining *metA* and *tauE* qRT-PCRs or combining *metA* qRT-PCRs with the widely used *ctrA* qRT-PCR for the diagnosis of this important pathogen may avoid any misidentification or false-negative results that may have severe consequences for the patient. The future development of a duplex or triplex PCR that detects multiple targets—i.e. both the *metA* and *tauE* genes—will improve the detection efficiency of *N. meningitidis*.

Data access

The whole genome shotgun sequence of the *N. meningitidis* strain CHUV is available in the European Nucleotide Archive under BioProject number PRJEB5731 with accession numbers CVTF01000001–CVTF01000144.

Transparency Declaration

This work was mainly supported by an institutional grant entitled 'Bacterial genomics of medical importance.' All authors report no conflicts of interest relevant to this article.

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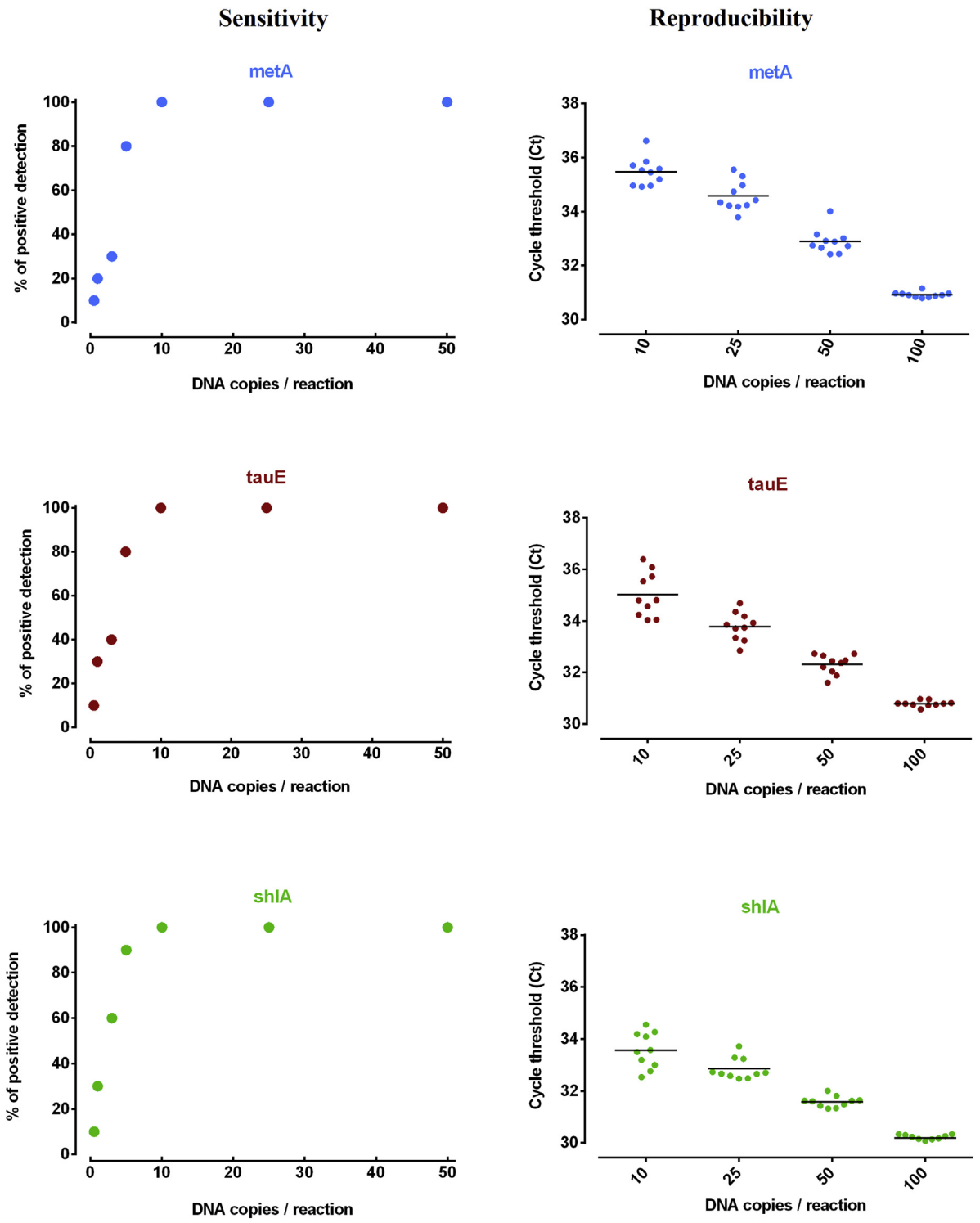

FIG. 1. Analytic sensitivity and reproducibility of three quantitative real-time PCRs. Analytic sensitivity was evaluated using each control plasmid (p*metA*, p*tauE*, p*shlA*) with ten replicates of seven different concentrations of plasmid DNA (0.5, 1, 3, 5, 10, 25 and 50 copies per reaction). Reproducibility was evaluated using same control plasmids with ten replicates of four different concentrations of DNA plasmid (10, 25, 50 and 100 copies per reaction).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2016.03.022.

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