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Density Distribution of Pharyngeal Carriage of Meningococcus in Healthy Young Adults: New Approaches to Studying the Epidemiology of Colonization and Vaccine Indirect Effects

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ABBREVIATED TITLE: Meningococcal Carriage Density Distribution

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CONFLICTS OF INTEREST AND SOURCE OF FUNDING

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Background: Improved understanding of *Neisseria meningitidis* (Nm) carriage biology and better methods for detection and quantification would facilitate studies of potential impact of new vaccines on colonization and transmission in adolescents.

Methods: We performed plate cultures on 107 oropharyngeal swabs stored frozen in STGG broth and previously positive for Nm. We compared quantitative (q)PCR detection of Nm in 601 STGG-swabs with culture. Using qPCR (n=87), a log-phase broth culture standard curve and semi-quantitative plate cultures (n=68), we measured density of carriage. We compared qPCR genogrouping of DNA extracts from STGG-swabs and from plate culture lawns (n=110) with purified isolates (n=80).

Results: Swab storage resulted in only 10% loss of culture sensitivity. Direct *sodC* qPCR Nm detection yielded more positives (87/601, 14.5%) than culture (80/601, 13.3%). Most samples (57/110) positive by culture were also positive by qPCR and vice versa, but discrepancies (single positives) were frequent among low density samples. *sodC* qPCR was positive in 79/80 isolates but in only 65 by *ctrA* qPCR. Density both by culture and qPCR varied across 4 orders of magnitude with the majority being low (<50 bacteria-gene copies/mL) and a minority being high (>1000). Genogrouping qPCRs yielded more positive results when performed on DNA extracts from lawn cultures.

Conclusions: We provide the first description of the distribution of Nm carriage density. This could be important for understanding transmission dynamics and population-level effectiveness of adolescent vaccine programs. Storage of swabs frozen in STGG for batched laboratory analysis facilitates carriage studies and direct *sodC* qPCR for Nm combined with qPCR genogrouping of lawn culture extracts provides accurate, detailed description of colonization.

INTRODUCTION

Pharyngeal carriage of the meningococcus (*Neisseria meningitidis* - Nm) has been extensively studied.[1] In contrast to several other occasionally invasive bacterial pathogens which are commonly found in the human upper respiratory tract, the peak age of colonization with Nm is in adolescence and early adulthood rather than early childhood and the bacterium is more commonly detected in the throat than in the nasopharynx.[1, 2] The evident importance of measuring and understanding the normally benign state of Nm carriage became apparent through the recognition that the effectiveness of group C Nm conjugate vaccines and, probably, those against serogroups A, W and Y resides primarily in their ability, when widely used, to prevent acquisition of invasive strains in the population. As a consequence, transmission to vulnerable individuals is reduced, importantly protecting young children,[3] in whom seroresponses to these vaccines are relatively short lived.[4-6] The arrival of a new generation of meningococcal vaccines containing non-capsular protein antigens therefore raises an important question: can they too deliver such indirect effects and thus control of disease caused by Nm strains expressing the antigens they contain? In order to study the effects of vaccines on Nm carriage effectively we need to optimize the methods used, in particular regarding identification of individuals most likely to be responsible for transmission of the bacteria to others and thus who are the most important targets for any vaccine program aiming to impact on this process.

Nearly all published studies of Nm carriage have involved the direct plating of pharyngeal swabs onto appropriate media, usually immediately after they have been taken,[2, 7] and have reported Nm colonization as a binary endpoint - either present or absent, based on standard culture and identification techniques. We have recently reported wide ranges in density of nasopharyngeal carriage of several bacterial species in pre-school children measured by single gene real time

quantitative polymerase chain reaction (qPCR) assays.[8] Although this methodology has been applied to the study of Nm carriage,[9] no information on colonization density of Nm has been reported. The presence of more bacteria may be associated with higher rates of transmission and thus be important for evaluation of vaccine indirect effects. qPCR assays may also be used to identify capsular groups more reliably than agglutination antisera in pharyngeal Nm in which levels of capsular expression are often downregulated.[9, 10]

We applied qPCR to the detection, quantification and characterization of Nm from oropharyngeal swabs, to compare the performance of this approach with conventional techniques and to work out how best to apply it to the study of the potential impact of new generation meningococcal vaccines on carriage and transmission. We describe for the first time the distribution of density of Nm oropharyngeal colonization in a sample of healthy young adults.

MATERIAL AND METHODS

Bacterial strains and storage media. A standard Nm serogroup B strain - Men B (H44/76) was used in experiments comparing bacterial survival frozen in different broths and as a generic positive control. Four different media, previously described for the preservation of viable Nm strains [Tryptic Soy Broth (TSB) (Becton Dickinson, UK), Brain Heart Infusion (BHI), Proteose Peptone, and LB (Maitland) Tryptone (all from Media Services, University of Bristol)],[11-14] and one medium commonly used in pneumococcal carriage studies [Skim milk Tryptone Glucose Glycerol (STGG) (made in house)],[15] were used.

For the Nm standard curve, serogroup A ATCC 53417 was used as well as in genogrouping assays along with B ATCC-BAA-335, C ATCC 53414, W ATCC 35559, X ATCC 35560 and Y ATCC 35561, as positive controls as previously described.[10]

Participants, oropharyngeal samples and storage. Oropharyngeal swabs were collected in two studies:

Study 1: a four center study performed in 2011-12 in the UK in school and first year university students, aged 10-25 years and reported previously.[9] Permission was obtained from the sponsor of the study to retain the heads of pharyngeal swabs collected from all subjects at the third study visit, after they had been used to inoculate agar plates immediately after being taken. Instead of discarding them, they were broken off into containers with 1.5mL STGG broth, all prepared in one batch. These samples were then held at 4°C for up to 3 hours before being frozen at minus 80°C until they were all shipped on dry ice to Bristol.

Study 2: a one center study conducted among 601 students in May 2012, at the University of Coimbra, Portugal and reported previously[10] each of whom had a single oropharyngeal swab that was placed directly into containers with 1.5mL STGG broth, stored as above and shipped on dry ice to Bristol.

In both cases samples were stored for a further period at minus 80°C until analysis.

Bacterial culture. Directly inoculated agar plates from study 1 were cultured and Nm identified and isolated in laboratories at each of the 4 UK centers using standard techniques[9] as described below. Later, 100µL STGG broth from all vials holding swabs from which *Neisseria* spp. were identified on immediate plating was cultured using the same methods employed for the direct plates. The same approach was used for experiments examining different broths and for samples from study 2.[10] Briefly, broth was inoculated onto G.C. agar plates (E&O Laboratories, Cumbernauld, UK) and after overnight growth at 37°C in 5% CO_2 and thereafter, if negative, at 24h intervals until 72h, plates were examined and oxidase-positive, Gram-negative diplococcal isolates were tested using the API NH strip test (bioMérieux, Lyon, France) for the identification

of Nm according to the manufacturer's instructions. In study 2, after removal of single colonies suspected to be Nm, for identification, the residual growth from each plate ("lawn culture") was removed using a plastic loop into 1mL of TSB with 15% glycerol and stored frozen at minus 80°C. Density by culture was assessed by allocating scores according to the number of colonies seen on the plate as follows: 0 – 0 colonies; 1 – 1-5; 2 – 6-20; 3 – 21-50; 4 – 51-100; 5 – >100. These scores (multiplied by 10 to get CFU/mL) define the ranges shown in figure 2.

Broth cultures were prepared in order to construct a standard curve for the *sodC* qPCR assay as follows: 3-4 colonies of the standard Nm strain from pure plate cultures were used to inoculate 10mL BHI (Oxoid, Basingstoke, UK) with 10% defibrinated horse blood and cultured at 37°C in 5% CO_2 at 50 rotations per minute in 25mL glass universals with the caps loosely fitted. After 1 hour, and subsequently at regular intervals until the stationary phase was reached, optical density (OD) at 600nm was measured (Thermo Spectronic Genesys 6, Thermo Electron Scientific Instruments LLC, WI, USA). When the OD_{600} was 0.4-0.6 (after approximately 270 minutes), 1mL broth culture was harvested and ten ten-fold serial dilutions prepared in STGG broth. 100µL aliquots of each dilution were plated out (as above) and aerobically incubated for 16-18h at 37°C in 5% CO_2 prior to colony counts being performed up to a maximum of 750 colony forming units (CFU) per plate. For qPCR, prior to DNA extraction, 350µL aliquots of each liquid culture dilution were heat inactivated at 100°C for 10 minutes using a digital block heater (Grant Boekel BBD, Grant instruments, Cambridge, UK). Successful inactivation was confirmed by appropriate plate cultures. *sodC* qPCR (see below) was run three times on each of the two log phase Nm broth culture dilutions and mean values used to construct a standard curve to convert the cycle threshold (C*t*) numbers at which a qPCR signal was detected into gene copies/mL (figure 1).

DNA extraction and PCR. Automated extraction of nucleic acids from samples was performed (QIAsymphony® QIAGEN, CA, USA) using DSP Virus/Pathogen Mini Kit version 1 (QIAGEN, CA, USA). In brief, after vortexing, 300µL of each STGG sample (350µL for standard curve broth culture samples), of 2 x 10^{4-6} dilutions of isolates and of 1 x 10^{3-5} dilutions of lawn cultures were used (the latter two underwent lysis in L6 bacterial cell lysis buffer (made in-house) which was found to kill bacteria more effectively than heat treatment) and total nucleic acids were captured on magnetic beads and eluted into 110µL elution buffer. DNA extract eluates were dispensed into 96-well elution microtubes (QIAGEN, CA, USA). After extraction, the plates were sealed and stored frozen at minus 80°C. Plates were thawed and centrifuged prior to qPCR. A QIAagility pipetting robot and software (QIAGEN, CA, USA) was used to prepare qPCR plates in reaction mixes containing Applied Biosystems Fast Universal Master Mix (10µL), primers (300nM), probe (100nM) and DNA extracts (5µL), to a total reaction volume of 20µL. The Applied Biosystems ViiA 7™ real time PCR system (Life Technologies, USA) was used for amplification and detection of DNA using MicroAmp optical 384-well reaction plates (Life Technologies, USA). Reaction conditions for DNA amplification were a 20 second hold stage at 95°C followed by 50 cycles of 95°C for 3 seconds and 60°C for 1 minute. Primers and probes designed to detect the Nm specific genes *sodC* 16 and *ctrA* 17 and capsular genogroups 18 were used. Samples with C*t* values ≤35 were considered positive with the exception of the *sodC* assay where the threshold was set at ≤36 cycles 19. ViiA™ 7 Software version 1.2.2 (Life Technologies, USA) was used for data analysis.

The bacteriophage T4 was used as an internal amplification control (successful DNA extraction and absence of PCR inhibition) in all qPCR assays. Two dilutions of DNA extracts of each target

reference strain were used as positive controls and DNA extracts of STGG broth and molecular grade water as negative reagent controls.

Ethics approval. Studies were approved by National Research Ethics Service Committee South Central (Oxford B: 10/H0605/80)[9] and by the Coimbra School of Medicine Ethics Committee[10] and all subjects provided written informed consent.

Statistical analysis. Pearson chi^2 for independence was used to assess differences in bacterial density category using culture and PCR methods. The Kruskal-Wallis test was used to assess the difference in Nm density distributions across serogroups simultaneously. All analyses were done in Stata v13.1.

RESULTS

Storage media. Initial experiments were performed comparing yield of Nm after storage frozen at minus 80°C of serial dilutions of a stock solution, frozen in five different media. Viable Nm were consistently cultured in high densities even when stored at low concentrations, with no detectable differences between broths (data not shown). STGG broth has been used in subsequent experiments and studies from our group and others.[10, 20]

Nm detection by culture following storage frozen. Culture of stored STGG samples containing the 109 swabs from which Nm had previously been isolated following immediate plating at the 4 UK contributing sites yielded Nm in 98 (89.9%). Swabs negative on direct plating were not retested. Corresponding results for *Neisseria lactamica* were 30/35 (85.7%). For some stored samples, cultures became positive only at 48-72h and, in general, isolates grew more slowly than from immediately plated swabs from which positive cultures were consistently evident at 24h. Of the 601 frozen STGG samples containing swabs from study 2, 80 (13.3%) yielded Nm.[10]

Nm detection by qPCR. The 601 samples from study 2 were subjected to DNA extraction and *sodC* qPCR. Detection rates of Nm in these frozen samples by this technique were slightly higher (87/601; 14.5%) than by culture (as above: 80/601; 13.3%), but were not statistically different at the 5% level (p=0.548 for difference in proportions). However, there were 30 qPCR positive, culture negative and 23 qPCR negative, culture positive samples.

When DNA extracts of the 80 isolates were subjected to qPCR, 79 were positive using primers for *sodC* but only 65 using primers for *ctrA,* which have been used in some previous studies.[9, 19] 13 of the 14 *sodC* positive *ctrA* negative isolates were not genogroupable (see below).

Bacterial density. It was hypothesized that these discordant results might be partly due to low quantities of bacteria and bacterial DNA in these samples therefore approaching the lower limit of detection of both techniques. In order to assess bacterial density, the culture-positive samples from study 2 were re-cultured after a further period of storage, yielding 68 positives upon which colony counts were performed. The standard curve conversion equation (figure 1) was used to generate gene copy density values for the 87 samples positive by *sodC* qPCR. These density distributions are both shown in figure 2. They span 4 orders of magnitude with the large majority of samples having low bacterial/gene concentrations. The discrepant qPCR positive, culture negative and qPCR negative, culture positive samples almost all showed low density by the technique that gave a positive result (figure 2), while both techniques reliably detected high-density carriage. The concordance of density estimations by the two methods in the 68 samples for which quantitative culture results were available is shown in table 1 and was generally good (no evidence to suggest the methods gave independent results $p<0.001$).

Genogrouping. Based on the results above, it was hypothesized that qPCRs performed on DNA extracts after amplification of viable *Neisseriae* by prior lawn culture of samples might match or

exceed the sensitivity of classical culture techniques for identification of different capsular genogroups. The 80 samples which had been culture-positive for Nm originally and the 30 additional samples positive only by direct *sodC* qPCR were assessed. The results of direct and lawn culture genogrouping qPCRs are shown in table 2 along with qPCRs performed on extracts from the 80 cultured isolates.[10]

Among the 80 culture-positive samples, qPCR of lawn culture DNA extracts identified the same Nm genogroups as DNA extracts from isolates in nearly all cases (the discrepancies being one additional group B and 4 fewer group Ys) and identified as many or more organisms of each genogroup than direct qPCR apart from group X (table 2). Among the 30 samples positive only by direct *sodC* qPCR, 6 more lawn culture samples were positive by qPCR for group B than by direct qPCR and one fewer for group W, so that overall 51 individual genogroups were identified among the 110 samples by this approach compared to 46 by culture and 24 by direct qPCR. The lawn culture qPCR approach also resulted in simultaneous detection of two genogroups (B and Y) in two subjects, representing either simultaneous colonization with meningococci of two capsular groups or possibly the presence of a single strain bearing both genes, while carriage of multiple genogroups was not detected by the classical culture approach and was detected by the direct qPCR approach in only one (different) case (table 2).

The median (range) Nm density values observed in subjects carrying (only) groups B, Y or NG, whose *sodC* detection qPCR was positive, were B - 12 gene copies/mL (2-3306) (n=31); Y - 16 (3-3048) (n=6); NG - 7 (2-638) (n=43). There was no evidence to suggest a difference in density distribution for positive samples between groups B, Y and NG (p=0.203). For groups C, W and X numbers were too small for meaningful evaluation.

DISCUSSION

In this paper we describe for the first time the distribution of colonization density among young adults carrying meningococcus in the oropharynx. Using both culture and qPCR we demonstrate that the bacterial load varies between carriers by up to 4 orders of magnitude and that the large majority carry the organism at lower densities. Clearly, although some sampling error is inevitable, it would not, by itself, generate a distribution that looks like this. The most immediate question or importance that this observation raises is: what is the relationship between density of carriage and efficiency of onwards transmission? Several other questions arise: do these different densities reflect sampling at different time points during colonization episodes, differences between individuals with some consistently carrying at higher densities than others, differences between bacterial strains with some reaching higher densities than others or interacting when simultaneously present, some combination of all three or even effects upon carriage density of other factors such as intercurrent respiratory viral infections? Much larger studies, which include frequent longitudinal sampling, are already in progress in order to address the latter questions using the methodologies described here. The design of studies to address the first question will depend to an extent upon the answers to the others.

The distribution of colonization density shown here contrasts markedly with those of nasopharyngeal bacteria in younger children,[8] where a wider range of and much higher average density is seen for the three most common culturable bacterial species *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, suggesting colonization transmission biology that may differ from Nm in more ways than just peak age and anatomical location. If, as might be expected, more efficient onward transmission of Nm occurs from individuals with higher density colonization, then impact of vaccination upon maximal density may be an important factor in dynamic

transmission models guiding vaccine policy,[21] replacing the simple presence or absence of detectable carriage used at present. Conversely vaccine studies designed to ascertain the impact of vaccines on carriage and transmission but which fail to measure possible impact on density of carriage[22] may underestimate potential population-wide effects.

Although it did not quantify density, the first study from which samples were used for this work did compare the performance of direct qPCR with culture in detection of Nm, targeting the *ctrA* gene.[9] This gene target has been shown to perform better for invasive isolates in some hands[19] but it tends not to detect non-groupable strains[23] and because of this, *sodC* performed better overall than *ctrA* in this sample of carriage isolates from young Portuguese adults. Both studies demonstrate that culture and qPCR deliver overlapping but distinct information and so complement one another, although qPCR was marginally more sensitive especially at low density (figure 2). If high density carriage proves to be a pre-requisite for efficient onward transmission, then our results suggest that either method will reliably detect it and so either would suffice. However, accurate quantification of very high density carriage by culture necessitates serial dilutions and multiple cultures whereas qPCR can be automated and is relatively inexpensive. Given that samples positive by one method and not the other usually show low bacterial density (figure 2), the most likely explanation for the number of samples positive by only one appears to be that they are around the lower limits of detection for both methods. In addition, qPCR might sometimes detect the DNA footprint of recent carriage after viable organisms have become undetectable. Although there is a common expectation for qPCR methods to be reliably more sensitive than culture, in reality the efficiency of qPCR reactions vary but can rarely distinguish single figure numbers of gene copies in a sample from any background signal, while culture is sometimes capable of picking up a single organism from a

sample of broth, manifest as a visible colony on agar. With regard to other relative merits of the two methods, culture is essential to generate the DNA necessary for efficient sequencing and thus epidemiologic molecular genetic studies of carriage isolates. In the context of new protein antigen "MenB" vaccines, culture of isolates in order to assess their expression of vaccine antigens may be important in the assessment of impact of universal vaccine use upon circulation of target strains.

Our data confirm that both dilutions of standard strains of Nm and bacteria collected on oropharyngeal swab tips can survive storage frozen in suitable media. Given that less than 10% loss in sensitivity was seen despite first plating the swabs taken in study 1 before storing the same tips for several months and shipping many of them between laboratories, higher rates of detection following storage and delayed culture might be expected with more optimal sampling (without prior plating) and handling conditions. Putting swabs into broth, storing them frozen and processing them later in batches has been the norm for many years in pneumococcal carriage studies and is now becoming established in meningococcal swabbing studies as well.[10, 20, 22] This approach is much easier logistically and less costly for clinical and laboratory teams and thus makes the conduct of such studies more accessible in settings where research resources are scarce. Storage of samples also creates an archive allowing re-analysis if repeats are needed or application of new or alternative laboratory techniques.

Reculture of culture positive samples yielded some negatives. These were samples amongst which many contained bacteria near the lower threshold of detection so this was an expected finding. However, other factors, such as an additional freeze thaw cycle and prolonged storage may also have played a part in reducing bacterial viability. The relative stability of DNA is an

additional reason why qPCR may be a better method for bacterial detection, particularly in samples which have been in prolonged storage or manipulated repeatedly.

Genogrouping is more efficient than conventional serogrouping techniques using antisera in studies of carriage bacteria, which often express only low levels of capsular antigens.[9, 10] The use of "lawn cultures" as a means to amplify DNA for molecular detection has been described in the literature on pneumococcal carriage studies.[24] In our hands it seems to compensate almost entirely for the deficits in sensitivity of genogrouping qPCRs when compared to standard culture and testing of isolates. When culture negative, qPCR positive samples are taken into account, this approach appears to be the best one for detection and genogrouping purposes, especially if one assumes that we would have detected further positive samples had we had resources to analyze all 601 samples this way. It can also detect multiple genogroups in some samples. However, lawn culture extracts alone cannot be used for accurate quantification of carriage density in the sample and do not yield pure single strain DNA for sequencing. Perhaps the ideal future portfolio of techniques would also include microarray detection of multiple strains as is becoming the preferred technique in pneumococcal colonization research.[24]

While methodologic refinements as described here can enhance our understanding of meningococcal epidemiology and transmission dynamics by increasing the information gained from cross sectional or longitudinal carriage studies, it is through their application to interventional studies using the growing number of meningococcal vaccines[22] that the optimal way to design and implement immunization programs that are maximally efficient at the population level can be defined. In addition, if studies can be done to identify the bacterial proteins, expression of which is associated with successful transit from one person to the next,

design of vaccines targeting interruption of spread rather than individual protection against invasive disease, may be achieved.

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FIGURE LEGENDS

Figure 1. *Neisseria meningitidis* qPCR Standard curve: qPCR cycle threshold (C*t*) values plotted *vs* culture results (real and calculated) in colony forming units(CFU)/mL broth. The curve represents the averages of the results of three qPCR runs performed on each of two dilution series of cultures

Figure 2. Bacterial density distributions: percentages of positive samples measured by culture (hatched-left) (n= 68) and qPCR (solid-right) (n= 87) within each density range are shown. The lighter upper sections of each bar represent proportions of samples that were negative by the other method (i.e. qPCR-negative hatched, culture-negative solid)

Table 1. Comparison of density distribution by culture and direct swab *sodC* qPCR. Data are shown for 68 culture-positive samples in which colony counts were performed.

		qPCR (gene copies/mL)					
		0	**<50**	**51-200**	**201-500**	**501-1000**	**>1000**
Culture (CFU/mL)	**<50**	13	**21**	2	2	1	0
	51-200	3	11	**2**	0	0	0
	201-500	1	3	1	**0**	0	1
	501-1000	0	0	3	1	**0**	0
	>1000	0	0	1	0	0	**2**

Table 2. Comparison of results of genogrouping qPCRs performed on DNA extracted directly from swab-in-STGG samples (n=100), from "lawn" cultures (n=110) and from *Neisseria meningitidis* cultured isolates (n= 80). NG – non groupable.

	Direct swab (n= 110)		**Lawn culture (n= 110)**		**Cultured isolates (n= 80)**
	Culture+ (n= 80)	**Culture -, qPCR+ (n= 30)**	**Culture + (n= 80)**	**Culture -, qPCR + (n= 30)**	
B	14	2	33	8	32
C	1	0	2	0	2
W	0	1*	1	0	1
X	2	0	1	0	1
Y	4	0	6**	0	10
NG	59	28	39	22	34

* both B and W detected in sample, only B detected from lawn; ** both B and Y detected in 2 samples, respective isolates and direct swab samples showed only Y and only NG.

Figure 1.

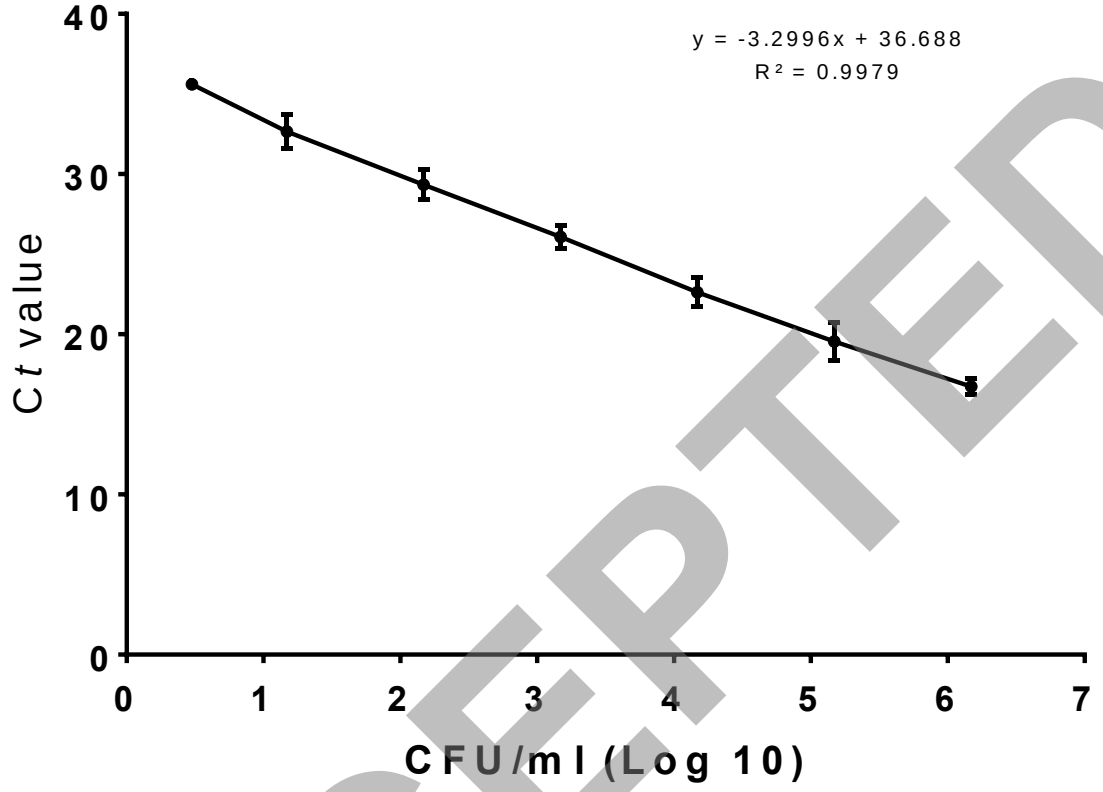

Figure 2.