Theo A. Schuurs[a], Rob Koelewijn, Eric A.T. Brienen[a], Titia Kortbeek[a], Theo G. Mank[a], Bert Mulder[a], Foekje F. Stelma[a], Lisette van Lieshout[a] and Jaap J. van Hellemond[a,*]

Harmonization of PCR-based detection of intestinal pathogens: experiences from the Dutch external quality assessment scheme on molecular diagnosis of protozoa in stool samples

https://doi.org/10.1515/cclm-2017-1057
Received November 14, 2017; accepted January 23, 2018; previously published online February 16, 2018

Abstract

Background: Real-time PCR methods are increasingly used in routine patient care settings not only to determine the presence or absence of pathogens in patient materials, but also to obtain semiquantitative results to estimate the pathogen load. However, it is so far unknown how well these methods are harmonized among different laboratories.
Methods: Sets of stool samples were distributed three to four times per year to ca. 25–40 participating laboratories within the European Union as part of an external quality assessment scheme (EQAS) for the detection of gastrointestinal protozoa. This paper presents the results obtained over a 3-year period for *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Cryptosporidium* species and *Dientamoeba fragilis*.
Results: Although both false-positive and false-negative results were reported, the overall sensitivity and specificity were high. The substantial differences in the quantitative output of the real-time PCR assays could be traced back to differences in DNA isolation procedures between different laboratories.
Conclusions: Participation in an EQAS proved to be important as it provides information on how the real-time PCR methods used by the participant compares to the generally reported results and indicates how procedures could be improved. Semiquantitative results of real-time PCR methods are not exchangeable between laboratories as long as the diagnostic procedures are not harmonized. Intralaboratory comparison of semiquantitative real-time PCR results seems only possible by the use of calibration curves derived from well-validated standards in clinical material and not by spiking solutions with purified DNA.

Keywords: *Cryptosporidium*; *Dientamoeba fragilis*; *Entamoeba histolytica*; external quality assessment scheme; *Giardia lamblia*; intestinal protozoa; real-time PCR.

Introduction

Classical methods used for detection of pathogens in clinical microbiology are mainly based on culture (viruses and bacteria) and microscopy (parasites). However, these time-consuming and labor-intensive methods are more and more replaced by molecular methods, of which many are based on RNA or DNA amplification. Because real-time PCR methods show high sensitivity and specificity and generate results fast, they are abundantly used for detection of pathogens in patient materials, such as blood, spinal fluid, urine and stool [1]. For some infectious diseases, it is sufficient that the method provides a reliable qualitative answer; i.e. is the pathogenic microbe present or absent, as treatment strategy is not influenced by the detected concentration of microbes. However, quantification might be relevant in specific cases. For example, the intensity of infection might reflect the severity of the infection (e.g. *Plasmodium falciparum*). Microbes can also be present in low quantities in immune-competent patients not causing any symptoms, while causing severe symptoms or even fatal disease in immune-compromised

[a]Current board members of the Parasitology Section of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML).
***Corresponding author: Dr. Jaap J. van Hellemond,** Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Centre, PO Box 2040, 3000 CA Rotterdam, the Netherlands, Phone: +31 10 7033510, Fax: +31 10 7033875, E-mail: j.vanhellemond@erasmusmc.nl
Theo A. Schuurs: Centre for Infectious Diseases Friesland, Izore, Leeuwarden, the Netherlands
Rob Koelewijn: Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Centre, Rotterdam, the Netherlands
Eric A.T. Brienen and Lisette van Lieshout: Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands
Titia Kortbeek: Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
Theo G. Mank: Regional Laboratory for Medical Microbiology and Public Health, Haarlem, the Netherlands
Bert Mulder: Department of Medical Microbiology, Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands
Foekje F. Stelma: Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands

patient (e.g. *Pneumocystis jiroveci*). Therefore, real-time PCR assays are currently used in routine patient care settings also to semiquantitatively estimate the pathogen load as an indicator for disease severity [2–5]. In addition, semiquantitative PCR assays are also used for epidemiological studies in which the detected pathogen load is used to estimate the infection intensities, for instance, during eradication programs for helminth infections [6, 7]. Although real-time PCR methods beyond any doubt provide semiquantitative results, it is so far unknown how well these methods are harmonized among distinct laboratories, in particular when dealing with the detection of parasitic infections.

In 2013, the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) launched an external quality assessment scheme (EQAS) for detection of gastrointestinal parasitic protozoa in stool by molecular methods based on DNA amplification, in order to gain insight into the qualitative as well as quantitative performance of this assay in routine patient care. In contrast to other EQAS organizations, which distribute purified DNA in an artificial matrix, the SKML-EQAS exclusively uses unpreserved clinical stool specimens in their distribution rounds. Hence, by using this commutable stool material, the entire laboratory procedure used for clinical specimens can be evaluated, thereby providing a reliable indication of the qualitative performance of methods used as well as the quantitative variation between different laboratories. Here we report the results over a 3-year period from the SKML-EQAS for the detection of the gastrointestinal parasitic protozoa *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Cryptosporidium* species and *Dientamoeba fragilis* in stool and provide recommendations on how to improve molecular detection of these protozoa.

Materials and methods

Distribution of stool samples in a quality control scheme

Sets of three to four stool samples have been distributed three to four times per year by regular mail to ca. 25–40 participating laboratories within the European Union. This EQAS has been part of a broad EQAS panel provided by the SKML. Hence, the distribution fulfills all regulations applying to a proper EQAS, such as anonymization, ethical approval and standardization (see www.skml.nl/en for details). To obtain a performance score, participants had to report their results, i.e. the protozoa species detected with the corresponding quantification cycle (Cq)-value (Cq; the PCR-cycle at which the fluorescence from PCR amplification exceeds the background fluorescence), within 2 weeks after receiving the samples via an Internet-based entry point. Here we report the participants findings of the period 2013–2015.

Materials

Four weeks prior to each scheduled distribution round, seven Dutch expert-laboratories for parasitology, all members of the parasitology section of the SKML-EQAS foundation, were requested to select stool samples containing one or more of the following protozoa in sufficiently high concentrations; *E. histolytica*, *E. dispar*, *G. lamblia*, *Cryptosporidium* species or *D. fragilis*. These selected stool samples were all derived from a routine diagnostic laboratory setting. They were send to the SKML-EQAS coordinating Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Centre, Rotterdam, the Netherlands, for further processing in order to prepare a stable and homogeneous stool suspension. From there, samples were further distributed in 0.5-mL aliquots.

Validation of materials

Inter- and intralaboratory variation was evaluated for each selected stool suspension by sending one aliquot to each of the seven expert-laboratories, as well as five aliquots to a single laboratory. All testing was done blinded, and the results were reported to the coordinating laboratory without knowing the outcome of the other expert-laboratories. To label a sample as being positive for a specific target, all expert reference laboratories had to be unanimous in their qualitative result. A suspension was considered to be homogeneous when the test results of the five aliquots showed an acceptable intralaboratory variation, which was defined as follows: all Cq-values should fall within the range of the average Cq ± 2 times the standard deviation, or otherwise the difference between the lowest and the highest test result of all five measurements should not be more than 2 Cq. In some of the suspensions, one or more protozoa species were present in such a low concentration that detection by all participating laboratories could not be guaranteed. In such a case, the suspension was still distributed, but examination of that particular target was considered 'educational', which means that no performance score was awarded to the participants for that particular target.

The stability of the protozoa in the unpreserved stool samples was thoroughly investigated before the EQAS was launched. Analysis of stool, which had been stored at room temperature for 2 weeks before distribution to the expert-laboratories, demonstrated a decrease of <2 Cq-values for all examined protozoa, including *D. fragilis*, over that period. Subsequently, the stability of targets in the stool samples was monitored by the expert-laboratories by examining these samples not only prior to distribution to the participants but also as actual participants in the EQAS. Substantial differences in reported Cq-values between these two examination time points were never observed.

On one occasion participants received a purified DNA sample of a stool that contained *G. lamblia* in addition to a sample of the original stool suspension. This was to explore possible causes of reported differences in Cq-values, in particular to discriminate within the entire diagnostic procedure the outcome of DNA isolation from DNA amplification.

Finally, a questionnaire was send out asking for details on the used analytical procedures, including applied dilution factors, the volume of diluted stool from which DNA was isolated, the volume of purified DNA eluted and the volume of eluted DNA used in the PCR.

This information was used to calculate per participant, the amount of stool from which the input DNA for the PCR was derived.

Results

Between 2013 and 2015, the number of participants in the SKML-EQAS for molecular detection of gastrointestinal protozoa laboratories increased from approximately 25 to 40 laboratories. Over these 3 years, participants received 27 stool samples divided over 11 distributions. The provided stool samples contained a variable composition of protozoa: zero to maximally three species. In total, the distributed samples comprised 10× *G. lamblia*, 4× *E. histolytica*, 6× *Cryptosporidium* species, 11× *D. fragilis* and 3× *E. dispar*. Some participants reported results for only a subset of these five targets, and therefore, the number of reported results varied from target to target (Table 1).

All gastrointestinal protozoa were detected with a high sensitivity (>96%), except for *E. histolytica*, which was detected with an overall sensitivity of 85% (Table 1). The frequency of false-negative results was correlated to lower pathogen loads (Figure 1). In addition, out of the 631 reported results, eight were false positive; *G. lamblia* (4×, 1.6%), *E. histolytica* (1×, 0.9%) and *D. fragilis* (3×, 2.5%). Two of these false-positive results were probably caused by an administrative error, as the intended results for sample 1 were reported for material 2 and vice versa.

The false-negative results suggest that the distinct methods used by the participants vary in sensitivity. Therefore, the semiquantitative results were analyzed by comparison of the reported Cq-values, which represents the number of DNA amplification cycles that are needed to produce a fluorescent signal that exceeds the threshold value of the real-time PCR. Because the amount of target DNA is amplified exponentially during the PCR, a difference of N cycles in Cq-value corresponds theoretically to a difference of 2^N in sensitivity to detect target DNA present in the examined stool specimen.

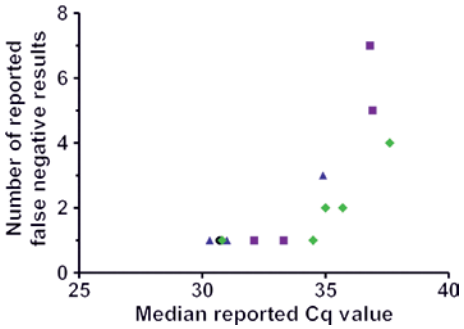

Figure 1: Correlation between the number of reported false-negative results and the parasite load.
Each dot represents a specimen for which the number of reported false-negative results is plotted on the y-axis and the parasite load on the x-axis (expressed as the median of reported Cq-values). Results are shown for *Giardia lamblia* (black dots), *Cryptosporidium* species (blue triangles), *Entamoeba histolytica* (purple squares) and *Dientamoeba fragilis* (green diamonds). Stool samples for which no false-negative results were reported are not included in the graph.

Despite the homogeneous load of protozoa in each distributed stool sample, as reflected by the small variations (<2 cycles) when examined in fivefold by one of the expert-laboratories, a difference was observed between participants of at least 10 amplification cycles in reported Cq-values (Figure 2). Variance in reported Cq-values (Cq) may, in part, be explained by the different ways of measuring these values (cycle threshold [Ct] versus crossing point [Cp] cycle is a different way to determine the cycle at which fluorescence from PCR amplification exceeds the background fluorescence) and/or laboratory (and apparatus)-specific settings and can also be indicative for sensitivity differences. Indeed, laboratories presenting relatively low Cq-values never reported false-negative results for that particular pathogen, as indicated by the green box in Figure 2A. Altogether, these findings suggest that some participants use a more sensitive method to detect the target DNA compared to other participants.

If the same analysis was performed for other targets, a similar pattern was observed (compare Figure 2A and B). On the other hand, participants that report relatively

Table 1: Sensitivity (%) of detection of gastrointestinal protozoa in distributed stool samples.

	Giardia lamblia	n	*Cryptosporidium* spp.	n	*Entamoeba histolytica*	n	*Dientamoeba fragilis*	n
2013	98.8	85	92.0	38	77.0	26	n.d.	–
2014	96.4	84	100.0	54	83.0	52	98.0	48
2015	98.8	85	96.0	57	97.0	29	100.0	73
Total	98.0	254	96.6	149	85.0	107	99.2	121

Data for *E. dispar* are not shown, as only five to seven participants reported results for this protozoa. n, number of reported results (number of participants × number of distributed samples validated positive of that particular protozoa); n.d., not distributed.

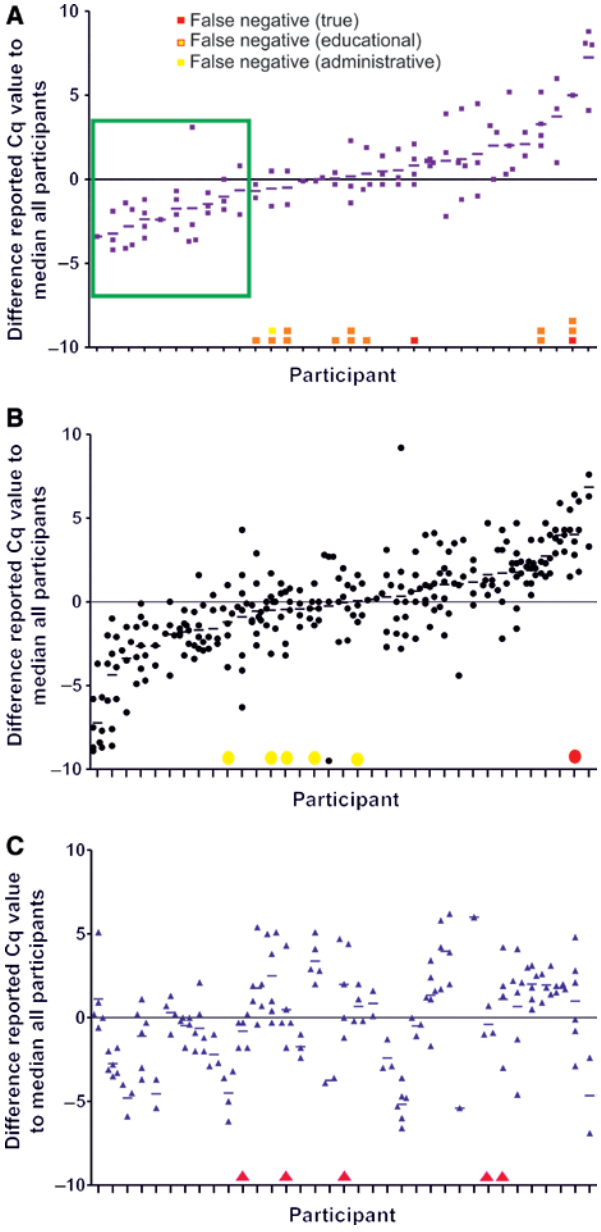

Figure 2: Reported Cq-values for stool samples positive of *Entamoeba histolytica* (A), *Giardia lamblia* (B) and *Cryptosporidium* spp. (C). Each dot represents a reported Cq-value by a single participant for a single stool sample. The reported Cq-values are plotted on the y-axis expressed as the reported Cq-value of the individual participant subtracted by the median of reported Cq-value of all participants. A dot with a negative y-axis value thus represents a reported Cq-value lower than the median of all participants and vice versa. Each lane on the x-axis represents an individual participant. Please note that the order in which the participants are plotted on the x-axis is identical in panels B and C but differs from that in panel A. Orange, red and yellow markers plotted on the x-axis indicate a reported false-negative result for a sample with a high parasite load (median of reported Cq-value <35; red marker), a low parasite load (median of reported Cq-value >35; orange) and errors presumably caused by sample exchange, respectively. The small horizontal lines indicate the average difference in reported Cq-value by the participant compared to the median reported Cq-value of all participants.

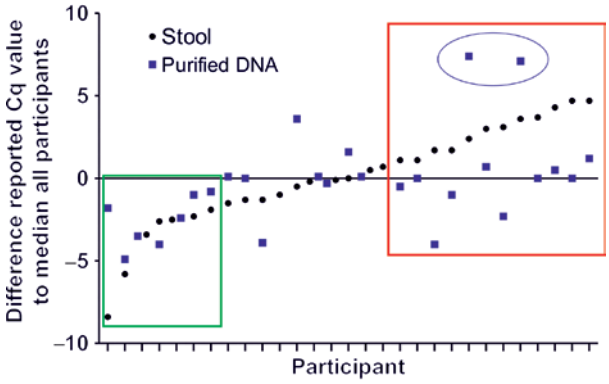

Figure 3: Reported Cq-values for *Giardia lamblia* in stool (black dots) and purified DNA (blue dots) specimens.
The reported Cq-values are plotted on the y-axis expressed as the reported Cq-value of the individual participant subtracted by the median of reported Cq-value of all participants. Each lane represents a single participant and the order in which the participants are plotted on the x-axis differs from that in Figures 1 and 2.

low Cq-values for one PCR target did not necessarily also reported low Cq-values for another target, as for instance the participant shown far right on the x-axis in Figure 2B reported relatively high Cq-values for *G. lamblia* but low Cq-values for *Cryptosporidium* spp. (Figure 2C). These findings suggest that the differences in reported Cq-values are caused by not only differences in efficiency of DNA extraction from stool but also differences in DNA amplification efficiency.

Figure 3 compares the outcome in reported Cq-values of the purified DNA sample of a stool that contained *G. lamblia*, to the original stool suspension. Similar to Figure 1, a range of more than 13 cycles was seen in the reported Cq-value for the analyzed stool sample. Likewise, a large difference (>12 cycles) was reported in the Cq-values for the purified DNA sample, illustrating a substantial difference in efficiency of the PCR methods used to amplify *G. lamblia* DNA. Participants that reported relatively low Cq-values for stool samples (Figure 3, green box), a result mirroring the entire diagnostic procedure of DNA isolation and amplification, also reported a relatively low Cq-value for the purified DNA sample. Participants that reported relatively high Cq-values for stool samples (Figure 3, red box) could be divided into two groups; some reported a relatively high Cq-value for the purified DNA sample (Figure 3, purple oval) but most reported a relative low Cq-value. In cases where the participant reported a high Cq-value for stool and low Cq-value for DNA, it seems that DNA is amplified efficiently and that the DNA isolation method could be improved. In cases where high Cq-values were reported for both the stool and the DNA sample, it seems that the DNA amplification method could be improved. It can be

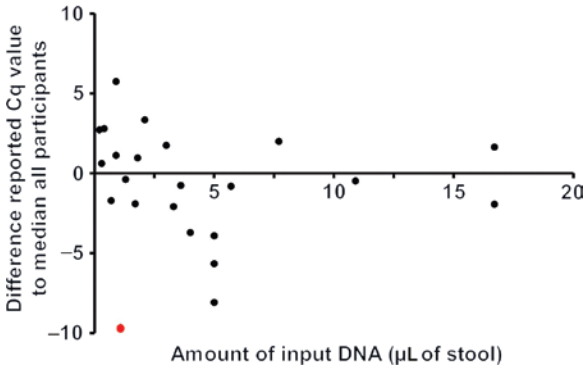

Figure 4: Correlation between the reported Cq-value and the amount of input DNA for the real-time PCR.
The reported Cq-values are plotted on the y-axis expressed as the reported Cq-value of the individual participant subtracted by the median of reported Cq-value of all participants. The amount of input DNA for the DNA is defined as amount of stool (in µL) from which the input DNA was derived. The red dot represents a reported false-negative result from a participant that used input DNA derived from 0.9 µL of stool.

concluded that that differences in sensitivity among participants are probably caused by differences in efficiency of both DNA extraction as well as DNA amplification methods.

Around 75% (23/32) of the participants responded to the questionnaire asking for details on the used analytical procedures. No correlation was seen between the reported Cq-values and the methods and machinery used for DNA isolation, the amplification procedure or any pretreatment protocol (e.g. bead beating, freeze-thawing). By contrast, an association was noticed between the reported Cq-values and the amount of stool from which the input DNA for the PCR was derived, which differed over 80-fold (corresponding to $>2^6$) among participants. The observed pattern in the relation between the amount of input DNA [defined as the amount of stool (in µL) from which the input DNA was derived] and the reported Cq-values suggests an optimal amount of used stool specimen, which was in this case circa 5 µL (Figure 4). The use of a smaller amount of stool, i.e. >4 µL of this specimen, or a larger amount of stool, i.e. more than 5 µL of this specimen, resulted in an increase of the reported Cq-value.

Discussion

To the best of our knowledge, this is the first study reporting the outcome of an EQAS for molecular detection of protozoa in unpreserved stool specimens. Analysis of the results of ca. 25–40 participating European laboratories over a 3-year period demonstrates high sensitivity of real-time PCR methods in routine diagnostic settings, confirms what has been shown before in numerous studies by comparing real-time PCR performance with more traditional microscopy-based methods [8–11]. Still, the occurrence of a few false-negative and false-positive results clearly demonstrated that participation within an EQAS is essential for proper quality control of each individual diagnostic laboratory, as suggested earlier [12].

Analysis of the reported semiquantitative results (Cq-values) demonstrated that the efficiency of the methods used for DNA isolation as well as target-DNA amplification varies substantially among distinct laboratories, as the interlaboratory variation was >10 Cq-values, whereas the intralaboratory variation was always small (<2 Cq-values). Variations in the amount of input DNA for the PCR, defined as the amount of stool used for DNA extraction, appeared to be a very important factor determining the interlaboratory variation. When the volume is too small, the amount of protozoa present might be limiting; when the volume is too large, DNA amplification inhibiting factors might interfere with the amplification process.

Besides the amount of stool used for DNA isolation, there are likely to be other causes of variation between participants such as the selected DNA target, the designed primers, the quality of the DNA polymerase, the actual PCR settings and the mathematical method used to determine the point at which the fluorescent signal exceeds the threshold. However, the number of participating laboratories was too low and the range of used molecular procedures was too high to pinpoint any additional key factors.

Our findings clearly illustrate that semiquantitative results of real-time PCR methods are not exchangeable between laboratories as long as diagnostic procedures are not harmonized. Most laboratories aim to standardize their overall molecular methods in order to combine the detection of a whole range of microorganisms. Such an intralaboratory standardization will lead to efficient use of materials, equipment and time and thus to a reduction in costs. On the other hand, interlaboratory standardization is less common. Laboratories are keen to continue using their own local settings for DNA isolation and performance of real-time PCR and are not easily persuaded to adapt procedures in full detail in order to harmonize protocols between laboratories. A possible way forward to achieve more comparable qualitative results between different laboratories could be the introduction of a calibration curve derived from well-validated standards. Based on our findings, it is recommended to prepare such validated standards by the use of actual clinical material

(stool, urine, serum, etc.) and not by spiking solutions with purified DNA.

In conclusion, by evaluating the EQAS data, we have shown that substantial differences in the quantitative output of real-time PCR for the detection of intestinal protozoa could be traced back to differences in DNA isolation procedures between distinct laboratories, and we confirmed the importance of participating in an EQAS when performing real-time PCR in a clinical routine laboratory setting.

Acknowledgments: Dr. D.L. Borin is kindly acknowledged for critically reading the manuscript, and Mrs. N. van der Ham is thanked for excellent technical support in preparation of the EQAS samples.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Verkerke HP, Sobuz SU, Petri WA, Jr. Molecular diagnosis of infectious diarrhea: focus on enteric protozoa. Expert Rev Mol Diagn 2014;14:935–46.
2. Fauchier T, Hasseine L, Gari-Toussaint M, Casanova V, Marty PM, Pomares C. Detection of Pneumocystis jirovecii by quantitative PCR to differentiate colonization and pneumonia in immunocompromised HIV-positive and HIV-negative patients. J Clin Microbiol 2016;54:1487–95.
3. de Freitas VL, da Silva SC, Sartori AM, Bezerra RC, Westphalen EV, Molina TD, et al. Real-time PCR in HIV/Trypanosoma cruzi coinfection with and without Chagas disease reactivation: association with HIV viral load and CD4 level. PLoS Negl Trop Dis 2011;5:e1277.
4. Alidjinou EK, Bocket L, Hober D. Quantification of viral DNA during HIV-1 infection: a review of relevant clinical uses and laboratory methods. Pathol Biol (Paris) 2015;63:53–9.
5. Gullett JC, Nolte FS. Quantitative nucleic acid amplification methods for viral infections. Clin Chem 2015;61:72–8.
6. Verweij JJ, Stensvold CR. Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic infections. Clin Microbiol Rev 2014;27:371–418.
7. Thiele EA, Cama VA, Lakwo T, Mekasha S, Abanyie F, Sleshi M, et al. Detection of Onchocerca volvulus in skin snips by microscopy and real-time polymerase chain reaction: implications for monitoring and evaluation activities. Am J Trop Med Hyg 2016;94:906–11.
8. van Lieshout L, Roestenberg M. Clinical consequences of new diagnostic tools for intestinal parasites. Clin Microbiol Infect 2015;21:520–8.
9. ten Hove RJ, van Esbroeck M, Vervoort T, van den Ende J, van Lieshout L, Verweij JJ. Molecular diagnostics of intestinal parasites in returning travellers. Eur J Clin Microbiol Infect Dis 2009;28:1045–53.
10. Cimino RO, Jeun R, Juarez M, Cajal PS, Vargas P, Echazu A, et al. Identification of human intestinal parasites affecting an asymptomatic peri-urban Argentinian population using multi-parallel quantitative real-time polymerase chain reaction. Parasit Vectors 2015;8:380.
11. Mejia R, Vicuna Y, Broncano N, Sandoval C, Vaca M, Chico M, et al. A novel, multi-parallel, real-time polymerase chain reaction approach for eight gastrointestinal parasites provides improved diagnostic capabilities to resource-limited at-risk populations. Am J Trop Med Hyg 2013;88:1041–7.
12. Frickmann H, Schwarz NG, Rakotozandrindrainy R, May J, Hagen RM. PCR for enteric pathogens in high-prevalence settings. What does a positive signal tell us? Infect Dis (Lond) 2015;47:491–8.