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Comparison between digital PCR and real-time PCR in detection of *Salmonella typhimurium* in milk

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ABSTRACT

As a kind of zero-tolerance foodborne pathogens, *Salmonella typhimurium* poses a great threat to quality of food products and public health. Hence, rapid and efficient approaches to identify *Salmonella typhimurium* are urgently needed. Combined with PCR and fluorescence technique, real-time PCR (qPCR) and digital PCR (ddPCR) are regarded as suitable tools for detecting foodborne pathogens. To compare the effect between qPCR and ddPCR in detecting *Salmonella typhimurium*, a series of nucleic acid, pure strain culture and spiking milk samples were applied and the resistance to inhibitors referred in this article as well. Compared with qPCR, ddPCR exhibited more sensitive (10^{-4} ng/μl or 10^{2} cfu/ml) and less pre-culturing time (saving 2 h). Moreover, ddPCR had stronger resistance to inhibitors than qPCR, yet absolute quantification hardly performed when target's concentration over 1 ng/μl or 10^{6} cfu/ml. This study provides an alternative strategy in detecting foodborne *Salmonella typhimurium*.

1. Introduction

Since infectious disease of foodborne caused a major crisis on human health, attentions have been given to rapid and accurate detections of causative pathogens in food products. Among all foodborne pathogens, *Salmonella typhimurium* is treated as a common bacterium causing foodborne illnesses around the world (Kirk et al., 2015). In China, regarded as the second major foodborne pathogen, *Salmonella* species were related to about 40% incidents of bacteria-related food poisoning (Chen et al., 2010; Xiao et al., 2015). The majority of people infected with *Salmonella typhimurium* suffer from diarrhea, fever, abdominal cramps and some severe patients need to be hospitalized. Possessing dramatically food reservoir, *Salmonella typhimurium* was associated with outbreaks of illnesses linked to contaminated cucumber, chicken, raw tuna, beef, pork, milk, egg, seafood and many other foods (Omiccioli et al., 2009). Hence, a reliable method detecting foodborne *Salmonella typhimurium* plays a crucial role in guaranteeing food safety and citizens' health.

The traditional method to detect *Salmonella typhimurium* is based on culture medium including pre-enrichment, selective culturing, isolation on selective medium, calculating positive colonies by biochemical and serological analysis (Schönenbrücher et al., 2008). Be deemed to time consuming and laborious, 3–6 days are requisite to complete the whole process (Wang et al., 2017). Furthermore, the traditional method showed a handicap in sensitivity of detecting foodborne pathogens, especially for those regarded as zero tolerance (Settanni and Corsetti, 2007). To overcome these drawbacks, PCR methods based on nucleic acid amplification tests (NAATs) are widely applied in detecting foodborne *Salmonella* species. Several articles demonstrated the effect of end-point PCR methods targeting different genes of *Salmonella typhimurium* in food products (Bansal et al., 2006; Kim et al., 2006; Maciorowski et al., 2008; Naravaneni, 2005; Radji et al., 2010). Thus, be subjected to ager analysis, end-point PCR method has obstacles in quantitative analysis (Borowsky et al., 2007).

Relying on calculating increment of fluorescence generated by amplified templates instead of agar gel analysis, abbreviating the time-consuming to 3 days or less (Corless et al., 2001) makes qPCR be widely applied in foodborne pathogens' measurement (Elizaquível et al., 2012; O'Regan et al., 2008; Zhang et al., 2011). Multiplex assays, minor grove binding (MGB) probe and propidium monoazide (PMA) applied in

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qPCR made it more flexible in practical application (Chen et al., 2010; Nocker et al., 2007; O'Regan et al., 2008). Owning to its superiority, qPCR becomes so common in detecting foodborne *Salmonella typhimurium* (Jung et al., 2005; Zheng et al., 2014), but limitations existing in measuring trace sample or absolute quantification.

As the latest generation of PCR and one of the most robust methods in molecular quantification, several articles in recent years have reported ddPCR in the field of food and drug testing, bacteria and viruses monitoring, gene modification and clinical diagnosis research (Cai et al., 2014; Day et al., 2013; Klančnik et al., 2015; Morisset et al., 2013; Porcellato et al., 2016). Differing from the traditional notion of ddPCR relying on multi-well plates, commercial ddPCR platforms developed droplets or silicon substrate approaches running tens of thousands of individual reactions in parallel. After PCR amplification and fluorescence signal collection, initial concentration of the target is calculated according to the pattern of Poisson distribution (Hindson et al., 2013). Compared with qPCR, ddPCR has advances in performing absolute quantification procedures without a standard, rapid data analysis, sensitivity, precision and resistance to inhibitors (Whale et al., 2012). Recent articles have demonstrated the sensitivity and precision of ddPCR in the quantitative detection of bacteria in sediment and commercial poultry processing water samples, yet its application for the detection of *Salmonella typhimurium* in food samples has so far not directly been defined (Rothrock et al., 2013; Singh et al., 2017).

To our knowledge, several methods have related to qPCR in detecting foodborne *Salmonella* spp. (Almeida et al., 2013; Tatavarthy et al., 2017) but rarely to ddPCR. Hence, whether ddPCR having advances in detecting and quantifying *Salmonella typhimurium* in milk was measured. Our results showed ddPCR exhibited stronger sensitivity and resistant to PCR inhibitors. Moreover, as a significant carrier of transmission of pathogenic bacteria, aseptic milk with *Salmonella typhimurium* culture was prepared to evaluate the efficacy of both systems, and the results manifested that ddPCR maintained lower limit of detection (LOD) and less enrichment time.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

Salmonella typhimurium and other control strains applied in this study were obtained from Shandong Institute for Food and Drug Control (Table 1). All tested strains were preserved in lysogeny broth media (Beijing Land Bridge Technology Co., Ltd.) at − 20 °C, activated in nutrient agar media (Beijing Land Bridge Technology Co., Ltd) for 24 h at 37 °C and enriched in nutrient broth media (Beijing Land Bridge Technology Co., Ltd) for 24 h at 37 °C. The number of *Salmonella typhimurium* suspension was calculated by plate count agar (PCA, Beijing Land Bridge Technology Co., Ltd).

2.2. DNA extraction

For all tested strains, 60 °C water bath for 15 min to promote bacterial cells lysis. Genomic DNA of *Salmonella typhimurium* was extracted by UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories) following manufacturer's instruction. Quality control of extracted genomic DNA samples was performed by Nanodrop 2000 (Thermo Fisher Scientific). Then, a series of 10-fold dilution DNA (concentration ranging from 10 ng/μl to 10^{-4} ng/μl) were prepared, and each gradient DNA sample was analyzed by qPCR and ddPCR (Fig. 1).

Table 1
List of bacterial species applied in specificity test.

Species	Origin	Strain number[a]	Results[b]
Salmonella typhimurium	Unknown	CICC 21483	+
Listeria monocytogenes	Unknown	ATCC 19111	−
Vibrio parahaemolyticus	Unknown	CICC 10552	−
Escherichia coli	Unknown	ATCC 25922	−
Enterobacter sakazakii	Unknown	CICC 21544	−
Micrococcus luteus	Unknown	ATCC 10240	−

[a] CICC: China Center of Industrial Culture Collection, ATCC: American Type Culture Collection.
[b] +: positive amplification, −: negative amplification.

2.3. Primer and probe

Based on FimY gene of *Salmonella* spp., a pair of primers and probe were selected from China food safety standard (CFDA.SN/T 1870 - 2016). The forward primer 5′-GCGGCGTTGGAGAGTGATA-3′, the reverse primer 5′-AGCAATGGAAAAAGCAGGATG-3′ and the taqman probe 5′-FAM-CATTTCTTAAACGGCGGTGTCTTTCCCT-MGB-3′ were purchased from Sangon (Sangon Biotech Co., Ltd.). Specificity of the primer pair and probe was measured by several pathogenic bacteria and blank control (Table 1).

2.4. qPCR assays and procedure

For each reaction, 1 μl DNA, 10 μl Premix Ex Taq (Takara Biotechnology Co., Ltd.), 1 μl of 10 μM forward and reverse primer, 0.8 μl of 10 μM probe, 0.4 μl Rox Reference Dye (Takara Biotechnology Co., Ltd.) and 6.8 μl nuclease-free water (Ambion, Thermo Fisher Scientific) were added to total volume of 20 μl. QuantStudio 5 Real-time PCR System (Life Technologies Inc.) was applied in thermal cycling: 50 °C hold for 2 min, followed by 95 °C for 30 s, then 40 cycles consisting of 95 °C for 5 s and 60 °C for 30 s per cycle, and finally cooled to 4 °C for 2 min. The output data were analyzed by associated software.

2.5. ddPCR assays and procedure

For each reaction, 1 μl DNA, 7.5 μl QuantStudio 3D Digital PCR Master Mix V2 (Life Technologies Corporation), 1 μl of 10 μM forward and reverse primer, 0.3 μl of 10 μM probe, 5 μl nuclease-free water were added to total volume of 14.8 μl. QuantStudio 3D Digital PCR System was used in thermal cycling: 96 °C hold for 10 min, followed by 39 cycles consisting of 56 °C for 5 s and 98 °C for 30 s, then a holding stage followed at 60 °C for 2 min. The output data were analyzed by associated software.

2.6. PCR inhibitor dilution series

Common PCR inhibition existing in milk (Schrader et al., 2012) or DNA extracted approaches, calcium ion and ethanol were added in PCR reaction with different DNA concentration. Diluted calcium chloride powder (Sigma Aldrich) in nuclease-free water or ethanol (Sinopharm Chemical Reagent Co., Ltd) was added, and the final concentration ranging from 4 mM to 6 mM or 0.5% to 2.5%. Then, each reaction performed qPCR and ddPCR procedure as described above.

2.7. Spiking sample

To prepare spiking milk samples, 1 ml of each concentration gradient of *Salmonella typhimurium* pure culture added in 24 ml sterile milk. Genomic DNA extracted by UltraClean Microbial DNA Isolation Kit from each spiking milk sample was analyzed by both platforms as described above (Fig. 1). In addition, 25 ml spiking milk mixed with 225 ml of buffered peptone water (BPW, Beijing Land Bridge Technology Co., Ltd) was prepared to estimate pre-cultured time-consuming (Fig. 1). Pre-enrichment process was promoted among samples out of LOD of two platforms for 2 h, 4 h, 6 h. Then, extracted DNA from each time phase was analyzed to measure time-consuming.

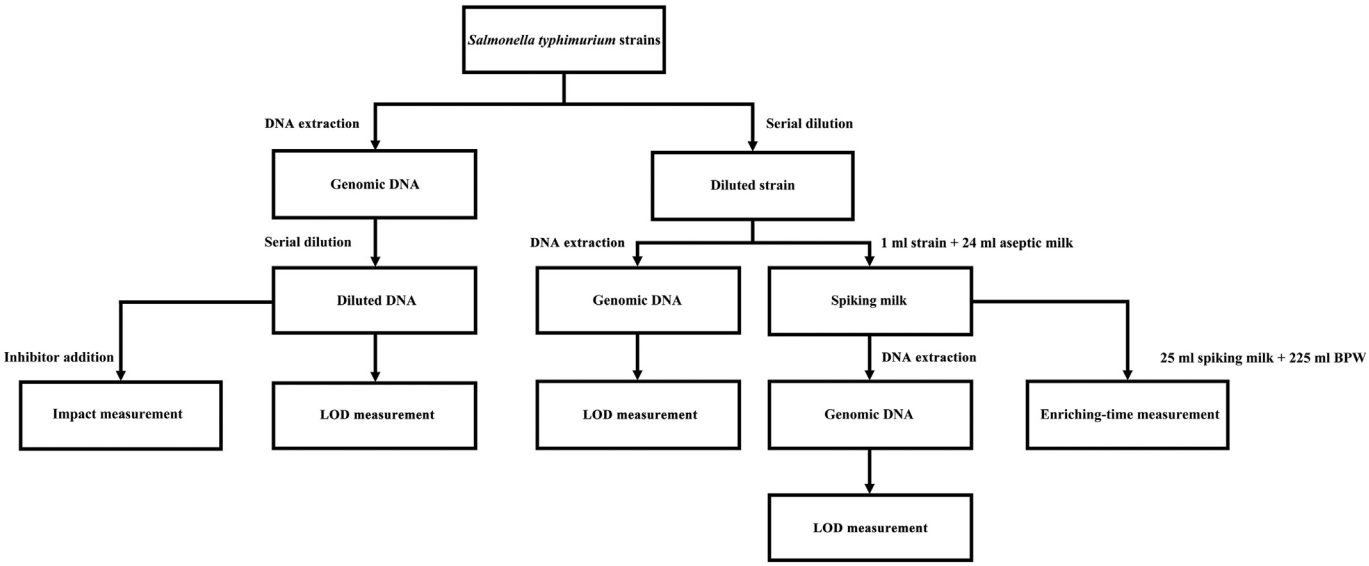

Fig. 1. Flow chart of this research.

3. Results

3.1. LOD of both platforms

3.1.1. Genomic DNA

To measure and compare LOD of two platforms, a serial dilution of genomic DNA samples extracted from *Salmonella typhimurium* pure culture was both analyzed by qPCR and ddPCR. The results showed the lowest detecting limit of qPCR reached 10^{-3} ng/μl. On contrast, the quantitative detecting range of ddPCR was from 10^{-4} ng/μl to 10^{-1} ng/μl (Table 2). Compared LOD of two platforms, ddPCR performed lower detecting limitation but may not quantify the target absolutely when its concentration over 1 ng/μl.

3.1.2. Pure culture

Prepared *Salmonella typhimurium* culture samples were measured by qPCR and ddPCR to calculate LOD of both platforms. Similar to the results of genomic DNA, ddPCR exhibited lower LOD (10^2 cfu/ml) than qPCR (10^3 cfu/ml) but may not perform absolute quantification when strain's concentration over 10^6 cfu/ml (Table 2). The results showed ddPCR had an advance in sensitivity but performed narrower dynamic range than qPCR.

3.2. Linearity and repeatability

Three repeat experimental results of each concentration were exhibited to reflect repeatability of two platforms. Based on linear analysis and calculated standard deviation value, we found no significant discrepancy in linearity between qPCR and ddPCR, but ddPCR was more stable applied in different samples (Fig. 2). Meanwhile, standard deviation of ddPCR and qPCR in each concentration gradient reflected no obvious divergence (Table S1). The results identified variation of target's concentration or source had rarely impact on the detecting performance of both instruments.

Table 2
LOD of two platforms in genomic DNA and pure culture of *Salmonella typhimurium*.

Conc.(ng/μl)	Genomic DNA		Conc.(cfu/ml)	Pure culture	
	qPCR	ddPCR		qPCR	ddPCR
1	+	−	10^6	+	−
10^{-1}	+	+	10^5	+	+
10^{-2}	+	+	10^4	+	+
10^{-3}	+	+	10^3	+	+
10^{-4}	−	+	10^2	−	+

+: performing quantitative analysis.
−: un-performing quantitative analysis.

3.3. Resistance to PCR inhibitors

Due to inhibitory action of calcium and ethanol to polymerase activity, PCR amplification efficiency always reduced, which generated negative impact on precision of quantitative analysis (Schrader et al., 2012). As a result, inhibitory activity was related to the amount of calcium or ethanol with inhibition of qPCR at occurring over 5.5 mM or 1.5%, yet no significant inhibition of ddPCR occurring (Tables 3 and 4). Although ddPCR exhibited stronger resistance to inhibitors, the amount of DNA had few impact on inhibitory ability of both platforms (Tables 3 and 4). Detailed data provided as a supplementary (Table S2).

3.4. Spiking samples

To estimate the effect and efficient of qPCR and ddPCR applied in practical samples, spiking milk with *Salmonella typhimurium* was prepared and analyzed by both platforms.

3.4.1. Un-enrichment

Un-enrichment spiking milk samples were prepared to measure the effect of two platforms in analyzing contaminated samples. Our results showed the absolute detection area of ddPCR was ranging from 2.5×10^6 cfu/ml to 2.5×10^2 cfu/ml and LOD of qPCR was 2.5×10^3 cfu/ml (Table 5), similar to the results of genomic DNA and pure culture.

3.4.2. Enrichment

Trace spiking samples out of LOD were selected to calculate the preculturing time of two platforms. Compared with qPCR, ddPCR saved 2 h of pre-culturing time (Table 6). So it had an advantage in saving test time and promoted detecting efficiency of *Salmonella typhimurium* in milk.

4. Discussion

In this article, the capabilities of qPCR and ddPCR in detecting *Salmonella typhimurium* in milk were measured and compared.

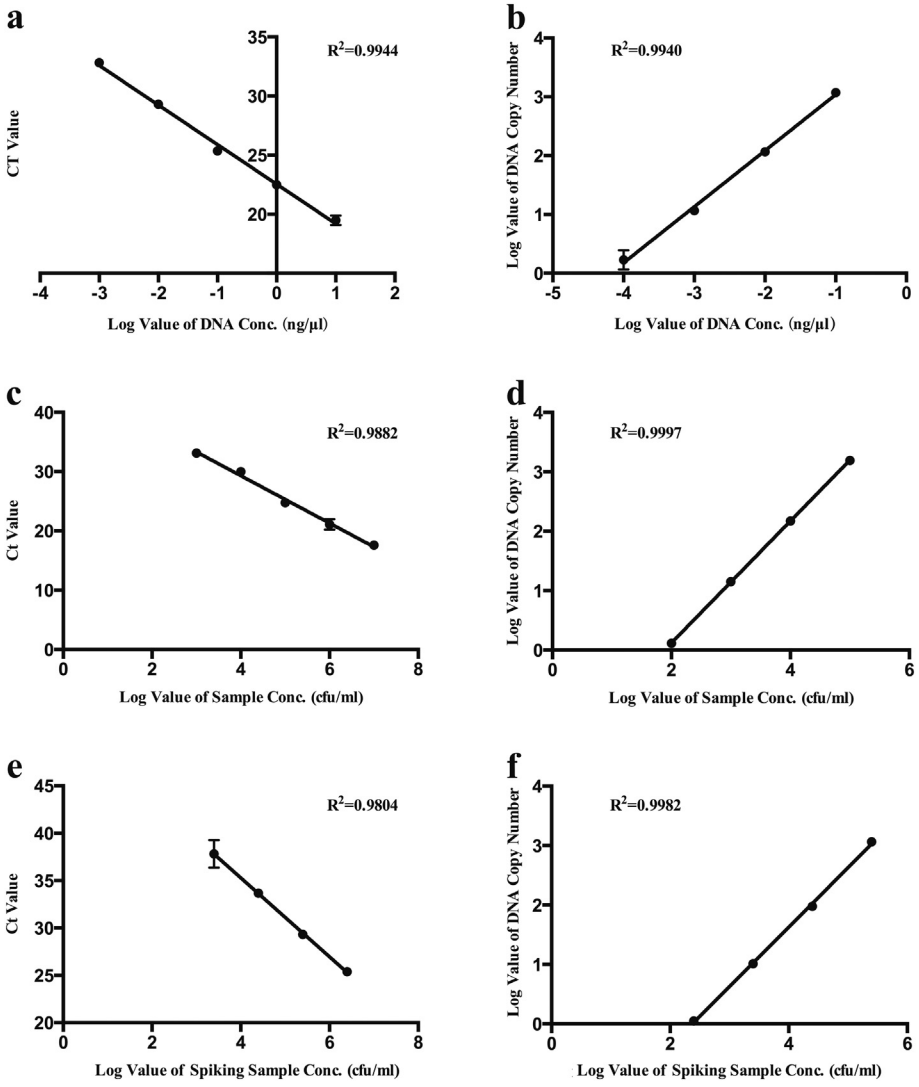

Fig. 2. Correlation curve of two platforms in several sources of *Salmonella typhimurium* a. qPCR in analyzing a serial dilution of DNA of *Salmonella typhimurium*. b. ddPCR in analyzing a serial dilution of DNA of *Salmonella typhimurium*. c. qPCR in analyzing a serial dilution of pure culture of *Salmonella typhimurium*. d. ddPCR in analyzing a serial dilution of pure culture of *Salmonella typhimurium*. e. qPCR in analyzing a serial dilution of spiking milk sample. e. ddPCR in analyzing a serial dilution of spiking milk sample.

Table 3
Impact of PCR inhibitors on qPCR.

DNA Conc.(ng/μl)	Ca^{2+} Conc.(mM)			Ethanol Conc.(%)		
	5	5.5	6	1.5	2	2.5
0.3 ng	+	+	−	+	−	−
0.1 ng	+	+	−	+	−	−

+: performing quantitative analysis.
−: un-performing quantitative analysis.

Table 4
Impact of PCR inhibitors on ddPCR.

DNA Conc.(ng/μl)	Ca^{2+} Conc.(mM)			Ethanol Conc.(%)		
	5	5.5	6	1.5	2	2.5
0.3 ng	+	+	+	+	+	+
0.1 ng	+	+	+	+	+	+

+: performing quantitative analysis.

Table 5
LOD of two platforms in spiking milk with *Salmonella typhimurium*.

Conc.(cfu/ml)	qPCR	ddPCR
2.5×10^6	+	+
2.5×10^5	+	+
2.5×10^4	+	+
2.5×10^3	+	+
2.5×10^2	-	+

+: performing quantitative analysis.
−: un-performing quantitative analysis.

Table 6
Time consuming of two platforms in spiking milk with trace *Salmonella typhimurium*.

Conc.(cfu/ml)	Cultured time (h)	
	qPCR	ddPCR
2.5×10^2	2	0
2.5×10^1	4	2
2.5×10^0	6	4

Similarity in repeatability and linearity reflects both ddPCR and qPCR are less affected by sample species and abundance. Compared with the conventional method, both qPCR and ddPCR have advances in sensitivity and efficiency. Also, our results indicate sensitivity of ddPCR with 10-flod higher than that of qPCR, and ddPCR saves 2 h of pre-enrichment time handling trace samples. However, it may hard to perform quantity analysis accurately when target's concentration exceeding

1 ng/μl or 10^6 cfu/ml. In addition, ddPCR shows stronger resistance to PCR inhibitors with few impact of DNA concentration.

Compared to qPCR, major strength of ddPCR in detecting foodborne pathogens in milk proves ddPCR is a suitable detecting and analytical tool especially for zero-tolerance bacteria in food control, which in agreement with previous findings (Porcellato et al., 2016; Velusamy et al., 2010). Owing to its superiority in LOD, less requisite for pre-enrichment time makes ddPCR more efficient in measuring *Salmonella typhimurium*, commonly saving 2 h compared to qPCR. Moreover, high resistant to PCR inhibitors makes ddPCR deal with DNA samples deriving from assorted sources more stable and reliable. As reported previously, ddPCR was not greatly affected until 35 mM of iron metal (The Effects of Known PCR Inhibitors on Droplet Digital PCR Performance, 2016), performing a huge advantage in resistant to PCR inhibitors. It also indicates the potential application of ddPCR in trace target detection, such as viruses causing human illness or genetically modified organism (GMO) detection. However, urgent requirements to move barriers in multiplex reaction and reduce the cost make ddPCR pursue several technical improvements to adapt the application in large scale.

Although qPCR is insufficient to sensitivity and more likely susceptible to influence of PCR inhibitors, its dynamic range of quantification is a worthy addition to ddPCR. As widely applied, multiplex assays make qPCR be a robust analytical approach in large-scale application. In addition, PMA combined with qPCR seems to be a potential way to distinguish living or dead cell, a puzzle in analytical technique relying on nucleic acid (Nocker et al., 2007).

Our findings identify the fact that ddPCR has advances in sensitivity, resistance and depletion of pre-culturing time, reflecting its well applicability in detecting foodborne pathogens especially for the zero-tolerance. In this research, the subject was only selected *Salmonella typhimurium* in milk and the applicable to other *Salmonella* species or sources were not estimated, yet discrepancy in strain or source may generate a limit impact on the conclusion (Porcellato et al., 2016). Thus, different treatment in DNA extraction and culture condition of strain may cause biases in results. In conclusion, detailed data provided by this research is important to select a suitable analytical tool according to experimental requirements, satisfying the urgent need in nucleic acids analysis and pointing to the requirement for foodborne pathogen detection. Supplying high sensitivity and wide dynamic range, the alternative of ddPCR or qPCR on the basis of experimental objective supplies a great assistant in food control and other fields.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2017.12.011.

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Competing interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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