



Short communication

Making standards for quantitative real-time pneumococcal PCR



Susan C. Morpeth^{a,b,*}, Jim F. Huggett^c, David R. Murdoch^{d,e}, J. Anthony G. Scott^{a,b,f}

^a KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya

^b Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom

^c Molecular & Cell Biology, LGC, London, United Kingdom

^d Department of Pathology, University of Otago, Christchurch, New Zealand

^e Microbiology Unit, Canterbury Health Laboratories, Christchurch, New Zealand

^f Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, United Kingdom

ARTICLE INFO

Article history:

Received 25 September 2014

Received in revised form

20 November 2014

Accepted 24 November 2014

Available online 4 December 2014

Keywords:

Quantitative

Real-time PCR

Streptococcus pneumoniae

Standards

ABSTRACT

Quantitative *lytA* PCR is often performed using in-house standards. We hypothesised equivalence when measuring a standard suspension of *Streptococcus pneumoniae* by colony-forming-units (CFU) or genome-copies. Median (IQR) ratio of CFU/genome-copies was 0.19 (0.1–1.2). Genome-copies were less variable than CFU, but the discrepancy between the methods highlights challenges with absolute quantification.

© 2014 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

1. Introduction

Research and Clinical Microbiology laboratories alike are embracing quantitative real-time PCR (qPCR) for detection of putative microbial pathogens where simply presence of a target organism is insufficient to determine pathogenicity [1–5]. For example, *Pneumocystis jirovecii* is known to cause severe pneumonia, but upon the advent of real-time PCR diagnostics, it was discovered that sensitive qualitative PCR is generally non-specific. It is frequently positive in well patients or those with another cause of illness. Quantitative PCR, however, may predict which samples are indicative of *P. jirovecii* pneumonia [2]. Likewise, detection of *Streptococcus pneumoniae* from respiratory tract samples by qualitative real-time PCR does not distinguish nasopharyngeal carriage of commensal *S. pneumoniae* from pneumococcal pneumonia. There is increasing interest globally in quantitative real-time PCR for detection of pneumococcus from respiratory tract samples, particularly utilising the *lytA* assay targeting the autolysin gene [4,6]. qPCR requires the use of standards of known concentration,

serially diluted to form a linear relationship between the quantification cycle (Cq) (also known as cycle threshold) value and the logarithmic value of the standard concentration. Methods for making these standards are not always described in publications [7].

2. Methods

We set out to explore methods for making standards for an in-house quantitative real-time *lytA* PCR assay. One possibility was to make a suspension of pneumococcus, plate out serial dilutions of the suspension, perform colony counting, and calculate the concentration of the original suspension in colony-forming-units (CFU)/mL from which DNA would be extracted for the standards. Another method was to extract the DNA from a suspension of pneumococcus, measure the DNA concentration in ng/μL, from which the concentration in genome-copies/mL would be calculated based on molarity (using Avogadro's number). These methods are unlikely to be equivalent because they assume a single viable bacterial cell produces a single colony-forming unit; may be perfectly extracted by DNA extraction methods and will contain only one genome, with only one gene copy [8]. If they were the same then the ratio of the concentration in CFU/mL to the concentration in genome-copies/mL would be one. To investigate the impact of pneumococcal autolysis, we used *Escherichia coli* as a comparator organism. For the same reason, we used bacterial growth in broth

* Corresponding author at: KEMRI-Wellcome Trust Research Programme, PO Box 230, Kilifi 80108, Kenya. Tel.: +254 41 7522063.

E-mail address: smorpeth@kemri-wellcome.org (S.C. Morpeth).

¹ Present address: Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, United Kingdom.

at log phase as well as bacterial growth harvested from solid media, to make the starting-point suspensions.

S. pneumoniae ATCC 49619 and *E. coli* ATCC 25922 were grown to log phase in brain heart infusion broth, or harvested from an overnight culture plate on blood agar and suspended in normal saline, and adjusted to 3.0 McFarland. The suspensions were serially diluted 1:5 nine times and 100 μ L of each of the last three dilutions plated in duplicate on blood agar for incubation overnight at $35 \pm 2^\circ\text{C}$ in 5% CO_2 . In addition, 1 mL of the suspension was used for immediate extraction. To estimate CFU/mL, all countable plates underwent colony counting on the following day to calculate the concentration of the original suspension.

DNA was extracted (QIAamp DNA mini kit, Qiagen, Germany) with two elution steps as described by the manufacturer to optionally maximise DNA recovery, and DNA concentration estimated in $\eta\text{g}/\mu\text{L}$ using the NanodropTM spectrophotometer (ThermoScientific, USA). Genome copies were estimated using the formula $\text{mass} = \text{DNA size (base pairs)} \times 1 \text{ mole}/6.023 \times 10^{23} \text{ molecules} \times 660 \text{ g/mole}$. $N = 12$ experiments were performed per organism, by the same operator, on different days.

The ratio of quantities derived from colony counting/DNA concentration (CFU/genome-copies) were compared by suspension method and by organism, using the Wilcoxon rank sum test.

3. Results and discussion

The distribution of bacterial concentrations by colony counting (mean log [sd] concentration for *S. pneumoniae* 8.4 [0.7] CFU/mL and for *E. coli* 8.9 [0.4] CFU/mL) was greater than the distribution of bacterial concentrations as calculated by DNA concentration (mean log concentration for *S. pneumoniae* 8.9 [0.2] genome-copies/mL and for *E. coli* 8.6 [0.2] genome-copies/mL). See Fig. 1. There was poor correlation between the bacterial concentration as measured by colony counting and the bacterial concentration as calculated from DNA concentration. For *S. pneumoniae* Spearman's rank correlation was 0.69 ($p = 0.014$) and for *E. coli* this was 0.10 ($p = 0.746$). See supplementary Fig. 1. The ratio of quantities derived from the two methods was almost never one. See Fig. 2.

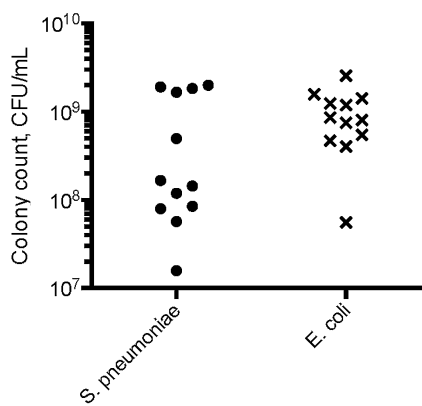
Supplementary Fig. 1 related to this article can be found, in the online version, at [doi:10.1016/j.bdq.2014.11.003](https://doi.org/10.1016/j.bdq.2014.11.003).

Overall, combining broth and solid media culture, the median (IQR) ratio (CFU/genome-copies) for *S. pneumoniae* was 0.19 (0.1–1.2) and for *E. coli* was 1.74 (1.1–2.9), $p = 0.007$. The ratio, whether the suspension was made in saline from growth on solid media or from broth culture, tended to be lower for *S. pneumoniae* than for *E. coli*. See Fig. 2. This supports a possible role of pneumococcal autolysis in lowering the ratio of concentration in CFU/mL to concentration in genome-copies/mL, because the autolysed nonviable cells in suspension could contribute to the quantity measured by DNA extraction but not to the quantity measured by colony-counting. Additionally, more than one genome may be present per cell, depending on the phase in the cell division cycle. It is not realistically possible for a single genome-copy to give rise to more than one CFU but ratios of >1 in CFU/genome-copies serve to highlight the difficulty in accuracy with either of the measurement methods.

For either *S. pneumoniae* or *E. coli*, there was no difference in the ratio (CFU/genome-copies) whether culture on solid media or log phase growth in broth was used to make the suspension. See Fig. 2. This lack of a difference between suspension methods for *S. pneumoniae* implies that log phase growth does not sufficiently overcome the problem of autolysed cells in suspension, or that pneumococcal autolysis is not the only problem.

The DNA concentration method of quantification was less variable than the colony counting method. Poor correlation between methods may be due to inherent differences in measuring bacterial

Spread of bacterial concentration as measured by colony counting



Spread of bacterial concentration as measured by DNA concentration

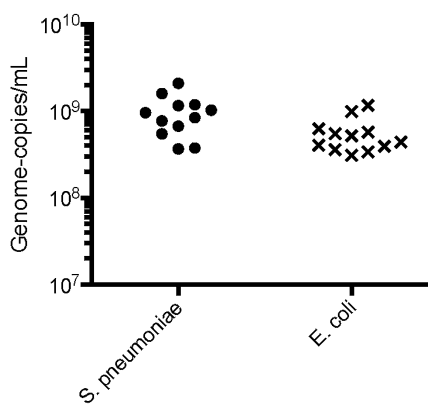


Fig. 1. Spread of bacterial concentrations of standard suspensions of *S. pneumoniae* and *E. coli* obtained by colony counting in CFU/mL and by DNA concentration in genome-copies/mL.

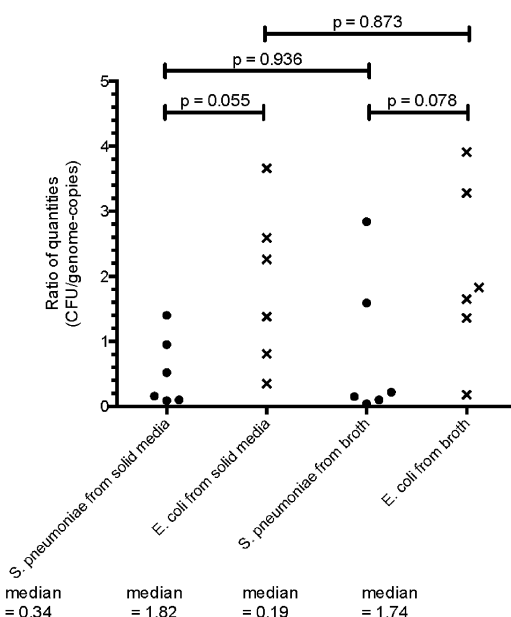


Fig. 2. Ratio of quantification methods: colony counting/DNA concentration in CFU/genome-copies, by organism and culture medium.

cells versus measuring their genomes, rather than simply pneumococcal autolysis as it was more pronounced for *E. coli* than for *S. pneumoniae*. Growing the suspension to log-phase before quantification did not alter the results, but the median ratio of concentrations was lower for *S. pneumoniae* than for *E. coli*, also suggesting that pneumococcal autolysis is not the sole cause of poor correlation between quantification methods. DNA extraction may have been more efficient at a lower starting concentration; this could be the subject of further experiments, taking care not to use a starting concentration too low to be useful as some clinical samples could have concentrations above the resulting range of quantitative standards.

It is key to note that while the DNA concentration method for making standards may appear to be a good choice for quantitative real-time PCR in a single laboratory, that there may be considerable intra-assay variation between batches of standards by either method. What is more, the lack of correlation between methods means that absolute quantification of samples between laboratories using different methods would not give comparable results.

Readers of the medical literature should be aware that the method used for assigning values to quantitative real-time PCR standards will affect the results obtained. This is particularly pertinent when applying thresholds or cut-offs that rely on quantification from an assay designed in one laboratory and then used in another laboratory.

Acknowledgements

The authors would like to thank Dr. Nicole Wolter of the National Institute for Communicable Diseases in Johannesburg, South Africa,

for helpful discussions about making standards. This work was supported by the PneumoADIP through a grant from GAVI. JAGS is funded by a fellowship from the Wellcome Trust of Great Britain (098532). This paper is published with the permission of the Director of the Kenya Medical Research Institute.

References

- [1] Hiramata T, Yamaguchi T, Miyazawa H, Tanaka T, Hashikita G, Kishi E, et al. Prediction of the pathogens that are the cause of pneumonia by the battlefield hypothesis. *PLoS ONE* 2011;6(9):e24474.
- [2] Alanio A, Desoubreux G, Sarfati C, Hamane S, Bergeron A, Azoulay E, et al. Real-time PCR assay-based strategy for differentiation between active *Pneumocystis jirovecii* pneumonia and colonization in immunocompromised patients. *Clin Microbiol Infect* 2011;17(10):1531–7.
- [3] Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, Nguyen AT, et al. Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. *Pediatr Infect Dis J* 2011;30(1):11–8.
- [4] Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, Cutland C, et al. Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. *Clin Infect Dis* 2012;54(5):601–9.
- [5] Werno AM, Anderson TP, Murdoch DR. Association between pneumococcal load and disease severity in adults with pneumonia. *J Med Microbiol* 2012;61(Pt 8):1129–35.
- [6] Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, et al. Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* 2007;45(8):2460–6.
- [7] Bustin SA, Benes V, Garson JA, Hellems J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;55(4):611–22.
- [8] Huggett J, Laver T, Tamisak S, Nixon G, O'Sullivan D, Elaswarapu R, et al. Considerations for the development and application of control materials to improve metagenomic microbial community profiling. *Accred Qual Assur* 2013;18(2):77–83.