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ORIGINAL PAPER

Is Immuno-PCR Better than ELISA Test for Detection of *Toxoplasma gondii* IgG Antibody?

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Abstract
Introduction IgG antibodies against *T. gondii* persist for years, and can act as a reliable serological biomarker for the diagnosis of previous exposure to this parasite. Hence, the current investigation was designed to compare diagnostic power of immuno-polymerase chain reaction (iPCR) and enzyme-linked immunosorbent assay (ELISA) methods for detection of *T. gondii* IgG antibody.
Methods Immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies against *T. gondii* were measured by the ELISA method in 81 participants. In addition, detection of acute and chronic toxoplasmosis was performed via the ELISA IgG avidity. The set-up of iPCR was carried out and then, serum IgG of subjects were detected using the iPCR method.
Results Of 81 samples, 4 (4.9%) and 30 (37%) cases were be found positive for IgM and IgG against *T. gondii* in the ELISA method, respectively. Moreover, of 81 specimens, 42 (51.9%) and 39 (48.1%) samples had low-avidity IgG and high-avidity IgG by the IgG avidity kit, respectively. While, 59 (72.8%) of 81 samples were detected positive using the iPCR technique. Kappa (κ) value coefficient, between the iPCR and ELISA (for IgG) showed a strong agreement (0.360, p value < 0.001). A value of 0.25 I.U./ml for serum IgG [area under curve (AUC) = 0.720 (CI = 0.613–0.827); p = 0.002] was the cut-off value to differentiating between positive and negative toxoplasmosis (with sensitivity 66.0% and specificity 60.0%).
Conclusion Our findings indicated despite a strong agreement shown between iPCR and ELISA methods, the diagnostic power of iPCR technique was more sensitive than ELISA test for detection of *T. gondii* IgG antibody. However, more complementary investigations are widely needed in this regard.

Keywords Immuno-PCR · ELISA · *Toxoplasma gondii* · Sensitivity · Specificity

Introduction

Toxoplasma gondii (*T. gondii*) is a protozoan that results in a zoonotic disease known toxoplasmosis [1]. Sero-epidemiological studies have shown that the parasite antibodies are in serum of one-third of the world adult population [2]. The clinical signs of this disease lead to a substantial disease burden in humans worldwide, including ocular, cerebral, and congenital toxoplasmosis [1, 3]. Furthermore, this parasite can result in clinical manifestations in

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animal hosts of *T. gondii*, leading to lesser welfare for the affected hosts as well as major losses in livestock industry [1]. However, signs in people with healthy immune system are transient and mild which include swollen lymph and mild fever glands [2].

Fruits, vegetables, water and soil contaminated with oocysts play a key role in both humans and animals toxoplasmosis [4–6]. In both intermediate (human) and definitive hosts, can also be infected through the congenital transmission of tachyzoites and the consumption of raw meat of intermediate hosts containing tissue cysts [7–9]. Congenital toxoplasmosis may result in some disorders in the fetus, such as abortion, hydrocephalus, cerebral calcifications, chorioretinitis, lymphadenopathy and microcephaly. It is important to be noted toxoplasmosis may even cause mortality in immunocompromised humans [4, 10, 11].

The diagnosis of this parasite can perform through indirect and direct routes. Direct tests detect parasitic DNA using polymerase chain reaction (PCR), or the diagnosis of parasitic stages by microscopic examination and/or isolation of the parasite by bioassays [12, 13]. These methods are highly specific, but, since they rely on the presence of the parasite in the sample; they often have limitations in test sensitivity [13]. Whereas, indirect tests are related to the serological identification of parasite-specific antibodies and are often used as the common method for the detection of this disease in both humans and animals [14, 15]. IgG antibodies (unlike IgM antibodies) against *T. gondii* persist for years, and can act as a reliable serological biomarker for the diagnosis of previous exposure to this parasite [13, 16, 17]. Several serological methods, including the modified agglutination test (MAT), direct agglutination test (DAT), Sabin–Feldman dye test (DT), indirect immunofluorescence test (IFAT), Western blot (WB), latex agglutination test (LAT), indirect hemagglutination assay (IHA), and enzyme-linked immunosorbent assay (ELISA) have been applied to identify *T. gondii*-specific antibodies in both humans and animals [14, 18]. ELISA is widely used for the diagnosis of exposure to this protozoan. This method is the most reliable, economical and practical among all these techniques [13, 19]. However, ELISA technique has some limitations [20, 21].

Immuno-PCR (iPCR) is a molecular technique that combines the versatility of enzyme immunoassays (EIA) with the amplification power of PCR which it was introduced by Sano *et al.* [22] in 1992 and leading to improved diagnostic sensitivity [23]. The iPCR method can improve detection limit of the EIA up 100- to 10,000-fold [24]. This technique has mainly been applied to identify bacterial antigens [25], bacterial toxins [26], viral antigens [27], parasitic antibodies and antigens [28], and/or prions [29]. To the best our knowledge, there are no studies about the power of iPCR technique in detection of *T. gondii* IgG antibody. Hence, the current investigation was designed to compare diagnostic power of iPCR and ELISA methods for detection of *T. gondii* IgG antibody.

Materials and Methods

Ethical Statement

The protocol of the study was reviewed and approved by the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences (Approval No. IR.AJUMS.REC.1398.418). Before sampling, an informed written consent was obtained from all participants.

Participants and Sampling

Among the subjects who referred to Ahvaz hospitals for toxoplasmosis test from July 2020 to January 2021, eighty-one subjects with no history of any diseases were enrolled in the present study. At first, characteristics of subjects, such as sex and age, were obtained and then, two milliliters peripheral blood sample was obtained from each subject. Serum samples were isolated, frozen, and kept at −80 °C until analysis.

ELISA (Enzyme-Linked Immunosorbent Assay)

The anti-toxoplasma immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies were detected using the commercial ELISA kits (Vircell, Granada, Spain) according to the manufacturer's instructions. Moreover, to differentiate acute and past infections, the semi-quantitative analysis of IgG anti-*T. gondii* IgG was carried out by Avidity *T. gondii* IgG quantitative (NovaTec Immundiagnostica GmbH, Dietzenbach, Germany) according to the manufacturer's instructions [30].

Immuno-PCR

Preparation of DNA

Table 1 shows the designed DNA and primers used for iPCR. We used the nested PCR method to detection of targeted DNA. First DNA fragment with length of 250 bp was synthesized that sequence was related to non-parasite plant DNA. The first PCR was performed using the designed DNA and primers of outer R and outer biotinylated F. Two PCRs were conducted in a final volume of 20 µl [6 µl of deionized distilled water, 1 µl of each of forward and reverse primers, 10 µl of Master Mix RED (Ampliqon-Biomol, Hamburg, Germany), and 2 µl of template DNA] in 35 cycles. The first cycle was carried out at 95° C for five minutes, denaturation at 95° C for 30 s, annealing at 58° C for 30 s and extension and the final cycle (extension) at 72° C for 30 s and 7 min,

Table 1 The oligonucleotide and primers used for iPCR

Primers/template	Sequences
Outer biotinylated F	AATCACATGGTCGGGATCCGC
Outer R	AGCTTTGCCAGCACCAAGAC
Inner F	GTCCAACCATGGCGGTGATG
Designed DNA	AATCACATGGTCGGGATCCGCTGCATAGGAGCTGGTTATGTG GGTGGTCCAACCATGGCGGTGATGGCTCTTAAGTGTCCT GAGATTGAAGTAGTCGTTGTGGATATCTCTGAACCAAGG ATCAATGCTTGGAACAGTGATAGGCTTCCTATTTACGAG CCGGGATTGGAAGATGTGGTGAAACAATGCAGAGGGAAA AACCTCTTCTTTAGCACAGACGTGGAGAAACATGTATTT GAGAGTGATATTGTATTTGTCTCAGTTAACACTCCAACC AAAACACAAGGTCTTGGTGCTGGCAAAGCT

respectively. After amplification, the PCR products were purified by the serum/plasma kit (Qiagen GmbH, Hilden, Germany) and stored at – 80 °C until next experiment.

iPCR

The ELISA plates coated with *T. gondii* antigens containing 1 µg/ml RH strain, biotin-conjugated monoclonal antibody/anti-human biotin-conjugated antibody were purchased from Pishtazteb Co. (Pishtazteb, Iran), lifespan biosciences, Inc. (LSBio, USA) and Padza Co. (Padza, Iran), respectively. Table 2 indicates serial dilution used for the best dilution of streptavidin (Thermo Fisher, USA) and biotin-conjugated monoclonal antibody/anti-human biotin-conjugated antibody. At first, the ELISA plate was incubated with 100 µl of the serial dilution of biotin-conjugated monoclonal antibody at 37 °C for 1 h and washed five times by the wash solution (PBS containing 0.05% Tween 20 five times). For serum IgG obtained from the subjects, the ELISA plate was incubated with 100 µl of diluted serum (1:100) and then, 50 µl of the serial dilution of anti-human biotin-conjugated antibody was added to any wells and incubated at 37 °C for 1 h. After washing, 50 µl of free streptavidin was added to the wells and incubated at 37 °C for 1 h and washed five times by the wash solution. In the next stage, 50 µl of the PCR products

Table 2 The checkerboard used for the best dilution of streptavidin (shown in red) and biotin-conjugated monoclonal antibody/anti-human biotin-conjugated antibody (shown in black). The proper dilution was 1:256 for the streptavidin (shown in red) as well as 1:256,000 and 1:32,000 for the biotin-conjugated monoclonal antibody and anti-human biotin-conjugated antibody (shown in black), respectively

	1	2	3	4	5	6	7	8	9	10	11 (Negative control)
A	1/4000 1/2	1/8000 1/2	1/16000 1/2	1/32000 1/2	1/64000 1/2	1/128000 1/2	1/256000 1/2	1/512000 1/2	1/1024000 1/2	1/2048000 1/2	1/2
B	1/4000 1/4	1/8000 1/4	1/16000 1/4	1/32000 1/4	1/64000 1/4	1/128000 1/4	1/256000 1/4	1/512000 1/4	1/1024000 1/4	1/2048000 1/4	1/4
C	1/4000 1/8	1/8000 1/8	1/16000 1/8	1/32000 1/8	1/64000 1/8	1/128000 1/8	1/256000 1/8	1/512000 1/8	1/1024000 1/8	1/2048000 1/8	1/8
D	1/4000 1/16	1/8000 1/16	1/16000 1/16	1/32000 1/16	1/64000 1/16	1/128000 1/16	1/256000 1/16	1/512000 1/16	1/1024000 1/16	1/2048000 1/16	1/16
E	1/4000 1/32	1/8000 1/32	1/16000 1/32	1/32000 1/32	1/64000 1/32	1/128000 1/32	1/256000 1/32	1/512000 1/32	1/1024000 1/32	1/2048000 1/32	1/32
F	1/4000 1/64	1/8000 1/64	1/16000 1/64	1/32000 1/64	1/64000 1/64	1/128000 1/64	1/256000 1/64	1/512000 1/64	1/1024000 1/64	1/2048000 1/64	1/64
G	1/4000 1/128	1/8000 1/128	1/16000 1/128	1/32000 1/128	1/64000 1/128	1/128000 1/128	1/256000 1/128	1/512000 1/128	1/1024000 1/128	1/2048000 1/128	1/128
H	1/4000 1/256	1/8000 1/256	1/16000 1/256	**1/32000** **1/256**	1/64000 1/256	1/128000 1/256	**1/256000** **1/256**	1/512000 1/256	1/1024000 1/256	1/2048000 1/256	1/256

was added to any wells and incubated at 37 °C for 1 h and washed eight times. Afterward, fifty µl of BamH1 solution (Thermo Fisher, USA) that containing 0.5 µl BamH1 enzyme, 5 µl BamH1 buffer and 44.5 µl sterile distilled water was added to the wells. Then, 50 µl of any wells was isolated and amplified using the primers of outer R and inner F (Table 1). Finally, the PCR products were visualized on 1.5% agarose as described previously [30]. For proper dilutions, no bands were observed during the electrophoresis (Fig. 1).

Fig. 1 The products of PCR electrophoresed on 1.5% agarose. For proper dilutions, no bands were observed during the electrophoresis. Line 1: Ladder 100 bp, Line 2–11: Improper dilutions, Line 12: Proper dilution: Streptavidin (1:256), Ab monoclonal (1:32,000), Line 13: Improper dilutions, Line 14: Negative control

Ipcr-Based Detection

As shown in Table 2, the best dilution of streptavidin and biotin-conjugated monoclonal antibody/anti-human biotin-conjugated antibody was confirmed using the checkerboard. The proper dilution was 1:256 for the streptavidin as well as 1:256,000 and 1:32,000 for the biotin-conjugated monoclonal antibody and anti-human biotin-conjugated antibody, respectively. For detection of serum IgG, briefly, the ELISA plate was incubated with 100 µl of diluted serum (1:100) and then, 50 µl of a dilution (1:32,000) of anti-human biotin-conjugated antibody was added to any wells. After adding 50 µl of free streptavidin, 50 µl of the PCR products was added to any wells. Afterward, BamH1 solution was subsequently added to the wells and then, 50 µl of any wells was amplified using the PCR technique. Finally, the products of PCR were electrophoresed on 1.5% agarose as described previously [30]. The existence of bond in the electrophoresis was considered as IgG-positive (Fig. 2).

Statistical Analyses

The data were analyzed using IBM SPSS version 21 (SPSS Inc., Chicago, IL, USA). Categorical data were presented as frequency and percentage. The difference between the ELISA test and iPCR was evaluated using the Chi-square test. Moreover, concordance between the ELISA test and iPCR was assessed by the kappa test. The receiver operator characteristic (ROC) curve analysis was conducted for evaluate the ability of serum IgG in distinguishing the positive and negative subjects. Significance was $p < 0.05$.

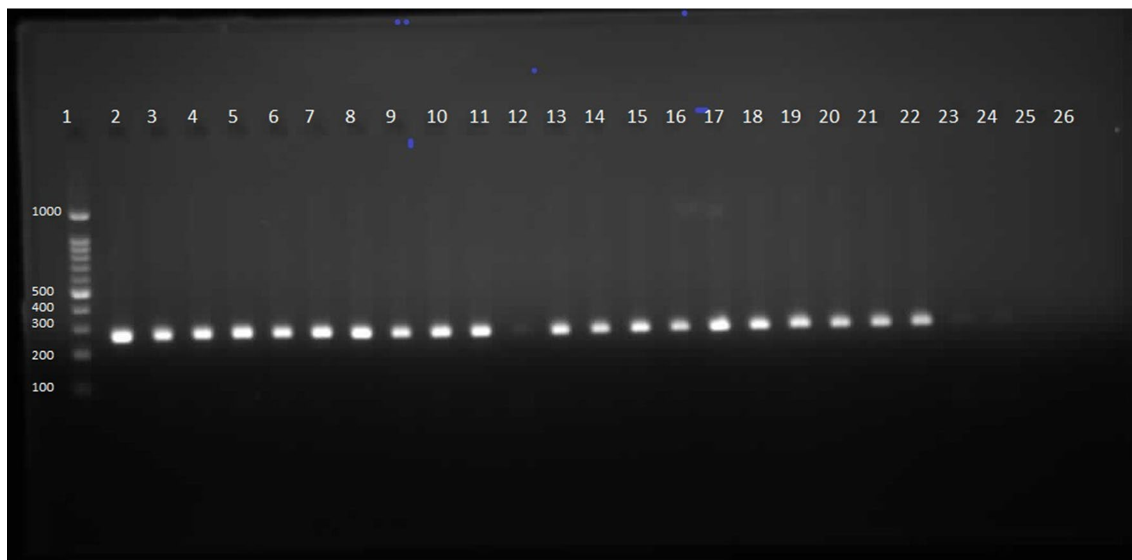

Fig. 2 The products of PCR electrophoresed on 1.5% agarose. The existence of bond in the electrophoresis was considered as IgG-positive. Line 1: Ladder 100 bp, line 2: Positive control, lines 3–11: Positive samples, line 12: Negative sample, lines 13–22: Positive samples, lines 23–25: Negative samples and line 26: Negative control

Results

Table 3 demonstrates the comparison of ELISA, iPCR and IgG avidity methods in the detection of *T. gondii*. Of 81 samples, 4 (4.9%) and 30 (37%) cases were be found positive for IgM and IgG against *T. gondii* in the ELISA method, respectively. Moreover, of 81 specimens, 42 (51.9%) and 39 (48.1%) samples had low-avidity IgG and high-avidity IgG by the IgG avidity kit, respectively. Also, 59 (72.8%) of 81 samples were detected positive using the iPCR technique (Table 3). Kappa (κ) value coefficient, between the iPCR and ELISA (for IgG) showed a strong agreement (0.360, p value < 0.001).

In addition, Table 4 indicates the diagnostic power of ELISA (for IgG) test in comparison with the iPCR method. The comparison of the results of iPCR and ELISA showed that the sensitivity of ELISA test was 50.85% (CI = 37.50–64.11), while the specificity was 100% (CI = 84.56–100) for this method. In addition, the positive and negative predictive values were observed 100% and 43.14% (CI = 36.92–49.58) for the ELISA (for IgG) method. In total, the accuracy of this test was 64.20% (CI = 52.77–74.55) in comparison with the iPCR method. On the other hand, Fig. 3 shows the receiver operating characteristic (ROC) curve for serum IgG. A value of 0.25 I.U./ml for serum IgG [area under curve (AUC) = 0.720 (CI = 0.613–0.827); $p = 0.002$] was the cut-off value to differentiating between positive and negative toxoplasmosis (with sensitivity 66.0% and specificity 60.0%).

Discussion

Developing cost-effective, early, and rapid diagnostic tests is necessary for prevention, control, early screening and treatment of toxoplasmosis [31]. ELISA is widely used for the diagnosis of exposure to *T. gondii*. This method is the most reliable, economical and practical [13, 19]. However, ELISA technique has some limitations, such as time-consuming, laborious, expensive as well as requires special equipment and materials and expert operators [20, 21]. Nowadays, interests have widely been elevated in immuno-diagnosis of parasitic and microbial diseases using novel methodologies with high specificity and sensitivity [23]. For this reason, the current investigation was designed to compare diagnostic power of iPCR and ELISA methods for detection of *T. gondii* IgG antibody. We have shown the first application of this technique for identification of IgG antibody against *T. gondii* in human serum.

Table 3 The comparison of ELISA, iPCR and IgG avidity methods in the detection of *T. gondii*

Methods	Results	Frequency (%)
ELISA (for IgM)	Negative	77 (95.1)
	Positive	4 (4.9)
ELISA (for IgG)	Negative	51 (63)
	Positive	30 (37)
IgG Avidity	Primery infection (with low-avidity IgG)	42 (51.9)
	Past infection or reinfection (with high-avidity IgG)	39 (48.1)
iPCR	Negative	22 (27.2)
	Positive	59 (72.8)

Table 4 The diagnostic power of ELISA test in comparison with iPCR

Properties	Value	95% CI
Sensitivity	50.85%	37.50% to 64.11%
Specificity	100.00%	84.56% to 100.00%
Positive Likelihood Ratio	–	–
Negative Likelihood Ratio	0.49	0.38 to 0.64
Disease prevalence	72.84%	61.81% to 82.13%
Positive Predictive Value	100.00%	–
Negative Predictive Value	43.14%	36.92% to 49.58%
Accuracy	64.20%	52.77% to 74.55%

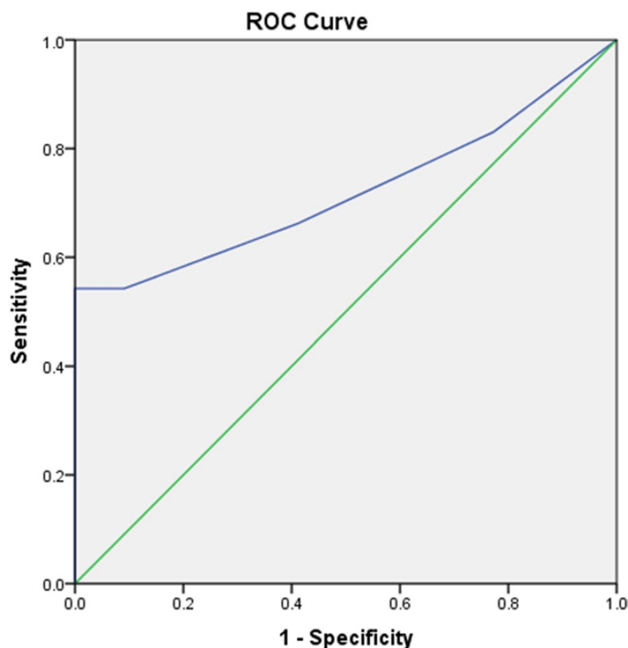

Fig. 3 Receiver operating characteristic (ROC) curve for serum IgG. A value of 0.25 I.U./ml [area under curve (AUC) = 0.720 (CI = 0.613–0.827); $p = 0.002$] was the cut-off value to differentiating between positive and negative toxoplasmosis (with sensitivity 66.0% and specificity 60.0%)

Our findings indicated despite a strong agreement shown between iPCR and ELISA methods, the diagnostic power of iPCR technique was more than ELISA test for detection of *T. gondii* IgG antibody. The iPCR is a widely powerful technique in identification and measurement of low abundance biomarkers (antigens and/or antibodies) which exist in biological specimens (e.g., urine and serum) [22, 32]. This technique acts as a link between signal amplification and immuno-reaction [22, 32] and it can improve detection limit of the EIA up 100- to 10,000-fold [24]. However, strategies available to link the DNA and the antibody include covalent conjugation through chemical cross-linkers [33] as well as non-covalent conjugation such as linking via streptavidin–biotin [22]. In the present study, we used from non-covalent conjugation. The tetrameric structure of streptavidin results in the formation of heterogeneous antibody–DNA conjugates that may diminish the reproducibility of this technique [32]. Although, chemical cross-linker reacts with the lysine/cysteine residues, and the changes may affect binding affinity of the antibody [32].

To the best our knowledge, there are no studies about the power of iPCR technique in detection of *T. gondii* IgG antibody. However, several investigations have evaluated this method in detection of other biomarkers. In this regard, Xiaohua *et al.* [34] in 2010 indicated that the diagnostic threshold for ricin in feces and serum was 10 ng/mL and 1 pg/mL by ELISA and iPCR, respectively. So, the iPCR technique was 10,000-fold more sensitive than ELISA test. In another study, Monteiro *et al.* [35] examined detection of *Helicobacter pylori* in human stool using magnetic iPCR. They showed that of the 57 *H. pylori*-negative and 47 *H. pylori*-positive patients, 66 (no false positive) and 38 (giving 9 false negatives) were negative and positive by the iPCR assay, respectively. The specificity and sensitivity for this protocol were observed 100% (95% CI, 92.1 to 100) and 80.9% (95% CI, 66.3 to 90.4) as well as predictive values for a negative or a positive result were 86.4% (95% CI, 75.2 to 93.2) and 100% (95% CI, 88.6 to 100), respectively. Moreover, Zhang *et al.* [26] showed that EIA identified 1 ng/ml of purified Stx2 (Shiga toxin-producing *Escherichia coli*), compared to 10 pg/ml Stx2 detected via PCR assay. They reported that iPCR was a highly specific and sensitive test for the diagnosis of trace amounts of the toxin. In addition, a type of iPCR has recently been introduced for detection of coronavirus disease 2019 (COVID-19) [36].

Conclusion

In the present study, we performed the set-up of iPCR to compare diagnostic power of iPCR and ELISA methods for detection of *T. gondii* IgG antibody for the first time. Our findings indicated despite a strong agreement shown between iPCR and ELISA methods, the diagnostic power of iPCR technique was more sensitive than ELISA test for detection of *T. gondii* IgG antibody. However, more complementary investigations are widely needed in this regard.

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Availability of Data and Materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no conflict of interest, financial or otherwise.

Human and Animal Rights No animals were used in this study. All human research procedures were in accordance with the standards set forth in the Declaration of Helsinki https://www.wma.net/policiespost/wmadeclarationof-helsinki-ethical principles for medical research-involving animals subjects/ principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

Consent for Publication Not applicable.

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