Comparative Clinical Study of Different Multiplex Real Time PCR Strategies for the Simultaneous Differential Diagnosis between Extrapulmonary Tuberculosis and Focal Complications of Brucellosis

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Abstract

Background: Both brucellosis and tuberculosis are chronic-debilitating systemic granulomatous diseases with a high incidence in many countries in Africa, Central and South America, the Middle East and the Indian subcontinent. Certain focal complications of brucellosis and extrapulmonary tuberculosis are very difficult to differentiate clinically, biologically and radiologically. As the conventional microbiological methods for the diagnosis of the two diseases have many limitations, as well as being time-consuming, multiplex real time PCR (M RT-PCR) could be a promising and practical approach to hasten the differential diagnosis and improve prognosis.

Methodology/Principal Findings: We designed a SYBR Green single-tube multiplex real-time PCR protocol targeting bscp31 and the IS711 sequence detecting all pathogenic species and biovars of Brucella genus, the IS6110 sequence detecting Mycobacterium genus, and the intergenic region senX3-regX3 specifically detecting Mycobacterium tuberculosis complex. The diagnostic yield of the M RT-PCR with the three pairs of resultant amplicons was then analyzed in 91 clinical samples corresponding to 30 patients with focal complications of brucellosis, 24 patients with extrapulmonary tuberculosis, and 36 patients (Control Group) with different infectious, autoimmune or neoplastic diseases. Thirty-five patients had vertebral osteomyelitis, 21 subacute or chronic meningitis or meningoencephalitis, 13 liver or splenic abscess, eight orchiepididymitis, seven subacute or chronic arthritis, and the remaining seven samples were from different locations. Of the three pairs of amplicons (senX3-regX3+ bscp3, senX3-regX3+ IS711 and IS6110+ IS711) only senX3-regX3+ IS711 was 100% specific for both the Brucella genus and M. tuberculosis complex. For all the clinical samples studied, the overall sensitivity, specificity, and positive and negative predictive values of the M RT-PCR assay were 89.1%, 100%, 85.7% and 100%, respectively, with an accuracy of 93.4%, (95% CI, 88.3—96.5%).

Conclusions/Significance: In this study, a M RT-PCR strategy with species-specific primers based on senX3-regX3+IS711 sequences proved to be a sensitive and specific tool, useful for the highly efficient detection of M. tuberculosis and Brucella spp in very different clinical samples. It thus represents an advance in the differential diagnosis between some forms of extrapulmonary tuberculosis and focal complications of brucellosis.

Introduction

Brucellosis remains one of the most widespread anthropozoonoses in the world, especially in the Mediterranean basin, the Middle East, India, Mexico and some countries of Central and South America [1]. Much evidence supports the conclusion that in countries without strong health systems, official data likely underestimate the true burden [2]. The high morbidity associated with brucellosis, together with its prolonged course and great tendency to produce relapses account for an important consumption of health care resources [3,4].

The global burden of tuberculosis (TBC) remains enormous [5]. Recent data in the WHO Global Tuberculosis Report 2012 confirm that TBC remains a major infectious killer today. In 2011, there were an estimated 8.7 million new cases and 1.4 million people died from TBC [6].

Like TBC, brucellosis can cause focal complications in any organ or system. The larger studies place the rate of focal complications of brucellosis at around 25–35% of all cases [3,7,8], similar to the rate of extrapulmonary complications in TBC, 15–40% [9]. Moreover, whilst in many countries there has been a reduction in the overall incidence of pulmonary tuberculosis, the
number of extrapulmonary tuberculosis cases has increased in some industrialized countries [10–13].

When tuberculosis or brucellosis affect specific sites, e.g., the CNS, or osteoarticular or genitourinary systems, the differential diagnosis between the two entities is virtually impossible based solely on clinical, haematological, biochemical or imaging studies. Furthermore, as both tuberculosis and brucellosis are granulomatous diseases, the pathological findings of focal complications of brucellosis and extrapulmonary tuberculosis can be very similar.

Both Mycobacterium tuberculosis complex (MTC) and Brucella spp are slow-growing microorganisms. Classical methods for determining the presence of these microorganisms are time-consuming and labor-intensive. Hence, molecular methods, which offer speed, sensitivity and specificity, have been developed to address this problem. Multiplex real time PCR (M RT-PCR) is increasingly used in various fields of microbiology for the rapid differentiation of microbial species involved in specific syndromes [14–16].

Our group has shown that M RT-PCR is a useful strategy for the rapid differential diagnosis between extrapulmonary tuberculosis and brucellosis when they affect specific locations [17]. Later, we simplified the technique to make it more accessible to any clinical laboratory [18]. This study compared experimentally, in both monoplex and multiplex forms, the PCR combinations of three different targets for each microorganism, optimizing and simplifying the technique using SYBR Green, determining the sensitivity and reproducibility in a small sample of patients.

The aim of the present study was to analyze comparatively the diagnostic yield of different strategies of M RT-PCR in a very representative sample of patients with focal complications of brucellosis or extrapulmonary tuberculosis and assessed the analytical specificity against a wide panel of microorganisms that included most of the non-tuberculous Mycobacteria related with human diseases, and the most important species and biovars of Brucella.
DNA extraction
All samples destined for M RT-PCR were maintained at −20°C until processing. The amount or volume used varied depending on the type of sample. To monitor contamination, negative controls were included during each DNA extraction procedure. DNA was extracted using the Quiamp DNA Mini (Qiagen, UK). Prior to DNA extraction, homogenized samples from the different tissues, CSF, synovial fluid, urine, purulent collections and strains were resuspended in 1 ml of molecular biology water, mixed and centrifuged at 13,000 × g for 10 min. The supernatant was discarded and the pellet was resuspended with the volume of buffer outlined in the manufacturer’s instructions. DNA pellets were resuspended in 30 μl molecular biology water and stored at 4°C until use. The concentration and purity of DNA were estimated by measuring the absorbance at 260 and 280 nm with a ND-1000 spectrophotometer (Nanodrop ThermoFisher, USA).

Primer design and Multiplex Real Time PCR assay conditions
For detection of members of MTC, the primer sets IS6110f/IS6110r (5′ TCAAGGAGGCATCAGGCC3′/5′TCAAGGTT-CAGGGTAgCC3′) and M1f/M3r (5′CGGCTTAATCAG-GACGGCAC3′/5′CCTCCTCCTCTGGTTGTACGCTTT 3′) were used to amplify 82 and 164 bp fragments of IS6110 and senX3-regX3, respectively. For Brucella, fragments of 152 and 142 bp of the bscp31 gene and IS711 were amplified using primers bscp31f/bscp31r (5′ GCATTCTTCACATCCAGG 3′/5′ CACCGGGATTTCCATATTCT 3′) and IS711f/IS711r (5′ TACAAGGAAAGCCATCAGA 3′/5′ GCATTCAAGGGAC-GAGA) [18]. The three real time reactions were monitored using a Light-Cycler 2.0 (Roche Diagnostic, Indianapolis, IN) with the LC FastStart DNA Master SYBR-Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). The M RT-PCR assays for MTC and Brucella were performed as described previously [18]. Briefly, the mixture included 1 × master mix, 3–3.5 mM MgCl₂, 0.5 μM primers, variable concentrations of DNA as template (150–250 ng depending on type of sample analyzed) and nuclease free dH₂O adjusted to a final volume of 20 μl. Each run included positive controls consisting of dilutions of Brucella spp and MTC DNA, and negative controls with all the elements of the reaction mixture except template DNA. The reactions were cycled 45 times, after an initial hold at 95°C for 10 min, between 95°C for 10 s, 60°C for 5 s, and 72°C for 6 s with programmed transitions of 20°C/s. The melting curves were acquired on the SYBR channel by heating momentarily at 95°C, collecting fluorescence continuously at a ramping rate of 0.1°C/s until 105°C. To minimize experimental variability the Ct values, the threshold cycle where the fluorescence signal rises significantly above background in the exponential phase of the amplification, were determined by the second derivative maximum method. In order to avoid potential observer bias, the clinical and microbiological diagnoses of the patients were unknown to the technician who performed the M RT-PCR assay.

Primer specificity
The specificity of the primers was first tested in silico using the BLASTn program in order to prevent non-specific amplifications. The analytical specificity was then tested against the 59 microorganisms listed in Table 1.

Sequencing of M RT-PCR product
To confirm the identities of the amplified fragments, some of the strains used as positive controls of Brucella spp and MTC and different clinical samples were sequenced. The ABI PRISM Big Dye Terminator Cycle sequencing reaction kit v. 3.0 (Applied Biosystems, Madrid, Spain) was used for the sequencing analysis, by capillary electrophoresis, in an ABI PRISM, model 3100 automated sequencer (Applied Biosystems).

Statistical analysis
Quantitative variables are represented as mean ± standard deviation and qualitative variables as percentages. Sensitivity, specificity, positive and negative predictive values, accuracy, likelihood ratios (LR) and 95% confidence intervals (CI) were calculated using the TwoByTwo 1.0 analyzer program.

Accession numbers
Brucella: bscp31 (M20404), IS711 (AE017223)
MTC: IS6110 (BX842574), senX3-regX3 (BX842573).

Results
The three M RT-PCR strategies based on amplification of the target sequences senX3-regX3+ bscp31, senX3-regX3+ IS711 and IS6110+ IS711 correctly identified all the species and biovars of Brucella as well as all the species belonging to MTC. The target based on the IS711 sequence did not amplify any of the bacteria serologically or phylogenetically related with Brucella spp, and the amplicons of the genes of the bscp31 protein gave a false positive result with Ochrobactrum anthrophium and intermedium. Likewise, the amplicons of the intergenic region senX3-regX3 were negative in all NTM tested, but those of the IS6110 sequence amplified various NTM: M. fortuitum, M. scrofulaceum, M. simiae and M. intracellulare (Table 1). Figure 1 shows the similar melting temperatures (Tm) of two of these NTM (as an example) and the two strains of Ochrobactrum for bscp31 compared with the control Tm for the two pathogens.

Of the 91 clinical samples included in the study, 35 were vertebral or paravertebral tissue from patients with vertebral osteomyelitis, 21 were CSF from patients with subacute or chronic meningitis or meningoencephalitis, 13 were tissue or abscess aspirates from patients with liver or splenic abscesses, 8 were urine samples from patients with orchiepididymitis, 7 were from synovial fluid from patients with subacute or chronic arthritis, and the remaining 7 were from different locations: two samples of purulent fluid from patients with neck abscesses, two bone biopsies from patients with osteomyelitis of the femur and sternum, respectively, two kidney biopsies from patients with chronic pyelonephritis, and a sample of seminal fluid from a patient with chronic orchiepididymitis. Table 2 summarizes the sample type and the final diagnosis of the study patients.

Of the 30 patients with brucellosis, 25 (83.3%) were primary infections and 5 (16.6%) had had a previous episode of infection. Brucella melitensis was isolated in 17 (56.6%) of the 30 patients with brucellosis; 13 (43.3%) in blood culture, 8 (26.6%) in non-blood samples (three vertebral tissue and one each of the following: urine, CSF, hepatic tissue, synovial fluid and thyroid abscess) and in 4 (13.3%) in both blood and non-blood samples. In 12 of the other 13 patients (40%) the diagnosis of brucellosis was based on clinical and serological criteria. One 43-year-old woman, who habitually consumed non-homogenized dairy products and who had osteomyelitis with thoracic segment involvement and whose biopsy showed non-caseating granulomas, constantly had negative cultures and absence of serological response, and was diagnosed with brucellosis based on her epidemiologic exposure and clear response to treatment with doxycycline plus streptomycin.
| Species          | Strain   | Origin | M RT-PCR | ATCC | HCH | H37Rv | MTC | Brucella | MTC | Brucella | MTC | Brucella | MTC | Brucella | MTC | Brucella | MTC | Brucella |
|------------------|----------|--------|----------|------|-----|-------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|
| Mycobacterium strains |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. tuberculosis  | H37Rv    | ATCC   | +        |  89.32 ± 0.02 | + 89.34 ± 0.02 | + 87.31 ± 0.04 |
| M. caprae       | 1040     | HCH    | +        |   89.11 ± 0.29 | + 89.06 ± 0.04 | + 87.32 ± 0.38 |
| M. caprae       | HCH      | +      |  89.21 ± 0.29 | + 89.75 ± 0.08 | + 87.47 ± 0.19 |
| M. africanum    | 25420    | ATCC   | +        |   89.52 ± 0.44 | + 89.76 ± 0.03 | + 87.83 ± 0.02 |
| M. africanum    | HCH      | +      |   89.38 ± 0.36 | + 89.07 ± 0.01 | + 87.72 ± 0.03 |
| M. bovis BCG    | Pasteur  | ATCC   | +        |   90.18 ± 0.37 | + 89.48 ± 0.14 | + 87.75 ± 0.22 |
| M. bovis        | 19210    | ATCC   | +        |   89.32 ± 0.14 | + 89.12 ± 0.03 | + 87.36 ± 0.34 |
| M. bovis        | XDR      | HCH    | +        |   90.12 ± 0.06 | + 89.17 ± 0.09 | + 87.81 ± 0.03 |
| M. microti      | 8710     | ATCC   | +        |   89.06 ± 0.02 | + 89.42 ± 0.09 | + 87.52 ± 0.05 |
| M. pinnipedi    | 13288    | ATCC   | +        |   89.27 ± 0.08 | + 89.40 ± 0.37 | + 87.82 ± 0.18 |
| Genetically related bacteria |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| NTM             |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. avium        | 1062     | ATCC   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. xenopi       | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. kansasii     | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. chelonae     | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. gordonae     | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. fortuitum    | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. scrofulaceum | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. szulgai      | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. marinum      | 7091     | CECT   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. celatum      | 342      | ATCC   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. intracellulare |       | ATCC   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. simiae       | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. smegmatis    | 3017     | CECT   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. flavencens   | 3027     | CECT   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. phlei        | 3016     | CECT   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. brumae       | 3022     | CECT   | –        |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. abscessus    | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. mucogenicum  | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| M. peregrinum   | HCH      | –      |          |     |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| Other acid-fast microorganism |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| Nocardia spp    |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| Brucella spp (22) |          |        |          |      |     |       |     |          |     |          |     |          |     |          |     |          |     |          |
| B. melitensis 1 | 16 M     | FMV    | –        |  86.84 ± 0.78 | + 84.59 ± 0.69 | + 84.25 ± 0.46 |
| B. melitensis 1 | Rev 1    | CAJA   | –        |  86.24 ± 0.06 | + 84.78 ± 0.26 | + 84.42 ± 0.07 |
| B. melitensis 2 | 63/9     | FMV    | –        |  86.08 ± 0.10 | + 84.72 ± 0.09 | + 84.77 ± 0.07 |
| B. melitensis 2 | AC       | FMV    | –        |  86.62 ± 0.12 | + 84.72 ± 0.20 | + 84.40 ± 0.21 |
| B. melitensis 3 | Ether    | FMV    | –        |  86.95 ± 0.04 | + 84.94 ± 0.07 | + 84.54 ± 0.09 |
| B. abortus 1    | AC       | FMV    | –        |  86.26 ± 0.02 | + 84.12 ± 0.11 | + 84.77 ± 0.39 |
| B. abortus 1    | B19      | CAJA   | –        |  86.49 ± 0.08 | + 84.67 ± 0.21 | + 84.85 ± 0.73 |
| B. abortus 2    | 86/859   | FMV    | –        |  86.56 ± 0.37 | + 84.67 ± 0.21 | + 84.48 ± 0.02 |
| B. abortus 3    | Tului    | FMV    | –        |  86.31 ± 0.22 | + 84.46 ± 0.14 | + 84.37 ± 0.04 |
| B. abortus 4    | 292      | FMV    | –        |  86.22 ± 0.03 | + 84.30 ± 0.04 | + 84.40 ± 0.01 |
| B. abortus 5    | B3196    | FMV    | –        |  86.32 ± 0.11 | + 84.37 ± 0.24 | + 84.48 ± 0.21 |
Of the 24 patients with extrapulmonary tuberculosis, *M. tuberculosis* was isolated in 18 (75%) and the other six (25%) had necro-tizing granulomas in their biopsies, with or without acid-fast bacilli. Only four (16.6%) of the 24 cases had smear-positive samples.

The three M RT-PCR strategies were positive in 49 (89.1%) of the 55 samples from patients with tuberculosis or brucellosis; 28 (90.3%) of the 31 focal complications of brucellosis and 21 (87.5%) of the 24 extrapulmonary tuberculosis. M RT-PCR was negative in the 36 samples from the control group patients. Thus, the overall sensitivity of the M RT-PCR was 89.1% (95% CI, 80.9–97.3) and the specificity 100%. The overall diagnostic yield of the M RT-PCR is shown in Table 3.

Of the six patients who had a false negative result with the M RT-PCR, two had received prolonged antimicrobial treatment of the 24 extrapulmonary tuberculosis. M RT-PCR was negative in the 36 samples from the control group patients. Thus, the overall sensitivity of the M RT-PCR was 89.1%, (95% CI, 80.9–97.3) and the specificity 100%. The overall diagnostic yield of the M RT-PCR is shown in Table 3.

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**Table 1.** Cont.

| Species                  | Strain | Origin | M RT-PCR senX3-regX3+bcsp31 | M RT-PCR senX3-regX3+IS711 | M RT-PCR IS6110+IS711 |
|--------------------------|--------|--------|-----------------------------|-----------------------------|------------------------|
|                          | MTC    | Brucella | Tm (°C)                    | MTC                        | Brucella               | Tm (°C)                |
| *B. abortus* biovar 6    | 870    | FMV    | –                           | +                          | –                      | 87.22±0.35             | 84.29±0.50             | 84.76±0.47             |
| *B. abortus* biovar 7    | 63/75  | FMV    | –                           | +                          | –                      | 86.79±0.64             | 84.37±0.31             | 84.55±0.25             |
| *B. abortus* biovar 9    | C/68   | FMV    | –                           | +                          | –                      | 86.81±0.53             | 84.47±0.22             | 84.92±0.35             |
| *B. suis* biovar 1       | 10036  | FMV    | –                           | +                          | –                      | 86.76±0.18             | 84.89±0.19             | 84.28±0.56             |
| *B. suis* biovar 2       | 10510  | FMV    | –                           | +                          | –                      | 86.89±0.03             | 84.74±0.43             | 84.83±0.20             |
| *B. suis* biovar 3       | 10511  | FMV    | –                           | +                          | –                      | 86.73±0.02             | 84.60±0.02             | 84.72±0.09             |
| *B. suis* biovar 4       | 40     | FMV    | –                           | +                          | –                      | 86.35±0.27             | 84.42±0.72             | 84.67±0.36             |
| *B. suis* biovar 5       | 10980  | FMV    | –                           | +                          | –                      | 87.18±0.01             | 84.68±0.67             | 84.86±0.04             |
| *B. neotomae*            | 10084  | FMV    | –                           | +                          | –                      | 86.96±0.44             | 84.59±0.01             | 85.05±0.32             |
| *B. ovis*                | Reo198 | FMV    | –                           | +                          | –                      | 86.96±0.07             | 84.96±0.10             | 84.68±0.05             |
| *B. canis*               | 10854  | FMV    | –                           | +                          | –                      | 86.52±0.02             | 84.36±0.16             | 84.69±0.16             |

**Antigenically related bacteria**

- *Escherichia coli* O157:H7 CECT
- *Moraxella osloensis* 460 CECT
- *Pasteurella multocida* 962 CECT
- *Yersinia enterocolitica* O:9 CECT
- *Vibrio cholerae* Inaba CECT

**Genetically related bacteria**

- *Ochrobactrum anthropi* 4426T CECT
- *Ochrobactrum intermedium* 3301 FMN

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**Figure 1.** Specificity of PCR products based on the Tm. Panel A, M RT-PCR assay IS6110+IS711. Black triangles lines, positive control of *B. abortus*; Black stars, positive controls of *M. tuberculosis*; blue lines and orange lines, false positive results due to *M. intracellulare* and *M. simiae*, respectively. Panel B, M RT-PCR assay senX3-regX3+bcsp31. Black triangles lines, positive control of *B. abortus*; Black stars, positive controls of *M. tuberculosis*; green lines, false positive results related with *Ochrobactrum anthropi*.

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before drawing the sample. The first of these was a 24-year-old woman with a kidney transplant and brucellar pyelonephritis in the transplanted organ, treated for two weeks before taking the renal biopsy with ciprofloxacin, meropenem and piperacillin-tazobactam. The second was a 33-year-old man with tuberculous vertebral osteomyelitis treated during the four months prior to taking the vertebral biopsy with rifampicin/isoniazid/ethambutol for the first two months and with rifampicin/isoniazid the second two months. In both cases the cultures were also negative. If these cases had been withdrawn from the analysis of efficacy, the sensitivity of the M RT-PCR for the overall sample would have risen to 92.5%. The other four false-negative results corresponded to two patients with brucellosis (one brucellar orchiepididymitis with positive blood cultures and a negative urine culture and the other vertebral osteomyelitis with negative blood and vertebral tissue cultures) and two patients with tuberculosis (one meningitis and one vertebral osteomyelitis, both with positive cultures and negative microscopic study). Table 4 shows the results of the M RT-PCR according to the type of microorganism, culture result and sample type. The M RT-PCR was positive in the four cases of extrapulmonary tuberculosis with smear-positive samples and in 17 (85%) of the 20 cases with smear-negative samples.

The mean Ct values of the senx3-regx3+ bcsp31, senx3-regx3+ IS711 + IS6110+ IS711 assays varied according to the type of sample, ranging from 31.03–34.85, 26.99–33.00 and 29.95–34.74 cycles respectively for the samples from patients with extrapulmonary tuberculosis to 24.68–30.57, 16.13–31.73 and 28.17–32.07 cycles for the samples from patients with focal complications of brucellosis.

The amount and purity of total DNA (microbial DNA and eukaryotic DNA) differed significantly depending on the type of eukaryotic DNA) differed significantly depending on the type of microorganism, culture result and sample type. The M RT-PCR was positive in the four cases of extrapulmonary tuberculosis with smear-positive samples and in 17 (85%) of the 20 cases with smear-negative samples.

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The amount and purity of total DNA (microbial DNA and eukaryotic DNA) differed significantly depending on the type of sample. Because of its high sensitivity, molecular diagnosis has now become a very useful tool for the diagnosis of many viral, bacterial and fungal infections.

Clinical microbiology is now directed more towards syndromic diagnosis, in which the most common causative agents of a particular clinical syndrome are all studied together at the same

**Table 2.** Sample type and diagnosis of the study patients.

| Clinical Sample                  | Brucellosis | Tuberculosis | Control Group |
|----------------------------------|-------------|--------------|---------------|
|                                  | n (culture positive) | n (culture positive) | n |
| Vertebral or paravertebral tissue | 11 (3)      | 12 (9)       | 12*           |
| CSF                              | 5 (1)       | 6 (4)        | 10*b          |
| Hepatic or splenic tissue        | 6 (1)       | 0            | 7*c           |
| Urine                            | 4 (1)       | 2 (2)        | 2d            |
| Synovial fluid                   | 1 (1)       | 2 (2)        | 4*            |
| Other samples                    | 4 (1)       | 2 (1)        | 1f            |
| **Total Samples**                | **31**      | **24**       | **36**        |

*S. aureus, 4 cases, E. coli and S. epidermidis 2 cases, S. agalactiae, Peptoestreptococcus, S. intermedius, and M. xenopi one case respectively.

**Table 3.** Diagnostic yield of M RT-PCR in clinical specimens from patients with focal complications of brucellosis or extrapulmonary tuberculosis.

|                | Sensitivity | Specificity | PPV | NPV | Accuracy | Positive LR | Negative LR |
|----------------|-------------|-------------|-----|-----|----------|-------------|-------------|
| **All Samples**| 89.1, (80.9–97.3) | 100         | 100 | 85.7, (75.1–96.3) | 93.4, (88.3–96.5) | ND* | 0.11, (0.05–0.23) |
| **Focal Brucellosis** | 90.3, (79.9–100) | 100         | 100 | 92.3, (83.9–100) | 95.5, (90.6–100) | ND* | 0.10, (0.03–0.28) |
| **Extrapulmonary Tuberculosis** | 87.5, (74.3–100) | 100         | 100 | 92.3, (83.9–100) | 95.0, (89.5–100) | ND* | 0.13, (0.04–0.36) |

PPV, positive predictive value; NPV, negative predictive value; Positive LR, positive likelihood ratio; Negative LR, negative likelihood ratio; 95% CI = 95% confidence interval, ND*, not done for mathematical reasons (division by zero).
Table 4. Results of M RT-PCR according to clinical sample, microorganism, and culture result.

| Clinical Sample                  | Brucellosis |     | Tuberculosis |     |
|----------------------------------|-------------|-----|--------------|-----|
|                                  | Positive     |     | Positive     |     |
|                                  | culture      | M RT-PCR+ | M RT-PCR+     | M RT-PCR-- | M RT-PCR-- | M RT-PCR+ | M RT-PCR+     | M RT-PCR-- | M RT-PCR--     |
| Vertebral or paravertebral tissue| 3            | 0   | 7            | 1   |
| CSF                              | 1            | 0   | 4            | 0   |
| Hepatic or splenic tissue        | 1            | 0   | 5            | 0   |
| Urine                            | 1            | 0   | 2            | 1   |
| Synovial fluid                   | 1            | 0   | 0            | 1   |
| Other samples                    | 1            | 0   | 2            | 1   |
| Total Samples                    | 8            | 0   | 20           | 3   |

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time in a single test. As M RT-PCR can do this ever more efficiently, it has experienced exponential development in recent years [14–16,20,21].

In many underdeveloped and developing countries, tuberculosis and brucellosis are still the most frequent causes of bacterial lymphocytic meningitis, granulomatous vertebral osteomyelitis, subacute arthritis and subacute orchiepididymitis. In these clinical scenarios, among others, M RT-PCR could be a useful tool for the rapid differential diagnosis between two pathogens whose isolation in culture is difficult and time consuming. Previous studies from our group have shown that of the different candidate genes, three combinations of amplicons of bcsp31 protein gene and the IS711 in the case of Brucella spp and the senX3-regX3 intergenic region and IS6110 for MTC permit a highly sensitive and reproducible co-amplification [18].

In this study we analyzed the diagnostic yield of the three possible combinations of the ampiclons of bcsp31, senX3-regX3+ IS711 and IS6110+ IS711) in a representative sample of patients with extrapulmonary tuberculosis and focal complications of brucellosis.

The three primer combinations correctly identified all the species and biovarieties of Brucella and MTC, and there was no non-specificity with the strategy based on the sequence amplification of senX3-regX3+ IS711. The target based on bcsp31 did, however, show a false positive result with Ochrobactrum spp. This cross-reaction, which has been described previously [22–23], is not surprising if we consider that Ochrobactrum spp. is the closest known relative of the Brucella genus. Concerning MTC, the target senX3-regX3 showed no non-specificity with the panel of NTM, though the strategy based on IS6110 produced a cross-reaction with M. fortuitum, M. scrofulaceum, M. intracellulare and M. simiae. This lack of specificity has been previously described. Thus, a study analyzing the specificity of IS6110-based methods in nine laboratories from France demonstrated false-positive reactions with an average rate of 7%, most of them caused by NTM [24]. This explains why many authors request caution in designing and evaluating diagnostic PCR tests based on this element [25].

The overall sensitivity of our M RT-PCR method should be considered very good since it was 89.1%; 87.5% in extrapulmonary TB cases and 90.3% in cases of focal complications of brucellosis. These results are as good as or better than those with any of the monoplex PCR methods so far tried, sensitivities of which have ranged from 53–95% in clinical samples from patients with extrapulmonary tuberculosis [26–30] and from 92–94% for non-blood samples of focal complications of brucellosis [31].

The yield of molecular diagnostic techniques falls in patients with extrapulmonary tuberculosis with respiratory or nonrespiratory smear-negative specimens [26,32].

In our study, only 4 (16.6%) of the 24 extrapulmonary tuberculosis cases were smear-positive, a percentage similar to that reported by other authors [26,30]. This very small number of samples makes it difficult to draw conclusions about the sensitivity of our M RT-PCR assay in patients with extrapulmonary tuberculosis with smear-negative samples. Nevertheless, the results of this study (83% sensitivity in smear-negative samples) show the high sensitivity of the technique, even in paucibacillar specimens. This high sensitivity in smear-negative samples may be related with the fact that in our study most were aspirates from abscesses or tissue samples. Recently Mouré et al, in a large study including 108 smear-negative extrapulmonary samples, found that the sensitivity of the Xpert was just 40.5% in sterile fluids versus 76.3% in abscess aspirates [33].

The diagnosis of brucellosis does not normally present problems in acute non-complicated forms. In these cases, all the serological tests commonly used have a high sensitivity. However, this is not the case in patients who have a more prolonged evolution, as occurs in most patients who have focal complications, particularly if they are patients who are professionally exposed or patients with recurrences of the disease. In both scenarios, serological studies
have important limitations [34]. In addition, the sensitivity of the cultures, whether they are from peripheral blood or non-blood samples, does not usually surpass 50% in patients with focal forms of brucellosis. Other than our own studies, reports dealing with the usefulness of molecular techniques for the diagnosis of patients with focal complications of brucellosis are anecdotal, though they all show the superiority of these techniques as compared to cultures [35,36].

In clinical practice the volume of a sample sent to the laboratory for the diagnosis of patients with extrapulmonary tuberculosis or focal brucellosis can vary greatly, depending on the site of the complication and the form of obtaining the sample. In fine-needle aspiration biopsies this volume can be really small. In our study the amount of DNA extracted and its purity can be considered good in all types of samples, except for CSF, as mentioned by others [37]. Concerning the amount and purity of DNA, previous studies by our group [18] have shown the inhibitory effect that high concentrations of DNA have on the technique. Given these previous results, in this study we used DNA amounts no greater than 250 ng per reaction, both in tissue samples and in abscesses. The small variable volumes of CSF available in clinical practice together with the peculiar characteristics of subacute lymphocytic bacterial meningitis; mild or moderate pleocytosis, and paucibacillary samples meant that the volume of DNA for each assay varied, ranging between 2 and 8 µl for a final volume of 20 µl in the PCR reaction.

As is logical, the purity of the DNA differed widely depending on the type and location of the study sample, though this did not greatly affect the Ct or the Tm in comparison with what was seen in the collection strains of the two pathogens. From a qualitative point of view, neither the type of sample nor the amount or purity of the DNA influenced significantly the diagnostic yield of the M RT-PCR, independently of the strategy used, indicating the robustness of the three SYBR Green based M RT-PCR strategies.

Though the comparative study of the three pairs of amplicons used showed no differences in the samples used, the M RT-PCR strategy based on the amplification of senX3-regX3 IS711 seems to be the most suitable, as it avoids false positive results derived not only from cross-reactions of IS6110 with NTM but also from amplification of Ochrobactrum spp., as this microorganism lacks IS711 [38].

The Ochrobactrum spp. comprises a group of very ubiquitous microorganisms. Although its ecology is not well known, it has been isolated from soil, water, multiple hospital material, and different clinical specimens and it may be part of the normal flora of the large intestine. Ochrobactrum spp. would seem to occupy a microbial niche similar to that of Pseudomonas aeruginosa, as most infections in humans have been in patients with catheters, other foreign bodies, or severely immunosuppressed persons [39]. Indeed, it is always important to exclude possible cross-reactions with potentially colonizing microorganisms.

### Table 5. Tm (°C) and Ct (cycle) values with the three strategies of M RT-PCR studied by type of clinical sample.

| Clinical samples | M RT-PCR | M RT-PCR | M RT-PCR |
|------------------|---------|---------|---------|
|                  | senX3-regX3+bcsp31 | senX3-regX3+i5711 | IS6610+i5711 |
| Brucella         | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| Vertebral or paravertebral tissue | 86.64 ± 0.30 (10) | 30.57 ± 6.32 (10) | 90.07 ± 0.43 (10) |
|                 |         | 32.39 ± 5.67 (10) | 30.07 ± 5.54 (10) | 31.66 ± 5.32 (10) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| CSF              | 86.88 ± 0.32 (5) | 28.31 ± 5.34 (5) | 90.03 ± 0.61 (5) |
|                 |         | 32.68 ± 3.33 (5) | 28.58 ± 0.27 (5) | 26.94 ± 4.36 (5) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| Hepatic or splenic tissue | 86.68 ± 0.30 (6) | 29.31 ± 5.24 (6) | 86.86 ± 0.30 (6) |
|                 |         | 29.24 ± 5.24 (6) | 29.38 ± 0.25 (6) | 31.73 ± 0.25 (6) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| Urine            | 86.68 ± 0.21 (3) | 28.34 ± 7.83 (3) | 89.66 ± 0.20 (2) |
|                 |         | 31.78 ± 1.42 (2) | 26.26 ± 0.42 (2) | 31.62 ± 1.02 (2) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| Synovial fluid   | 86.95 ± 0.33 (6) | 24.68 ± 0.39 (6) | 89.94 ± 0.39 (6) |
|                 |         | 31.03 ± 0.64 (6) | 16.13 ± 0.39 (6) | 32.67 ± 1.02 (6) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |
| Other samples    | 86.51 ± 0.31 (3) | 26.85 ± 2.95 (3) | 90.50 ± 0.15 (2) |
|                 |         | 34.85 ± 1.32 (2) | 26.11 ± 0.59 (3) | 29.62 ± 1.79 (3) |
|                 | Brucella | MTC     | MTC     | MTC     |
| Tm (°C)          |         |         |         |

Results are given as mean ± SD with the number of samples assayed in parentheses. doi:10.1371/journal.pntd.0002593.t005

### Table 6. Amplicons Tm of the three M RT-PCR assayed in different clinical samples and collection strains.

| Type of sample assayed | M RT-PCR (Tm, °C) |
|------------------------|-------------------|
|                        | senX3-regX3+bcsp31 | senX3-regX3+i5711 | IS6610+i5711 |
| Brucella               | MTC               | MTC               | MTC         |
| Clinical samples       | 86.64 ± 0.33 (28) | 90.05 ± 0.48 (21) | 84.64 ± 0.46 (28) |
|                        | 89.98 ± 0.44 (21) | 84.45 ± 0.30 (28) | 87.62 ± 0.46 (21) |
| Collection strains     | 86.62 ± 0.33 (22) | 89.45 ± 0.39 (10) | 84.55 ± 0.21 (22) |
|                        | 89.35 ± 0.25 (10) | 84.61 ± 0.21 (22) | 87.59 ± 0.21 (10) |
| Difference of Tm (°C)  | 0.02              | 0.60              | 0.09         |
|                        | 0.63              | 0.16              | 0.03         |

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In addition to its high sensitivity, other important aspects of single-tube M RT-PCR make it especially attractive to clinical laboratories for use in samples from patients in whom extrapulmonary TBC or focal complications of brucellosis are suspected. First, M RT-PCR provides results within 4 hours, which is much less than the time required for conventional methods to rescue a fastidious microorganism such as M. tuberculosis or Brucella spp.; second, the technique almost completely obviates the need for direct handling of the pathogen, thus drastically reducing the risk of infection of laboratory personnel; and third, the sample can either be processed immediately or easily stored at −20°C until processing.

In conclusion, a SYBR Green single-tube M RT-PCR assay based on senX3-regX3+ IS711 coamplification allows a rapid and efficient identification of M. tuberculosis complex and Brucella spp in different clinical samples. Based upon our own experience with M. tuberculosis complex and those of other authors, this new strategy is more specific than those previously reported, which, together with its high sensitivity, make it a very useful tool for the differential diagnosis between some forms of extrapulmonary tuberculosis and focal complications of brucellosis.

**Supporting Information**

**Checklist S1** STARD checklist for reporting of studies of diagnostic accuracy.

**(DOC)**

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**Author Contributions**

Conceived and designed the experiments: JDC PM RSJ. Performed the experiments: RSJ PM JDC. Contributed reagents/materials/analysis tools: RSJ PM MJR. Wrote the paper: RSJ JDC PM.

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