Isothermal Recombinase Polymerase Amplification Assay Applied to the Detection of Group B Streptococci in Vaginal/Anal Samples

Rana K. Daher,1,2 Gale Stewart,1 Maurice Boissinot,1 and Michel G. Bergeron1*

BACKGROUND: Group B streptococcal infections are the leading cause of sepsis and meningitis in newborns. A rapid and reliable method for the detection of this pathogen at the time of delivery is needed for the early treatment of neonates. Isothermal amplification techniques such as recombinase polymerase amplification have advantages relative to PCR in terms of the speed of reaction and simplicity.

METHODS: We studied the clinical performance of recombinase polymerase amplification for the screening of group B streptococci in vaginal/anal samples from 50 pregnant women. We also compared the limit of detection and the analytical specificity of this isothermal assay to real-time PCR (RT-PCR).

RESULTS: Compared to RT-PCR, the recombinase polymerase amplification assay showed a clinical sensitivity of 96% and a clinical specificity of 100%. The limit of detection was 98 genome copies and the analytical specificity was 100% for a panel of 15 bacterial and/or fungal strains naturally found in the vaginal/anal flora. Time-to-result for the recombinase polymerase amplification assay was <20 min compared to 45 min for the RT-PCR assay; a positive sample could be detected as early as 8 min.

CONCLUSIONS: We demonstrate the potential of isothermal recombinase polymerase amplification assay as a clinically useful molecular diagnostic tool that is simple and faster than PCR/RT-PCR. Recombinase polymerase amplification offers great potential for nucleic acid–based diagnostics at the point of care.

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Streptococcus agalactiae or group B Streptococcus (GBS)3 is one example of severe infectious diseases that cause sepsis and meningitis in neonates (1). Between 20% and 40% of pregnant women may be colonized by GBS (2). Vertical transmission from mother to the newborn accounts for 75% of neonatal GBS colonization, and approximately 1% of these infants will develop early-onset GBS sepsis (3). The 2002 CDC guidelines recommend prenatal screening of pregnant women at 35–37 weeks of gestation, with vaginal/anal culture and selective administration of intrapartum antibiotic prophylaxis to infected women for a minimum of 4 h (4). Implementation of these guidelines has reduced the incidence of early-onset neonatal GBS disease from 1.5 to 0.3 per 1000 live births. The current gold standard method for GBS detection, culturing a vaginal/anal swab in selective medium broth (5), requires at least 48 h for GBS identification (3). The culture method also has a lower clinical sensitivity than a molecular assay (6). Furthermore, negative culture results for prenatal women may turn positive after labor (4). Thus, in some cases the antibiotic treatment for GBS colonization may be either inappropriate or unnecessary (6). A screening test that could detect women carrying GBS during labor could eliminate the need for prenatal screening at 35–37 weeks and reduce the risk of antibiotic prophylaxis for noncolonized women (3, 6). The screening test must include a sample preparation method that ensures high recovery of nucleic acids and sufficient purity of clinical samples to control inhibitors (7, 8). Comparative performance studies of automated extraction platforms have demonstrated the direct correlation between the performance of the extraction system and the imprecision of a molecular assay (7–9).

Real-time PCR (RT-PCR) is useful for rapid and accurate GBS screening in pregnant women at the time
of delivery (10, 11). US Food and Drug Administration (FDA)-approved molecular tests based on RT-PCR, such as GBS GeneXpert, BD MAX GBS, and BD the GeneOhm™ StrepB assay can detect GBS and replace the standard culture (6, 10–15). PCR is useful, one limitations is the need for a thermocycler that ensures rapid heating/cooling temperature cycles (16). Efforts to overcome PCR limitations have identified alternatives such as isothermal amplification techniques that do not require thermal cycling but instead rely on enzymatic activity for DNA/RNA synthesis (17). Huy et al. (18) developed an isothermal amplification assay based on loop-mediated amplification (LAMP) for the screening of GBS along with 3 other bacterial pathogens responsible for meningitis. Recently, the FDA cleared the Meridian Illumigene GBS DNA amplification assay, which uses LAMP to detect the pathogen in clinical samples and produces results in less than 1 hour (19).

Among the existing isothermal amplification techniques, recombinase polymerase amplification (RPA) operates between 25 and 42 °C. It features a primer–recombinase complex in association with single-strand binding proteins (SSBs) to substitute heat cycles during the amplification process. RPA does not require an initial heat denaturation step to unwind dsDNA (double-stranded DNA), because the primer–recombinase complex along with SSBs ensure the unwinding the stability of nucleic acid during the various exchange processes (20). In addition, RPA primer design is simple and does not rely on sophisticated sequence design or on melting temperature considerations (21).

Owing to these advantages, we chose to evaluate an RPA assay as a potential point-of-care (POC) diagnostic method. Prior studies have applied RPA to aptamer ssDNA (single-stranded DNA) (22), positive control DNA (23), purified DNA (20, 24–28), human genomic DNA (20, 26, 29), double-stranded PCR product (30, 31), plasmid DNA (27, 28), cDNA (25, 26), and spiked plasma (25, 32), but none have tested RPA with human samples. In contrast, we used actual human samples in our study.

Materials and Methods

Table 1. Bacterial/fungal strains tested for specificity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATCC no.</th>
<th>RT-RPA result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus agalactiae</td>
<td>12973</td>
<td>+</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>19435</td>
<td>−</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>19615</td>
<td>−</td>
</tr>
<tr>
<td>Bifidobacterium breve</td>
<td>15700</td>
<td>−</td>
</tr>
<tr>
<td>Anaerococcus lactolyticus</td>
<td>51172</td>
<td>−</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>19606</td>
<td>−</td>
</tr>
<tr>
<td>Bordetella pertussis</td>
<td>9797</td>
<td>−</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>25285</td>
<td>−</td>
</tr>
<tr>
<td>Mobiluncus mulleris</td>
<td>35243</td>
<td>−</td>
</tr>
<tr>
<td>Mobiluncus curtisii subsp. holmesii</td>
<td>35242</td>
<td>−</td>
</tr>
<tr>
<td>Trichomonas vaginalis</td>
<td>30001</td>
<td>−</td>
</tr>
<tr>
<td>Gardnerella vaginalis</td>
<td>14019</td>
<td>−</td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>VR–9028</td>
<td>−</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>43069</td>
<td>−</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>10231</td>
<td>−</td>
</tr>
<tr>
<td>Candida kruzei</td>
<td>34135</td>
<td>−</td>
</tr>
</tbody>
</table>

To determine the analytical specificity of real-time RPA (RT-RPA) assay, purified genomic DNA from a variety of bacterial/fungal strains (Table 1) naturally found in vaginal/anal samples were tested at 0.1 ng of DNA per reaction (33).

Vaginal/anal samples were collected from women in labor in accordance with a protocol approved by the ethical review board of the Centre de recherche du CHU de Québec. The vaginal/anal samples were then eluted in Copan Transystem™ Liquid Stuart medium (Copan Italia International), mechanically lysed, and prepared according to the manufacturer’s protocol for the BD GeneOhmTM Strep B assay kit. The lysates were screened for GBS for a first time by the RT-PCR BD GeneOhmTM Strep B assay kit and the results recorded. These lysates were then stored at −80 °C. Fifty of the frozen lysates were chosen for the comparative study between RT-PCR and RT-RPA.

An RPA assay internal amplification control (ICRPA) was included in the RT-RPA assay with the clinical lysates. ICRPA was a lysate of Bacillus atrophaeus spores. The latter were mechanically lysed with glass beads according to a homemade protocol that consisted of vigorous shaking of a 50-μL spore dilution on a vortex-type mixer for 5 min followed by a brief centrifugation. The lysates were used at 1000 spores for each RT-RPA amplification reaction (34).
For the RT-PCR assay, the primers and probes for the target and internal control sequences were supplied in the BD GeneOhm™ Strept B assay kit (35).

For the RT-RPA assay, the primers and probes (Table 2) were designed according to the instruction manual using the TwistAmp™ exo kit (TwistDx). The forward and reverse primers as well as the probe for GBS were designed in silico to be specific for the cAMP factor (\(c^b\)) gene and generated a 234-bp product. The primers and probe for IC\textsubscript{RPA} (Table 2) were designed in silico to be specific for the \textit{atpD} (ATP synthase FO\textsubscript{1} subunit beta) gene of \textit{B. atrophaeus}. The generated product of the IC\textsubscript{RPA} was 227 bp long.

### RT-PCR ASSAY

The RT-PCR procedure was performed according to the manufacturer’s protocol for the BD GeneOhm Strept B assay kit. The amplification/detection of the samples was performed in a SmartCycler® instrument according to the SmartCyclerDx Software operator manual.

### RT-RPA ISOTHERMAL AMPLIFICATION ASSAY

The real-time isothermal amplification procedure was performed using the reagents and protocols from the TwistAmp™ exo kit.

To evaluate the LOD and analytical specificity of the simplex assay, RT-RPA was performed in a 25-µL total volume. First, a mastermix consisting of 29.5 µL rehydration buffer, 11.2 µL nuclease-free water, 2.1 µL forward primer (Sag59a) (420 nmol/L), 2.1 µL reverse primer (cfbSag263) (420 nmol/L), and 0.6 µL RT-RPA probe (cfbSag159-E1-A1) (120 nmol/L) were added to 1 freeze-dried reagent pellet and vortex mixed. The latter mix was separated into 2 reaction volumes of 22.75 µL each. One microliter of template DNA at the appropriate concentration was added to each reaction: template DNA for LOD analysis was GBS genomic DNA at a concentration corresponding to each of the serial dilutions. For analytical specificity analysis, template DNA was the genomic DNA of each of the chosen bacterial/fungal strains as well as GBS at 0.1 ng per amplification reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 1.25 µL of magnesium acetate solution (14 mmol/L) to each reaction. The reaction tubes were vortex-mixed briefly and then incubated in a Rotor-Gene™ 6000 (Corbett Life Science,) at 39 °C for 30 min. Fluorescence measurements were taken every 30 s. For the LOD analysis, the number of replicates for each dilution was at least 12. The number of replicates for the analytical specificity analysis was 4.

To evaluate the LOD of the multiplex assay, RT-RPA was performed in a 50 µL total volume. The reaction mixture was made of 29.5 µL rehydration buffer, 11.2 µL nuclease-free water, 1.05 µL of each forward primer (Sag59a and ABgl158b) (420 nmol/L), 1.05 µL of each reverse primer (cfbSag263 and ABgl345c) (420 nmol/L), and 0.3 µL of each RT-RPA probe (cfbSag159-E1-A1 and ABgl220-E1-M2) (120 nmol/L). One freeze-dried reagent pellet included in the kit was added to every reaction tube and vortex mixed. One microliter of GBS genomic DNA template at a concentration corresponding to each of the serial dilutions and 1 µL of \textit{B. atrophaeus} genomic DNA of 1000

### Table 2. Primers and probes used in RT-RPA assay.

<table>
<thead>
<tr>
<th>Name\textsuperscript{a}</th>
<th>Sequence (5’-3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sag59a</td>
<td>TTTACCAAGCCTAGTTAAGATCAGTCGATC</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>cfbSag263</td>
<td>ACTGTCGAGGCGGCGACGAATGGATC</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>cfbSag159-E1-A1</td>
<td>GTGCCAGAAGTAGATCCATCCTGGTGAATAA (FAMdT)(THF)(BHQ1dT)CAAAGATA</td>
<td>Biosearch Technologies</td>
</tr>
<tr>
<td>ABgl158b</td>
<td>AGAGGTCGATTCATTAGGGCAGATCT</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>ABgl345c</td>
<td>ACGGAGCTGCTGTAATCGGATCTTTC</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>ABgl220-E1-M2</td>
<td>GCCGGAAATGGGAGCGGGTGGAAAAGGCTTC</td>
<td>Biosearch Technologies</td>
</tr>
<tr>
<td></td>
<td>GCGCACGAAG TTTCATCCAGTGAGTAC</td>
<td>Integrated DNA Technologies</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Sag59a/cfbSag263, GBS forward/reverse primers; cfbSag159-E1-A1, GBS RT-RPA probe; ABgl158b/ABgl345c, IC\textsubscript{RPA} forward/reverse primers; ABgl220-E1-M2, IC\textsubscript{RPA} RT-RPA probe.\textsuperscript{b} FAMdT, fluorescein linked to thymidine; THF, tetrahydrofuran spacer; BHQ1dT, Black Hole Quencher 1 linked to thymidine; Spacer-C3, \textsuperscript{3} Spacer-C3 blocking elongation.\textsuperscript{c} M2dT, Texas Red linked to thymidine; phosphate, \textsuperscript{3} phosphate blocking elongation.
genome copies were added simultaneously to each reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 2.5 μL of magnesium acetate solution (14 mmol/L) to each reaction. The reaction tubes were vortex-mixed briefly and then incubated in a Rotor-Gene 6000 at 39 °C for 30 min. Fluorescence measurements were taken every 30 s. The number of replicates for each dilution was 10.

For analysis of the frozen vaginal/anal lysates, RT-RPA was performed in a 53-μL total volume. The reaction mixture was made of 29.5 μL rehydration buffer, 12.05 μL nuclease-free water, 1.05 μL of each forward primer (Sag59a and ABgl158b) (396 nmol/L), 1.05 μL of each reverse primer (cfbSag263 and ABgl345c) (396 nmol/L), and 0.3 μL of each RT-RPA probe (cfbSag159-E1-A1 and ABgl220-E1-M2) (113 nmol/L). One freeze-dried reagent pellet included in the kit was added to every reaction tube and vortex-mixed. Three microliters of lysate from every vaginal/anal sample and 1 μL of B. atrophaeus lysate of 1000 spores was added simultaneously to each reaction. The reaction mixture was set on an ice-cold block to start a synchronized amplification in all the reaction tubes. The amplification reaction was initiated by the addition of 2.65 μL of magnesium acetate solution (14 mmol/L) to each reaction. The reaction tubes were vortex mixed briefly and then incubated in a Rotor-Gene 6000 at 39 °C for 40 min. Fluorescence measurements were taken every 30 s.

**STATISTICAL ANALYSIS**

LOD data for the simplex and multiplex assays were statistically analyzed with homebrew software Lotlod (version 1.2.2) that used R software (version 2.14.1) (36) to determine LOD with their 95% CI from a logistic regression model.

**Results**

**LIMIT OF DETECTION**

For both the simplex and multiplex assays, RT-RPA detected as little as 15 genomic copies in 50% of cases (see Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/issue4). For the simplex assay, we determined with Lotlod that the LOD of the RT-RPA for GBS was 98 (20) genomic copies (95% CI). In the presence of IC_{RPA} genomic DNA, the LOD for the multiplex assay was 100 (20) genomic copies (95% CI). The time threshold varied among replicates of a same dilution as well as between other dilutions (see online Supplemental Fig. 1).

**ANALYTICAL SPECIFICITY**

The RT-RPA assay did not detect the genomic DNA of the 13 following bacteria: *Lactococcus lactis*, *Streptococcus pyogenes*, *Bifidobacterium breve*, *Anaerococcus lactolyticus*, *Acinetobacter baumannii*, *Bordetella pertussis*, *Bacteroides fragilis*, *Mobiluncus mulieris*, *Mobiluncus curtisi subsp. holmesii*, *Trichomonas vaginalis*, *Gardnerella vaginalis*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* (Table 1). It also did not detect the genomic DNA of 2 fungi, *Candida albicans* and *Candida krusei* (Table 1). These bacterial and fungal strains are naturally found in the vaginal/anal flora. These results show the analytical specificity of the RT-RPA assay with our designed set of primers and probe (Sag59a, cfbSag263, and cfbSag159-E1-A1, respectively) to GBS (Table 2).

**GBS SCREENING OF FROZEN CLINICAL LYSATES WITH RT-PCR AND RT-RPA**

Among the 50 frozen vaginal/anal lysates, 25 were identified as positive and 25 identified as negative for GBS by the RT-PCR with the BD GeneOhm StrepB assay kit. With this latter assay the time to result was approximately 45 min. The analysis made by the SmartCyclerDx Software showed no discordant nor unresolved samples. A sample was considered as unresolved if the IC_{RPA} failed to amplify.

Compared to the results obtained by RT-PCR, 5 clinical samples were unresolved by RT-RPA. On re-
testing, 1 became a false negative and 4 became true negatives for GBS because the IC<sub>RPA</sub> was detected in all 5 samples. Among the 25 PCR-positive samples, 24 were positive with RT-RPA, showing a clinical sensitivity of 96% (Table 3). Among the 24 GBS-positive samples, the time threshold was <10 min for 15 samples, <15 min for 8 samples, and 15.5 min for 1 sample. The mean time threshold for GBS-positive samples was 9.0 min with an SD of 2.9 min. For IC<sub>RPA</sub> the mean time threshold for GBS-positive samples was of 11.5 min with an SD of 2.6 min (see online Supplemental Fig. 2A).

With respect to the RT-PCR–negative samples, RT-RPA agreed 100% with the RT-PCR results (Table 3). Among the 25 GBS-negative samples, the time threshold for IC<sub>RPA</sub> was <10 min for 3 samples, <15 min for 15 samples, and >23 min for 7 samples (see online Supplemental Fig. 2B). For IC<sub>RPA</sub> the mean time threshold for GBS-negative samples was 16.0 min with an SD of 8.3 min. The positive predictive values (PPV) and negative predictive values (NPV), for the RT-RPA assay (Table 3) showing a PPV of 100% and an NPV of 96%. 

**SPEED OF REACTION OF RT-RPA VS RT-PCR**

In this study, we also evaluated the difference in amplification time-to-result between RT-RPA and RT-PCR by calculating the mean time threshold, which was the time (min) at which an amplification signal was detected. For this purpose, we converted the cycle threshold obtained by SmartCycler for a whole run into minutes so that it matched the time threshold for an RT-RPA assay (data not shown). We then calculated the mean with the corresponding SD of the time threshold for each of the 24 GBS-positive clinical lysates obtained with RT-RPA and RT-PCR. We observed that the mean time threshold for the same positive clinical samples for the RT-RPA assay (9 min) betters the RT-PCR assay (29 min) in time-to-result by approximately 20 min (Fig. 1). In addition, because both RT-PCR and RT-RPA used the same sample preparation method, the total time-to-answer was determined by the amplification/detection time. For the RT-RPA assay, it was at least 20 min faster than RT-PCR with all the steps carried out at a single temperature.

**Discussion**

In this study, we evaluated the clinical performance of isothermal amplification technique RT-RPA compared to a reference RT-PCR assay. We chose the RPA method for 2 reasons. First, RPA allows a real-time detection of amplification via fluorescent probe. Second, RPA freeze-dried reagents pellets are stable at room temperature for days, which makes its POC application easier (26, 27).

In our study, the LOD for the RT-RPA simplex assay was 98 (20) and 100 (20) genomic copies (95% CI) for the multiplex assay. The presence of 1000 genomic copies of the IC<sub>RPA</sub> in the same reaction tube with the template did not impact the LOD of the GBS template. The reported analytical sensitivity of the BD GeneOhm strep B RT-PCR assay is 10–50 genome copies. We expect that the analytical sensitivity of the RT-RPA assay could be improved by providing a proper mixing of RPA reagents after a few minutes of amplification. This has worked with the TwistAmp exo kit protocol (37), especially when working with low DNA concentrations. Lutz et al. (30) ensured a proper mixing of the amplification reagents with a modified Rotor-Gene 2000 by removing the reaction tubes from the instrument after 3 min of amplification for an additional vortex-mixing step. The reaction tubes were then placed again in the instrument for real-time detection. However, we were not able to add a mixing step after 3 min with the Rotor-Gene 6000, and real-time monitoring could not be interrupted without disruption of fluorescence acquisition. This may explain both the LOD and the variability in time threshold between replicates of different dilution samples that we observed.

We designed an RT-RPA assay to specifically amplify a sequence of the cfb gene for GBS. Amplification did not take place with the tested bacteria and fungi naturally found in vaginal/anal flora. The high analytical specificity of the RT-RPA makes the assay amenable to clinical applications.

In clinical applications, molecular methods are generally subject to the problem of contaminants and inhibitors from crude samples (38). Some of our preliminary results obtained with 15 vaginal/anal crude samples spiked with 1000 copies of GBS genomic DNA showed that an initial heat denaturation step at 95 °C for 2 min after sample lysis may reduce the inhibition and enhance the speed of the reaction for RT-RPA (data not shown). As with RT-PCR, the omission of

### Table 3. Clinical performance of RT-RPA.

<table>
<thead>
<tr>
<th>RT-RPA</th>
<th>BD GeneOhm StreptB (reference assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>24 (TP)*</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (FN)</td>
</tr>
<tr>
<td>%</td>
<td>96 (sensitivity)</td>
</tr>
</tbody>
</table>

* TP, true positive; FP, false positive; FN, false negative; TN, true negative.
heating at 95 °C to denature PCR inhibitors decreased the efficiency of detection of B. atrophaeus DNA (35). Our study with the 50 frozen clinical lysates demonstrated a good performance of RT-RPA compared to the RT-PCR. The only false-negative sample obtained was not inhibitory for B. atrophaeus IC_{RPA}, suggesting that the RPA reaction was not inhibitory for GBS either. This led us to assume that the bacterial load of this clinical lysate was probably below the LOD of RT-RPA. When we compared the cycle thresholds (Ct) obtained with the SmartCycler for the 50 clinical lysates tested, we found that the false-negative sample was among the samples with high Ct, demonstrating that it was among the lowest bacterial load samples (data not shown). Because mixing after few minutes of amplification is recommended, the false-negative sample may be explained by the performance of RPA in the absence of proper mixing. Among the GBS-negative clinical lysates, 7 were shown to be inhibitory, because the IC_{RPA} amplification signal was delayed by 10–20 min compared to other samples. In addition, we showed the feasibility of multiplexing with the RT-RPA assay, in which 2 different DNA targets, GBS, and B. atrophaeus, were simultaneously amplified with multiplex sets of primers and probes. Different amplicons were generated and detected in the same reaction tube at the same time. Therefore, B. atrophaeus could serve as an internal control as well as a process control (34).

Considering GBS from a clinical perspective, the PPV and NPV values show that a positive test with RT-RPA is a strong indication that GBS colonization is present (100%). However, with a negative test it is possible that GBS colonization could be undetected (4%). It will be important to verify the performance of RT-RPA GBS assay with fresh vaginal/anal swabs collected directly from women in labor.

The RT-RPA assay shows some advantages over RT-PCR. It is faster in terms of runtime and obviates the need for a sophisticated instrument for thermal management. Combined with the avoidance of labor costs because of the requirements for trained personnel in a certified laboratory, a POC RPA assay becomes more cost-effective. The BD GeneOhm strep B RT-PCR assay used in this study costs approximately $35 per test just for the disposable version and $48 600 for the SmartCycler instrument. We predict that an RPA-based POC assay would cost approximately $10 per disposable and $10 000 or less for a dedicated instrument. We consider this work promising for a future application of an RPA method as an alternative tool in clinical settings for the screening and detection of multiple infectious agents.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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References


