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A Comparative Retrospective Study

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Accurate tools for *Toxoplasma gondii* detection and quantification can be valuable for the early and effective management of toxoplasmosis. Droplet digital PCR (ddPCR) is a next-generation end-point PCR technique with high performance. The objective of the study was to evaluate the performance of ddPCR for the detection and absolute quantification of *T. gondii*. From January 2019 to October 2020, DNA samples collected at the Laboratory of Parasitology and Mycology of Pitié-Salpêtrière Hospital in Paris were retrospectively analyzed by ddPCR and real-time quantitative PCR (qPCR). To detect *T. gondii* with the best sensitivity possible, the REP-529 multicopy target was used. For absolute quantification of *T. gondii*, a specific single-copy target of α-tubulin was designed. *T. gondii* detection by ddPCR and qPCR was strongly correlated ($R^2 = 0.93$), with a total concordance of 96.7% ($n = 145/150$). Quantification of *T. gondii* using ddPCR was successful for 15 of 35 samples showing a parasite load ≥170 copies/mL of DNA eluate using the α-tubulin target. The qPCR REP-529 quantification based on a standard curve was approximate and dependent on the strain genotype, which led to an estimate of parasite copy number 14- to 160-fold superior to the ddPCR result. In total, ddPCR is an effective molecular method for *T. gondii* detection that shows equivalent performance to qPCR. For robust *T. gondii* quantification, ddPCR is clearly more accurate than semiquantitative qPCR methods. *(J Mol Diagn 2023, 25: 467—476; https://doi.org/10.1016/j.jmoldx.2023.03.006)*

Toxoplasmosis is an infectious disease caused by *Toxoplasma gondii*, a protozoan parasite.[1] Up to one-third of the world's human population is infected by *T. gondii*. However, toxoplasmosis is considered a neglected disease in many countries.[2] The diagnosis of toxoplasmosis is challenging. Real-time quantitative PCR (qPCR) techniques are recommended for a precocious diagnosis of prenatal, neonatal, cerebral, disseminated, and retinochoroiditis infections.[3] Despite scarce and contradictory literature,

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parasitic load estimation by qPCR could prove useful as a prognostic marker of disease severity. Indeed, for congenital infections, the presence of clinical signs in fetuses has been correlated with a higher concentration of *T. gondii* parasites in amniotic fluid.[4,5] In immunocompromised patients, quantitative qPCR was useful for posttherapeutic follow-up to monitor the decrease in parasite loads.[6,7]

To date, routine *T. gondii* qPCR quantification methods are still approximate, with most of them using a standard curve from a biological sample spiked with a *T. gondii* reference strain.[8] To increase sensitivity, REP-529, a repeated noncoding DNA sequence, is the standard molecular target used in France and most European countries.[9–11] However, the number of copies of REP-529 varies 200- to 300-fold,[12] and it may have an impact on quantification. The method of extraction and the efficacy of the qPCR assay may also cause result variability.[11,13] Therefore, a rapid, accurate, and easy to standardize absolute quantification method is required. Droplet digital PCR (ddPCR) is a next-generation end-point PCR that exhibits high performance for the detection and absolute quantification of specific targets in various human biological samples,[14] including major applications in oncology, particularly for liquid biopsies.[15,16] It is promising for absolute quantification of bacterial, viral, and parasite loads in clinical samples without a standard curve and for the calibration of reference standards for qPCR assays.[17–19] In parasitology, although successfully applied for a range of protozoa and helminths,[20] it has never been evaluated for *T. gondii* diagnosis in humans.

This study aimed to evaluate the performance of ddPCR for the detection and absolute quantification of *T. gondii*. A first assay was aimed at detecting *T. gondii* with the best sensitivity possible using the REP-529 multicopy target, and a second assay was aimed at absolute ddPCR quantification of *T. gondii*, due to a specific single-copy region of α-tubulin.

Materials and Methods

Sample Collection

From January 2019 to October 2020, DNA samples ($n = 150$) were collected at the Laboratory of Parasitology and Mycology of Pitié-Salpêtrière Hospital in Paris and stored at −80°C following the routine diagnosis of toxoplasmosis. The DNA samples were obtained from blood ($n = 98$, including one placental cord blood), amniotic fluid (AF) ($n = 10$), placenta ($n = 4$), cerebrospinal fluid (CSF) ($n = 7$), aqueous humour (AH) ($n = 20$), and bronchoalveolar fluid (BAL) ($n = 7$). Four quality controls (2 AF and 2 blood samples) were included from the molecular biology associated laboratory of the National Reference Centre for Toxoplasmosis (Montpellier, France). Two reference strains of *T. gondii* were used: the ME49 strain (TgA 00001, type II) provided by the Biological Resource Centre for Toxoplasma (*http://www.toxocrb.com*, last accessed January 2023) and the RH strain (type I) provided by the Montpellier laboratory. The ME49 strain was obtained from an infected mouse brain, whereas the RH strain was obtained from lyophilized spiked AF. The RH strain was used for the elaboration of a standard curve (initial concentration of 10^5/mL of *T. gondii* genome equivalents), Production of the RH stock suspensions has been described and validated elsewhere.[8] Briefly, tachyzoites were grown *in vitro* by serial passage in human foreskin fibroblast monolayers in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum using standard procedure. Harvested tachyzoites were washed twice, resuspended in sterile RPMI 1640 medium, and counted in a hemocytometer. Means and SDs were calculated from 10 values, and the parasites were then diluted in AF to obtain 10^5/mL of *T. gondii* genome equivalents.

DNA Extraction Protocol

The sample volume of blood was 200 μL. For CSF, BAL, AF, placenta, and AH samples, a centrifugation step at 11,180 × *g* for 2 to 5 minutes was implemented before DNA extraction. For the AH samples, the resultant supernatant was discarded and 200 μL of phosphate-buffered saline was added to the centrifugation pellet. For the other samples, the pellet was resuspended in 200 μL of initial matrix. DNA was manually isolated using a Qiamp DNA mini kit (Qiagen, Hildesheim, Germany) or an Emag (bioMérieux, Paris, France) automatized DNA extraction following the manufacturer's recommendations. The elution volume was 200 μL for manual DNA extraction or 100 μL for automated DNA extraction. DNA concentrations were determined using a Qubit double-stranded DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). The method of extraction used for each sample is detailed in Supplemental Table S1.

REP-529 qPCR Assay

T. gondii detection was performed by amplification of REP-529, a noncoding repeat element sequence located on chromosome IV (*http://www.ncbi.nlm.nih.gov/genbank*; accession number AF487550),[10,12] using previously described primers and probe.[21] Final concentrations were 0.5 μmol/L for primers and 0.2 μmol/L for probe. In each reaction, the sample volume of DNA extract was 5 μL if the DNA concentration was ≥2 ng/μL and 10 μL if <2 ng/μL. REP-529 was amplified using a 7500 Fast Real-Time PCR instrument (Applied Biosystems, Waltham, MA). The cycling conditions were as follows: 95°C for 20 seconds followed by 45 cycles of 2 seconds at 95°C and 20 seconds at 68°C. All the samples showing a characteristic amplification curve and an amplification threshold cycle (C_T) <40 were deemed positive. Negative (no DNA template) and

positive controls (ME49 and RH strains) were applied in each experiment. All experiments were repeated three times.

ddPCR Protocol

ddPCR was performed to detect and quantify *T. gondii* using a QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA). For ddPCR REP-529, the same volume of DNA extract as that used for qPCR REP-529 was used in each reaction for adequate comparisons. For ddPCR α-tubulin, a volume of 10 μL of DNA extract was used to gain sensitivity. Final concentrations of 2× ddPCR supermix (no dUTP) (Bio-Rad) were 900 nmol/L for primers and 250 nmol/L for probes in a total volume of ddPCR mix of 20 μL. The cycling conditions were as follows: 95°C for 10 minutes (polymerase activation), followed by 45 cycles of 95°C for 30 seconds, 60°C (REP-529), or 55°C (α-tubulin, see primers and probe below) for 60 seconds (denaturation and elongation), and 98°C for 10 minutes (droplet consolidation), with a final hold at 12°C (cooling and preservation). The data were analyzed using Quanta Soft software Pro version 1.7 (Bio-Rad) with the thresholds set based on the results obtained during the validation method (see *Results* section). For a normalization concern, quantification of *T. gondii* was expressed in copy numbers per milliliter of eluate, which was determined by multiplication of the mean copy numbers per microliters of reaction (absolute quantification provided by the ddPCR system) by a factor of ×1000. Quantification with the α-tubulin gene was performed for all samples, with an REP-529–positive signal obtained by real-time PCR and/or digital PCR. All experiments were repeated three times.

Validation Method of the ddPCR Assay

The validation method was performed following the Digital MIQE Guidelines.[22] This validation included positivity and intensity threshold assessments, DNA quantification according to the nature of the biological sample, correlation comparisons between ddPCR and qPCR, and repeatability.

Positivity and Intensity Thresholds

The total droplet number was determined, and only wells containing >10,000 droplets were accepted for analysis. To discriminate positive droplets from negative droplets, a positivity threshold was set in terms of the fluorescence intensity and number of positive droplets. The intensity threshold was calculated as the sum of the average of the fluorescence intensity of all droplets from 75 negative samples and values 3 times the standard deviation. To fix the droplet positivity threshold for *T. gondii* detection, samples of bronchoalveolar fluid and blood negative by qPCR were replicated 8 times and analyzed by ddPCR using REP-529 and α-tubulin.

DNA Quantification

The amount of DNA in nanograms was calculated in the test DNA extract submitted to ddPCR for various clinical samples, including AH, BAL, CSF, AF, and placenta, thanks to the direct measurement of DNA concentration in nanograms per microliter with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) (Supplemental Table S1).

Correlation Comparisons between ddPCR and qPCR

A regression line was performed with serial 2-, 5-, and 10-fold dilutions of a DNA extract from a reference strain of *T. gondii* RH (type I). Each dilution was analyzed in triplicate in two independent experiments. The correlation was established between log (mean concentration in copies/5 μL of REP-529) obtained by ddPCR and the mean C_T value obtained by qPCR (Supplemental Tables S2 and S3).

Repeatability

The repeatability of ddPCR was determined for six different biological samples (AF, BAL, blood, and AH) and replicated eight times in a single experiment (Supplemental Table S4).

Absolute Quantification by ddPCR

Absolute quantification was performed using the α-tubulin target, a one-copy gene of 1672 bp located on chromosome XI of the *T. gondii* genome.[23] New consensus primers and probes were designed, and a standard curve of the ddPCR signal targeting the α-tubulin gene was generated to assess the linearity. Standard curve was generated with serial 2.5- and 10-fold dilutions of BAL samples positive for *Toxoplasma gondii* (sample 3, type III strain, TgH29139A) (Table 1 and Supplemental Table S5). Comparisons were performed between *T. gondii* parasite loads measured by α-tubulin ddPCR absolute quantification and estimated by REP-529 qPCR relative quantification (Supplemental Table S6). The number of *T. gondii* parasites estimated by REP-529 qPCR was based on a standard curve of RH reference strain (Supplemental Table S7 and Supplemental Figure S1).

Genotyping of *T. gondii*

DNA samples from the patients and controls positive for *T. gondii* were genotyped at Limoges National Reference Centre Laboratory using 15 multilocus microsatellite markers distributed on 10 of 14 chromosomes, according to a previously published protocol.[24]

Statistical Analysis

GraphPad Prism software version 9 (GraphPad Software, San Diego, CA) was used for the generation of curves, histograms, and box plots. For each correlation test, the equation of the simple linear regression is provided, as well

Table 1 Clinicoepidemiologic Settings of the Patients along with Quantification of *Toxoplasma gondii* by α-Tubulin ddPCR Absolute Quantification and by REP-529 qPCR Relative Quantification

Patient	Age, years	Sex	Country	Key clinical points	Positive sample by qPCR and ddPCR REP-529	Quantified sample
A	58	M	Reunion Island	Reactivation, pulmonary toxoplasmosis, immunosuppressed, renal transplant, death	Blood, BAL	Blood Blood BAL
B	53	M	France	Reactivation, pulmonary toxoplasmosis, immunosuppressed, liver transplant, death	Blood, BAL	Blood BAL
C	49	F	France	Reactivation, cerebral toxoplasmosis, immunosuppressed, HIV, death	Blood, CSF	Blood
D	38	F	France	Congenital toxoplasmosis	AF	AF
E	33	F	France	Congenital toxoplasmosis	AF	AF
F	32	M	Gabon	Reactivation, pulmonary toxoplasmosis, immunosuppressed, renal transplant	Blood, BAL	Blood BAL
G	31	F	Cameroun	Reactivation, ocular toxoplasmosis, immunosuppressed, HIV	AH	AH
H	26	F	Mali	Congenital toxoplasmosis	AF	AF
I	15	M	France	No data available	Blood	Blood
J	27	F	DRC	Ocular toxoplasmosis, immunocompetent, panuveitis, hyalitis	AH	AH
K	63	F	France	Immunosuppressed, cerebral toxoplasmosis, hemopathy, death	Blood, CSF	CSF
					Controls	
						AF
						AF
						Mouse brain

(*table continues*)

**Toxoplasma* quantifications are expressed as the mean copies per milliliter of DNA eluate.

†The mean *Toxoplasma gondii* copies per milliliter estimated by qPCR REP-529 were based on a standard curve of an RH reference strain (Supplemental Tables S6 and S7 and Supplemental Fig. S1).

‡Number of amplified microsatellite markers is indicated into brackets.

F, female; M, male; AF, amniotic fluid; AH, aqueous humour; BAL, bronchoalveolar fluid; CSF, cerebrospinal fluid; C_T, threshold cycle; ddPCR, droplet digital; DRC, Democratic Republic of Congo; qPCR, real-time quantitative PCR.

as the linear determination coefficient (R^2), to determine the quality of the prediction of the linear regression. Agreement and correlation between ddPCR quantification and cycle threshold values obtained by qPCR were evaluated by the Bland-Altman method comparison test.[25] In this study, the Bland-Altman represents, for each sample, the difference between the log (mean concentration in copies per milliliter of REP-529) obtained by ddPCR and the mean qPCR cycle threshold values, plotted against the mean of these two values.

Ethical Statement

The clinical samples were deidentified before research and not linked to clinical records. Thus, the study does not fulfill criteria for human subjects research. At the time of sampling, none of the patients expressed opposition to the use of their samples for scientific purposes. The study is thus exempt from ethics committee approval, in accordance with French law 2004-806, dated August 9, 2004, on public health policy.

Results

Validation of the ddPCR Assays

The intensity threshold was set to 5160 fluorescence units for REP-529 and 2300 fluorescence units for α-tubulin. One droplet at most from one replicate of eight was observed above the selected intensity threshold in the two negative tested samples (Figure 1A). Therefore, samples with at least two droplets were considered positive. The amount of DNA varied greatly, depending on the type of biological sample (Figure 1B). Nevertheless, the amount of DNA did not impact parasite detection by ddPCR. A good correlation ($R^2 = 0.99$) was observed between the ddPCR and real-time qPCR C_T values using the REP-529 target and an RH strain (Figure 1C). The repeatability was high for the six

Table 1 (*continued*)

Sample no.	qPCR REP-529, mean C_T values	qPCR REP-529, mean copies/mL*,†	ddPCR α-tubulin, mean copies/mL)*	Genotype‡	Strain
1	28.89	85,564	1490	—	—
2	30.70	26,565	473	—	—
3	23.49	2,700,638	59,310	Type III (n = 14/15)	TgH29139A
4	26.38	422,251	6250	—	—
5	25.72	645,695	10,040	Type III (n = 15/15)	TgH29148A
6	23.30	3,045,884	29,667	Type II (n = 15/15)	TgH29147A
7	28.09	140,988	880	Type II (n = 10/15)	TgH29140A
8	27.30	235,015	2980	Type II (n = 14/15)	TgH29143A
9	28.38	117,251	1752	—	—
10	31.63	14,556	342	Type III (n = 15/15)	TgH29146A
11	28.90	84,095	651	Atypical (n = 11/15)	TgH29141A
12	24.31	1,593,862	22,277	Atypical (Africa 1) (n = 15/15)	TgH29142A
13	32.09	10,860	170	Not amplified	TgH29144A
14	30.33	33,510	292	Not amplified	TgH29149A
15	29.12	72,911	497	Type II (n = 12/15)	TgH29145A
16	24.18	1,738,988	21,423	Type I	RH
17	23.17	3,325,211	54,983	Type I	RH
18	28.2	131,676	9357	Type II	ME49

tested samples, and the good precision was reflected by low CV (Figure 1D).

Cohort Analysis

High Correlation of Detection of *T. gondii* by ddPCR or qPCR Using the REP-529 Target

A good correlation was observed between ddPCR and qPCR (R^2 = 0.92) (Figure 2A), and the overall concordance between ddPCR and qPCR was 96.7% (n = 145/150). The Bland-Altman plot and linear regression showed very good agreement with no sample outside the ±1.96 SDs area and a high level of correlation (R^2 = 0.97) (Figure 2B). Among the 150 samples of the study cohort, 35 tested positive for *T. gondii*, 110 tested negative, and 5 were discordant between the ddPCR and qPCR results. Positive samples by qPCR included blood (n = 13/100), AH (n = 10/20), AF (n = 6/12), BAL (n = 3/7), and CSF (n = 3/7). The four quality controls were concordant and valid. The five discordant results were positive only by ddPCR, and they were based on only two positive droplet signals. One of them was a CSF sample (patient B) (Table 1) that yielded one positive replicate by ddPCR, as confirmed in an independent experiment. Interestingly, for this patient, positive blood and BAL test results were recorded approximately 7 days later. The other discordant samples were more likely to correspond to false-positive results by ddPCR, with an AH sample obtained from a patient with negative serologic test results for *T. gondii* and three blood samples with only one positive replicate.

Accurate Absolute Quantification of *T. gondii* Using α-Tubulin

The PCR primers for the α-tubulin gene amplified a 144-bp fragment and showed good specificity for *T. gondii* (Figure 3A). Indeed, all the negative samples using REP-529 also tested negative using α-tubulin. In addition, good linearity of the ddPCR signal was obtained using the α-tubulin newly designed target (R^2 = 0.99) (Figure 3B).

Among the 35 positive samples detected by ddPCR using REP-529, absolute quantification using α-tubulin was achievable for 15 samples (42.8%) from 11 patients. Three positive controls for the RH and ME49 reference strains were also successfully quantified. Quantification results by ddPCR along with clinical issues and *T. gondii* genotypes are summarized in Table 1. Coefficient of correlation between the α-tubulin ddPCR absolute quantification and

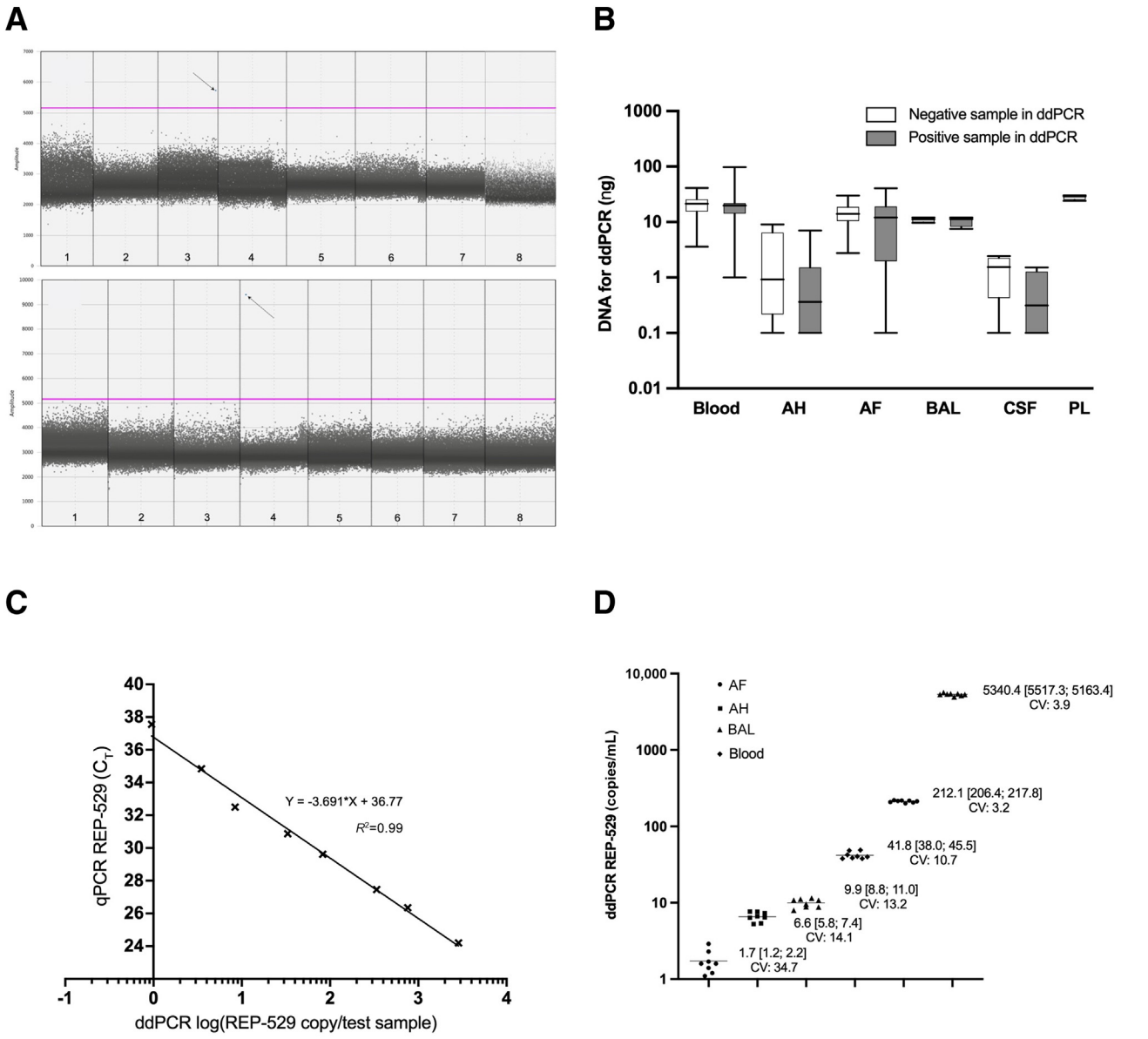

Figure 1 Validation of the droplet digital PCR (ddPCR) assays. **A:** Determination of ddPCR positivity and intensity thresholds for the REP-529 target. Bronchoalveolar fluid (BAL) (**top image**) and blood (**bottom image**). Each column represents a sample replicate numbered from one to eight, and the horizontal line represents the intensity threshold. The background signal is visible below the horizontal line. One droplet at most from one replicate of eight (indicated by the **arrows**) was observed above the selected intensity threshold in the two negative tested samples. Therefore, samples with at least two droplets were considered positive. **B:** DNA quantification according to the nature of the biological sample. The amount of DNA was estimated in the test DNA extract submitted to ddPCR. **C:** Correlation between ddPCR and real-time quantitative PCR (qPCR) using the REP-529 target and an RH strain. The regression line was performed with serial 2-, 5-, and 10-fold dilutions of a DNA extract from a reference strain of *Toxoplasma gondii* RH (type I). Each dilution was analyzed in quadruplicate in two independent experiments. The correlation was established between log (mean concentration in copies/5 μL of REP-529) obtained by ddPCR and the mean number of threshold cycle (C_T) values obtained by REP-529 qPCR. **D:** Repeatability of ddPCR using the REP-529 target. The repeatability of ddPCR was determined for six different biological samples [amniotic fluid (AF), BAL, blood, and aqueous humour (AH)] and replicated eight times in a single experiment. For each sample, the mean copy number per microliter in the reaction is indicated with the range in parentheses. CSF, cerebrospinal fluid; PL, placenta.

REP-529 qPCR cycle threshold results estimated regardless of the genotype was $R^2 = 0.91$ (Figure 3C). Compared with ddPCR absolute quantification, the parasite load was over-estimated using the REP-529 qPCR relative quantification method (Figure 3D). For the same patient strain but different clinical samples, the ratio between the theoretical parasite load by qPCR and the measured parasite load by ddPCR was close, as shown for patients A (46, 56, and 57), B (68 and 64), and F (67 and 43). Between the *T. gondii* strains, the ratio showed greater variations within and between

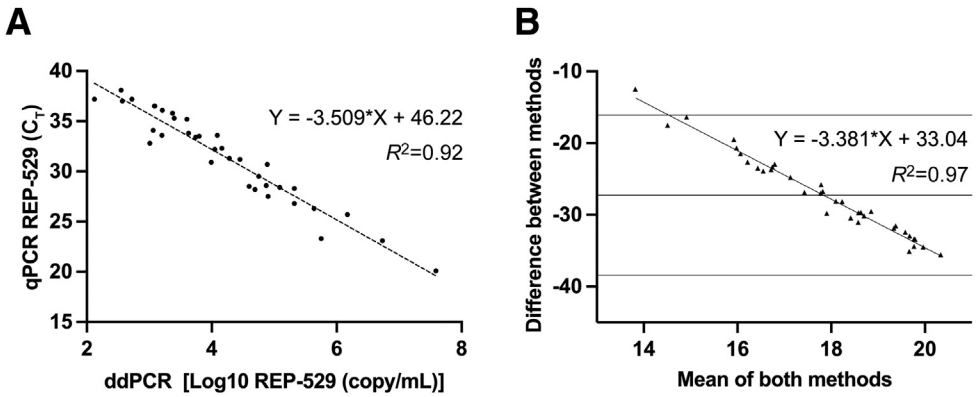

Figure 2 Cohort analysis by droplet digital (ddPCR) and real-time quantitative PCR (qPCR) using REP-529. **A:** Correlation between ddPCR and qPCR using REP-529. The regression was performed with all positive samples by qPCR and an RH reference strain. The correlation was established between log (mean concentration in copies per milliliter of REP-529) obtained by ddPCR and the mean number of threshold cycles (C_T) obtained by REP-529 qPCR. The obtained quantifications of REP-529 were only theoretical because of the repeated number of copies. **B:** Bland-Altman method comparison between ddPCR and qPCR using REP-529. The Bland-Altman plot is a graphical representation of the difference between the log (mean concentration in copies per milliliter of REP-529) obtained by ddPCR and the mean number of C_T values obtained by REP-529 for each sample, plotted against the mean of these two obtained values. The **dotted lines** represent the mean ± 1.96 SDs. $n = 35$ positive samples and 1 RH reference strain.

lineages. The largest variation was observed within the type II strains, with ratios from 14 to 160.

Discussion

This is the first study evaluating ddPCR for *T. gondii* detection and quantification in human clinical samples. A total of 150 samples were retrospectively studied. Good concordance and correlation were observed between the ddPCR and qPCR results for *T. gondii* detection using REP-529. Absolute quantification of *T. gondii* by ddPCR using an α-tubulin single-copy target was successful for 15 of 35 samples showing a parasite load ≥170 copies/mL of DNA eluate.

We have thus shown that ddPCR is a robust method with good linearity and good precision for *T. gondii* detection and quantification. This result is in agreement with other studies also reporting good linearity and precision results for other pathogen detection.[17,19] For *T. gondii* detection, ddPCR and qPCR shared a high performance, as indicated by the high level of agreement between the results, but ddPCR did not show clear superiority. Contrasting results were reported in terms of the higher accuracy of ddPCR compared with qPCR for parasitologic diagnosis. For example, a higher sensitivity of ddPCR compared with qPCR has been shown for all *Plasmodium* species detection of subpatent parasitemia samples in duplex ddPCR,[26] whereas another study observed a higher sensitivity of ddPCR to diagnose *Plasmodium falciparum* but equal sensitivity for *Plasmodium vivax*.[27]

This study presents a valuable assessment of the ddPCR method for *T. gondii* detection and quantification. The ddPCR results were compared with those of the qPCR reference method, which is currently used in the routine diagnosis of *T. gondii*. Both negative and positive samples for six different biological sample types that are commonly tested (blood, BAL, CSF, AF, placenta, and AH) were successfully evaluated. Among the positive samples, five different genotypes, including type I, type II, type III, and two atypical strains, were also successfully tested. Patients with *T. gondii* exhibited various typical clinical features, including reactivation, ocular toxoplasmosis, and congenital toxoplasmosis. Nevertheless, the study did not allow for the evaluation of the impact of absolute *T. gondii* quantification in the clinical management of *T. gondii* cases and in the prognosis of patients because of the low number of cases for each clinical entity. In addition, the quantification of parasite load using α-tubulin was achievable only for 43% of samples positive by qPCR REP-529 because of lower sensitivity of the single-copy target, require for absolute quantification. However, the lowest parasite load measured by α-tubulin ddPCR was 170 copies/mL of DNA eluate (ie, 0.17 copy/μL), which is a low limit of quantification, equivalent to that observed in the literature with other DNA targets. Although not previously reported for *T. gondii* using ddPCR, for *P. falciparum* protozoan parasite, the lowest measured parasite load was 0.7 copy/μL in mosquito midgut[28] and approximately 1 copy/μL in human blood.[27] For another protozoan parasite, *Trypanosoma cruzi*, it was 5 copies/μL,[29] whereas for *Aspergillus fumigatus* filamentous fungus, it was 0.2 and 0.3 copy/μL.[30] Interestingly, the limit of quantification of α-tubulin ddPCR was comparable to the limit of qPCR amplification of microsatellite markers of *T. gondii*, as a complete genotype could be obtained only above 292 copies/mL.

Diversity of strain genotypes did not appear to influence the accuracy of REP-529 qPCR detection and α-tubulin ddPCR quantification, as revealed by the high level of correlation between the ddPCR absolute quantification and

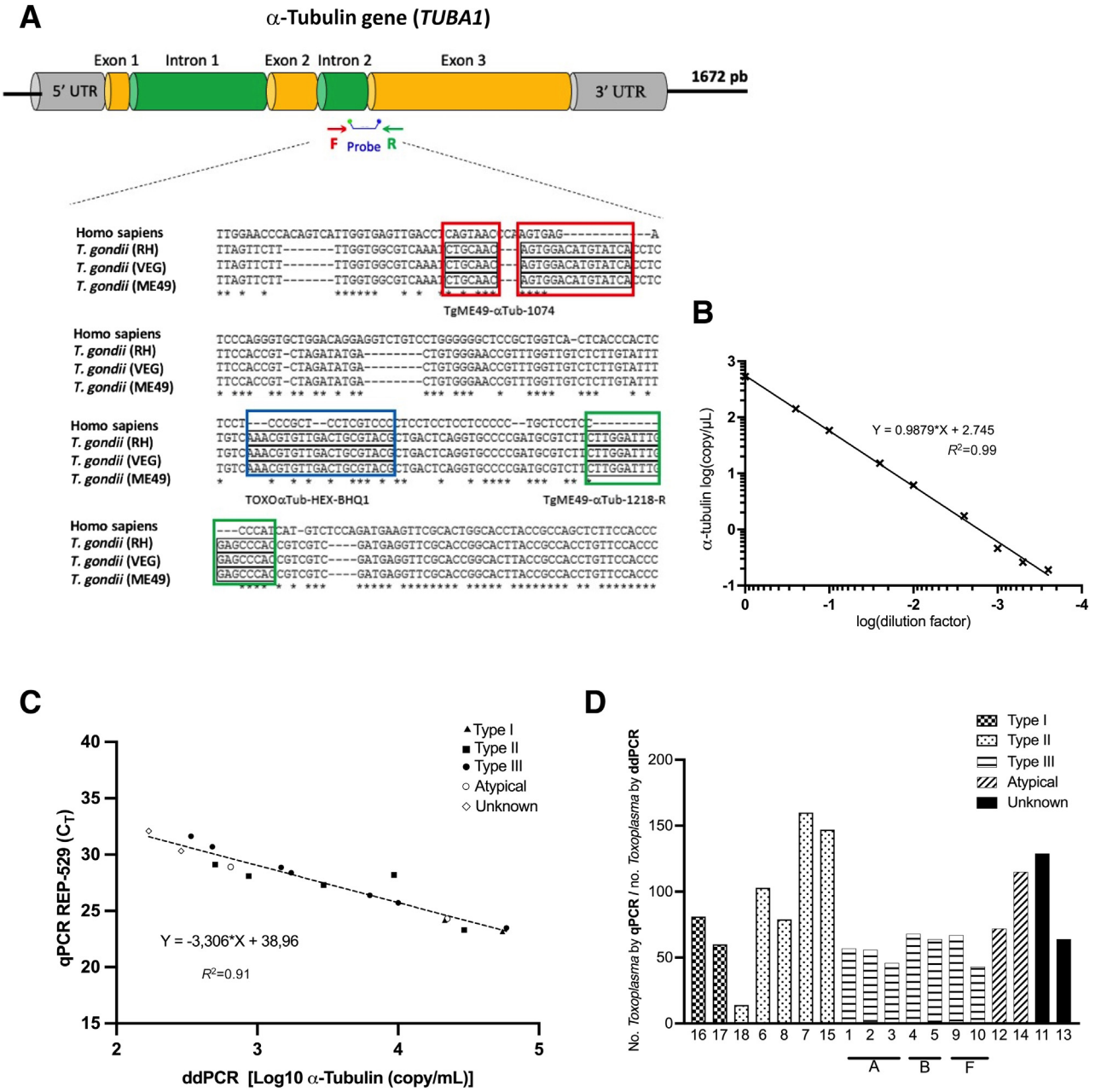

Figure 3 Droplet digital PCR (ddPCR) absolute quantification of *Toxoplasma gondii* using α-tubulin. **A:** Comparison of α-tubulin DNA sequences from human and *T. gondii*. Multiple alignments were performed using MUSCLE version 3.8[32] and the CLUSTAL algorithm among the α-tubulin complete gene from homo sapiens (*http://www.ncbi.nlm.nih.gov/genbank*; accession number X01703.1), the *T. gondii* RH strain (*http://www.ncbi.nlm.nih.gov/genbank*; accession number M20024.1), the *T. gondii* VEG type III strain (*http://www.ncbi.nlm.nih.gov/genbank*; accession number LN714501.1), and the *T. gondii* ME49 strain (*http://www.ncbi.nlm.nih.gov/genbank*; accession number NC_031479.1). The forward and reverse primers are located in intron 2 and exon 3 of the α-tubulin gene, respectively. The 3′ end of each primer was absent from the human α-tubulin gene sequence to avoid amplification of the human gene. The following names and sequences of the forward and reverse primers and probe are boxed in red, green, and blue, respectively, in the figure: TgME49-αTub-1074: 5′-CTGCAACAGTGGACATGTATCA-3′; TgME49-αTub-1218-R: 5′GTGGGCTCCAAATCCAAG-3′; and TOXO-αTub-HEX-BHQ1: AAACGTGTTGACTGCGTACG. **Dashes** indicate gaps in the sequence and the **asterisks** indicate that all the nucleotides in the corresponding column are identical. **B:** Standard curve of the ddPCR signal targeting the α-tubulin gene. Standard curve was generated with serial 2.5- and 10-fold dilutions of bronchoalveolar samples positive for *T. gondii* (sample 3, type III strain, TgH29139A) (Table 1). Each dilution was analyzed in quadruplicate. The correlation was established between the two log values (dilution factor of sample 3 and mean concentration in copies per microliter of α-tubulin obtained by ddPCR, respectively). **C:** Correlation between the α-tubulin ddPCR absolute quantification of *T. gondii* and REP-529 real-time quantitative PCR (qPCR). The correlation was established between log (mean concentration in copies per milliliter of α-tubulin) obtained by ddPCR and the mean number of threshold cycle (C_T) values obtained by REP-529 qPCR. The tested samples correspond to the samples shown in Table 1. Genotypes of the different *T. gondii* strains are indicated with different symbols. **D:** Comparison between *T. gondii* parasite loads estimated by REP-529 qPCR relative quantification and measured by α-tubulin ddPCR absolute quantification. The figure shows the ratio between the number of *T. gondii* parasites estimated by REP-529 qPCR based on a standard curve of an RH reference strain and the direct measurement of parasite load by α-tubulin ddPCR (Supplemental Tables S6 and S7 and Supplemental Fig. S1). Genotypes are indicated with different fill patterns. The numbers correspond to the samples, and the letters correspond to the patients, as shown in Table 1. $n = 18$ samples.

REP-529 qPCR C_T. A previous study also showed that the genotype did not impact the detection performance using the REP-529 target.[31] However, we demonstrated that the genotype impacted the quantification performance using REP-529 qPCR, probably because of the variable number of REP-529 copies. Nevertheless, the absolute number of copies of REP-529 per parasite strain could not be directly provided by absolute ddPCR quantification in this study because the target was multicopy. To assess the number of REP-529 copies per parasite using ddPCR, a prior restriction enzyme digestion is needed to separate each copy of REP-529 and to ensure the inclusion of a single copy of REP-529 by ddPCR reaction. In such conditions, the ratio between REP-529 gene concentration and α-tubulin gene concentration could provide the number of REP-529 copies per parasite.

Using our new absolute quantification assay targeting a specific region of α-tubulin of *T. gondii*, we demonstrated that ddPCR may be used if the qPCR REP-529 results show an approximately amplification C_T <32. From our perspective, it would be preferable to use qPCR as a screening tool for *T. gondii* and to perform ddPCR only for absolute quantification purposes of positive qPCR samples because of the higher complexity and lower throughput of ddPCR than qPCR. Absolute quantification for *T. gondii* could be useful for specific clinical scenarios, such as examining pathogen clearance after starting antitoxoplasma therapy in a severely immunocompromised patient who seems not to be responding adequately to treatment. Indeed, several concomitant syndromes, such as immune reconstitution inflammatory syndrome or the presence of another opportunistic infection, can be masking or misinterpreted as toxoplasma treatment failure. The assessment of parasitic burden could also be useful to evaluate the prognosis of the disease in the case of congenital toxoplasmosis and for transplant and hematologic immunocompromised hosts by providing a parasite load threshold correlating with prognosis, but further studies are needed. Finally, ddPCR could prove useful for laboratory practice for the precise quantification of standards. It also responds to the need for laboratory standardization of the quantification of *T. gondii*. This would greatly enhance comparability of parasite loads from different laboratories and facilitate multicentric studies.

Conclusions

ddPCR is an emerging method for the diagnosis and monitoring of microbial infections. This proof of concept shows the high performance of ddPCR for *Toxoplasma gondii* detection and absolute quantification for a large variety of clinical samples. We have shown that ddPCR offers a more accurate *T. gondii* quantification compared with qPCR. Thereby, molecular tools, such as ddPCR, open the door to new clinical applications by improving the therapeutic monitoring of toxoplasmosis and the molecular assessment of parasitic burden. To better assess the clinical impact of *T. gondii* absolute quantification, a multicentric, prospective study should now be performed, with samples from specific clinical cohorts, such as pregnant women and immunosuppressed patients after graft transplant in hematology or after solid organ transplant.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2023.03.006*.

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