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First detection of *Waddlia chondrophila* in Africa using SYBR Green Real-time PCR and Tunisian veterinary samples

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Abstract:

Waddlia chondrophila is a strict intracellular microorganism belonging to the order *Chlamydiales* that has been isolated twice from aborted bovine fetuses, once in USA and once in Germany. This bacterium is now considered as an abortigenic agent in cattle. However, no information is available regarding the presence of this bacteria in Africa and more especially in bovines from Tunisia. Given the low sensitivity of cell culture to recover such an obligate intracellular bacteria, molecular-based diagnostic approaches are warranted. This report describes the development of a quantitative SYBR Green real-time PCR assay targeting the *recA* gene of *Waddlia chondrophila*. Analytical sensitivity was 10 copies of control plasmid DNA per reaction. No cross-amplification was observed when testing pathogens that can cause abortion in cattle. The PCR exhibited a good intra-run and inter-run reproducibility. This real-time PCR was then applied to 150 vaginal swabs taken from Tunisian cows that have aborted. Twelve samples revealed to be *Waddlia* positive, suggesting a possible role of this *Chlamydia*-related bacterium in this setting. This new real-time PCR assay represents a diagnostic tool that may be used to further study the prevalence of *Waddlia* infection.

Key words: *Waddlia chondrophila,* SYBR Green, real-time PCR, Tunisian cattle, abortion, vaginal swabs

1. Introduction:

Abortion in cattle is a major cause of economic losses due to (i) fetus death, (ii) cost of maintenance of non-producing animals and (iii) cost of prophylaxis in the case of certain diseases. In Tunisia, studies conducted to estimate the true prevalence of certain microbial agents of miscarriage in cattle were only occasional and limited to certain farms or regions. Consequently, the control programs are not very effective, even for established agents of abortion in cattle. Therefore, the etiology remains unknown in many cases of bovine abortion.

In cattle, several disease syndromes caused by Chlamydiae infection, including abortion and other uro-genital tract infections, pneumonia, conjunctivitis, enteritis, polyarthritis, encephalomyelitis, and mastitis, as well as subclinical infections, have been reported around the world (Holliman et al., 1994; Idtse, 1984; Perez-Martinez and Storz, 1985; Shewen, 1980; Stroz and Kaltenboeck, 1993). During the last 15 years, several *Chlamydia*-related bacteria have been discovered (Everett and Andersen, 1997; Greub and Raoult, 2002). These *Chlamydia*-related bacteria are widely distributed in nature and exhibit a diverse host range across the animal kingdom. Many of them live in close association with free-living amoebae and are able to infect mammals (Horn, 2008). Many studies have shown the involvement of *Parachlamydia* spp and related Chlamydia-like organisms in bovine abortion (Borel et al., 2007). *Waddlia chondrophila* is another Chlamydia-like organism belonging to the *Waddliaceae* family (Corsaro and Greub, 2006). This bacterium was initially isolated from lung, liver, and other tissues of an aborted bovine fetus in the United States (Dilbeck et al., 1990). This organism is now considered as an abortigenic agent with a worldwide distribution in cattle, based on a report of *Waddlia*-related abortion in Germany (Henning et al., 2002). A recent serological study revealed a significant statistical association between anti-*Waddlia* antibody titers and cows that have aborted (Dilbeck et al., 2003). Moreover, infection of bovine fetuses with *Waddlia* was associated with their deaths within 2 weeks (Dilbeck et al., 2003).

Detection of this obligate intracellular bacterium by culture can be difficult because of its inability to grow on axenic media. Till now, no information is avaible about the presence of *Waddlia* in Africa and about the prevalence of *Waddlia chondrophila* in Tunisian cattle.

Thus, we developed a real-time diagnostic PCR assay to specifically detect *Waddlia chondrophila* DNA from veterinary samples and applied it to vaginal swabs samples taken from Tunisian cows presenting an abortion.

2. Materials and methods

2.1 Animals and samples:

To assess the diagnostic test performance, the assay was then validated on cattle samples collected in twenty dairy herds from different regions of Sfax in Tunisia that experienced reproductive disorders and abortions, from October 2010 to January 2012. Herd sizes ranged from 20 to 1500 cows. In all herds, 150 cows suffered from miscarriage. All these 150 cows were sampled as well as 64 healthy cows. Practically, vaginal swab samples were collected in duplicate from each cow, using sterile swabs after vulva disinfection with chlorhexidine solution. All swabs were put into tubes containing 1 ml of 2-sucrose phosphate medium (2-SP). They were kept at 4 °C during transportation in ice-pack containers, and then stored at -80 °C until use.

2.2 Primer design:

Using Primer3 software (Rozen and Skaletsky, 2000), a forward primer WadF (5′-AGGTCGAGCGCTGAAATTCT-3′), and a reverse primer WadR (5′-AATTCCGCAGCTCGAAAAG-3′) were selected to amplify a 150 bp fragment of the *recA* gene of *Waddlia chondrophila* strain (ATCC VR-1470). The region selected was 100% conserved among *Waddlia chondrophila strain 2032/99*. PCR primers were to exhibit optimal biophysical properties and no dimer formation with Oligoanalyzer 3.1. Primers were also blasted against the

nucleotide database of the NCBI website to ensure the absence of significant homology with other microorganism sequences.

2.3 DNA extraction for the real-time PCR test:

The vaginal swab samples collected in 2-SP medium were thawed; 1 ml was transferred to a new micro tube, and then centrifuged at 13,000 × g for 20 min. The pellet was resuspended in 200 µl sterile water. The total volume was extracted by Quick-gDNA MiniPrep D3006 Kit (Zymo Research) as recommended by the manufacturer. Extracted DNA was re-suspended in 50 µl of elution buffer and stored at −20°C until subsequent analysis.

2.4 Real-time PCR assay development:

The real-time PCR assay was performed on a CFX96™ real-time PCR cycler (Biorad). The optimal concentration of primers was assessed by testing different concentrations (0.05, 0.15, 0.3, and 0.5 µM) and by defining the one that gave the highest recorded fluorescence and the lowest Ct. Each reaction was run in duplicate in a mastermix containing 10 µl of SsoAdvanced™ SYBR® Green Supermix (Biorad), 0.15 µM of each primer, and 1 µl of purified DNA to a final volume of 20 µl using nuclease-free water. The thermal cycling conditions were assessed testing different annealing temperatures (between 54 °C and 60 °C) during different times (5, 10, 20, 30 and 60 sec.). The optimal qPCR efficacy was obtained using cycling profile included an initial denaturation at 95 ◦C for 3 min, then 40 cycles of 10 s at 95 °C and 10 s at 55.5 °C. A melting curve analysis was performed using the following cycling parameters: 60 °C for 30 s, and 5 °C temperature changes to the end temperature of 95 °C. The generated melt peak represented the specific amplified product. In all experiments, each PCR run included a negative extraction control (sterile water) and a negative PCR control, containing 5 µl Diethylpyrocarbonate (DEPC) treated H_2O instead of DNA extract, to detect any possible contaminating DNA. Samples and controls were run in duplicate.

2.5 Positive recombinant plasmid control:

To facilitate bacterial quantification, a plasmid containing the target gene was constructed. DNA was extracted from *Waddlia chondrophila* strain (ATCC VR-1470) and the target sequence was amplified with WadF and WadR primers. The final 50 µl reaction mixture contained 0.4 mM each primer, 1 U/µl ITaq$^{TM\ DNA}$ polymerase (Biorad), 5 µl 10× PCR buffer containing (20 mM Tris/HCl, 100 mM KCl, 3 mM MgCl2, 400 mM dNTPs) and 1 µl *Waddlia chondrophila* DNA. PCR was performed in a CFX96 (Biorad) according to the following procedure: 10 min at 95 °C, 40 cycles at 95 °C for 30 s, 55.5 °C for 1 min, 72 °C for 1 min. PCR products were then purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and cloned into pCR 2.1-TOPO vector (Invitrogen) according to the manufacturer's protocol. Isolation of recombinant plasmid DNA was performed with Zyppy™ Plasmid Miniprep Kit (Zymo Research), and the presence of the correct insert was confirmed by sequencing. Plasmids were then linearized and quantified with a NanoDrop ND-1000 Spectrophotometer. Copy numbers of the cloned *recA* gene were derived from the molecular weights of the cloning vector and insert, diluted in 10 mM Tris–HCl, pH 8.0 to generate standards ranging from 1 to 10^5 molecules and stored at -20 °C.

2.6 Detection of the amplification product and sequencing:

Ten µl of each PCR product were electrophoresed in 2% agarose gel, stained with ethidium bromide and observed under UV illumination. The fragments at correct size were excised and further purified for sequencing. Briefly, DNA was extracted using the QIAquick Gel extraction Kit (Qiagen), was then subjected to cycle sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and processed by the ABI 3100 Genetic Analyzer. The obtained sequences were compared with the sequences available in GenBank by using the BLAST server from the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST).

2.7 Analytical specificity, efficiency, and reproducibility of the PCR:

The specificity of the new quantitative PCR was tested using DNA extracted from different bacteria commonly found in cases of miscarriage (Table 1). Using the positive control plasmid, the analytical sensitivity and the reproducibility of the PCR was assessed on duplicates with 10-fold dilutions (10^5 to 10 copies/reaction) in 10 independent runs. Intra-run reproducibility was assessed on 50 duplicates by plotting the Ct (threshold cycle) values of both duplicates against each other on a graph, by plotting the differences between both duplicates against the mean of each duplicate and by calculating the 95% confidence interval using the Bland-Altman algorithm (GraphPad 5.0). To assess the inter-run reproducibility, the mean Ct of duplicates obtained in 10 independent runs were compared for each concentration of plasmid DNA.

Mean, standard deviation (SD), and coefficient of variation (CV) were calculated in Excel. In particular, the CV was calculated as the percentage of the ratio of standard deviation and the mean of the number of copies.

2.8 Ethical considerations:

Samples were collected by clinical veterinarians as part of the usual screening scheme on farms and Tunisian ethical guidelines and animal welfare regulations were strictly respected. All herd owners had given an informed consent prior to the study.

3. Results and discussion:

Genomic DNA of *Waddlia chondrophila* strain (ATCC VR-1470) was successfully amplified by this *recA* gene real-time PCR with Ct values of (19.16 ± 0.21) when 10^5 DNA copies/µl were tested in duplicate (Table 2). The analytical sensitivity of the real-time PCR was about ten copies of plasmid control DNA. Indeed, when testing ten-fold dilutions of plasmid, none of ten replicates were positive at a concentration of one copy, whereas all 10 replicates were positive at a concentration of ten copies (Ct=33.40 ± 1.034) (figure 1A). The linearity and efficiency of the

SYBR Green real time PCR were determined by generating a standard curve in which serial 10-fold dilutions of recombinant plasmid were tested. The standard curve was generated by plotting the real-time PCR threshold cycle numbers (Ct) of each dilution against the known copy numbers of recombinant plasmid (figure 1A). For accurate quantification of PCR products, the slope of the standard curve obtained with 10-fold dilutions should approach -3.3 in theory, but a slope from -3.1 to -3.6 was acceptable in practice. Furthermore, the corresponding correlation coefficient should be >0.95. For this real time PCR the slope was −3.468 with a coefficient of determination ($R2$) > 0.989 and a reaction efficiency (E) of 0.943, calculated from the slope (S) using $E = 10(-1/S) - 1$(figure 1A).

The specificity of the reaction was confirmed by a melting temperature of 83 ℃ for standard plasmid dilutions, indicating the formation of a single PCR product with no artifacts, such as nonspecific amplification products or primer dimmers (figure 1B). No cross amplification was observed when testing microorganisms listed in Table 1, which indicates that the specificity of primers ensured a high degree of discrimination between the amplicon of *Waddlia chondrophila* and those of the other tested bacteria. Furthermore, amplification products were also checked on agarose gel 2% stained with ethidium bromide in standard TAE buffer. A clear and well-defined specific band of approximately 150 bp was visualized with all replicates of recombinant plasmid dilutions, except the concentration of 1 copy/μL (figure 1C).

As shown in figure 2 (panel A), intra-run reproducibility was confirmed, with Ct of both duplicates being relatively similar with a correlation coefficient r^2 of 0.995. As expected, intra-run variability was slightly higher at very low concentration of 10 plasmid copies/µl (33.4±1.033) (Table 2).

In figure 2 (panel B), the Bland-Altman graph revealed that the 95% confidence interval was 1.338 cycles. The inter-run reproducibility is shown in figure 2 (panel C). The inter-run variability was low, as expected at very low concentration of 10 plasmid copies/µl. The standard deviation values reflect small deviation from the mean and indicate a good reproducibility among

different runs (Table 2). Thus, the quantification was highly reliable from 10 to 10^5 copies, with an average difference of 3.5 cycles per ten-fold dilution. The linear relationship of the Ct with copies numbers of recombinant plasmid/µl suggests that this method is a valuable and accurate tool for quantitative evaluation of *Waddlia*. Also data provided from coefficient of variation (CV) were reported to range around the value of 1% (Table 2). This percentage is quoted in most of the literature dealing with the field and should be considered satisfactory.

The *Waddlia chondrophila* real-time PCR optimized in this study showed strong qualities in terms of sensitivity, specificity and a reproducibility comparable to those of other diagnostic TaqMan PCR assays (Goy et al., 2009; Jaton et al., 2006). This real-time PCR is more sensitive than the 16SigF-Rp2Chlam broad range PCR (Thomas et al., 2006), which detects about only 1000 copies of DNA (Casson et al., 2008). Thus, this real-time PCR allows detection of *Waddlia* DNA at this amount that would not be detected by classical PCR. This increased sensitivity is likely a result of the real-time technology, which combines amplification of small length amplicons and detection with highly sensitive fluorescent intercalating agents.

Since several lines of evidence are supporting the role of *Waddlia chondrophila* as an agent of bovine abortion, we applied real-time PCR to 150 vaginal swabs obtained from cows suffering from abortion and to 64 samples taken from cows without abortion. *Waddlia* DNA was detected in twelve samples, all obtained from cows suffered a miscarriage ($p<0, 05$). Bacterial quantities in positive samples ranged from 10 to 1307 copies/µl (Table 3). Melting curve analysis showed a single peak at 83 °C for each positive vaginal sample (figure 3A). In conventional gel PCR, the twelve samples positive by real-time PCR produced amplicon of 150 bp when resolved on an agarose gel (figure 4B). To confirm that positive samples were due to the presence of *Waddlia chondrophila* DNA, PCR products were sequenced and analysed by BLAST web interface (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained presented 100% identity with *Waddlia chondrophila recA* gene (data not shown). The results for all 12 positive samples were confirmed by the 16S rRNA real-time PCR previously described by Goy et al (2009). In this

study, we thus found 12 (8%) bovine vaginal samples positive for *Waddlia chondrophila* DNA in the subset of 150 samples. These 12 samples tested also negative for *Chlamydia abortus, Chlamydia pecorum, Brucella* spp*, Brucella abortus, Brucella melitensis, Coxiella burnetii* and *Salmonella* spp. In two of the 12 cases (16.66%) positive for *Waddlia, Listeria monocytogenes* DNA, could be detected by a specific real time PCR. To our knowledge the combination of these two abortive agents has not been described yet. For the 10 remaining cases, no other agents have been detected and no other conditions explaining miscarriage was documented. Thus, our data strongly supports a role for *Waddlia* in miscarriage. A PCR contamination is unlikely expected, all samples have been extracted with similar protocols and none of the 64 samples taken from healthy cows were positive. In addition, all the extraction controls remained negative for *Waddlia*.

The current PCR may also help to detect the *Waddlia* bacteria in pets and this may be important in term of human health, given the zoonotic risk of *Waddlia* transmission from pets to humans, demonstrated by the association of anti-*Waddlia* seropositivity with exposure to pets (Baud et al., 2007) and given the role of *Waddlia* as an agent of miscarriage in humans (Baud et al., 2007; Baud et al., 2008; Baud and Greub, 2011; Baud et al., 2011). Some epidemiological data have been gathered on the epidemiology of *Chlamydia abortus* and *Chlamydia pecorum* infections in Tunisia (Rekiki et al., 2002; Berri et al., 2009; Mohamed et al., 2010), however no data on *Waddlia chondrophila* prevalence have yet been reported in Tunisia and even in any African country.

In conclusion, the present real-time PCR method provides an accurate tool for the molecular diagnosis of *Waddlia chondrophila* infection. The assay is reproducible and sensitive enough to detect concentration of 10 copies of bacteria in clinical veterinary samples and may be used in the future for further epidemiological investigations of *Waddlia* spread.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Mohamed Barkallah, Imen Fendri, Amina Dhieb and Yaakoub Gharbi designed the experiments, analyzed the data and drafted the manuscript. Gilbert Greub and Radhouane Gdoura conceived research and approaches and have given final approval of the manuscript to be published. All authors read and approve the final manuscript.

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Table 1: Bacterial species used to determine the specificity of the real-time PCR.

Bacterial species	**Source/strain**
Chlamydia suis	S45
Chlamydia abortus	S26
Chlamydia pecorum	W73
Chlamydia psittaci	T49
Chlamydia trachomatis	Clinical specimen
Parachlamydia acanthamoebae	ATCC VR-1476
Brucella abortus	S19
Escherchia coli	ATCC 8739
Listeria monocytogenes	ATCC 19115
Staphylococcus aureus	ATCC 25923
Neisseria ovis	Clinical specimen
Pseudomonas aeruginosa	ATCC 27853
Streptococcus bovis	ATCC 33317

Table 2: Intra and inter-run standard deviation (S.D.) and coefficient of variation (CV) of mean threshold cycle (Ct) for gene real-time PCR performed on 10 replicates in a single run or in ten different runs.

Reproducibility setting	**Control plasmid (copies/µl)**	**n**	**Ct mean**	**S.D. (±)**	**CV (%)**
Intra-run	1×10^5	10	19.16	0.21	1.04
	1×10^4	10	22.26	0.23	1.04
	1×10^3	10	25.61	0.34	1.35
	1×10^2	10	28.95	0.31	1.1
	1×10^1	10	33.4	1.033	3.1
	1	10		ND	
Inter-run	1×10^5	10	19.17	0.22	1.14
	1×10^4	10	22.3	0.26	1.17
	1×10^3	10	25.62	0.4	1.55
	1×10^2	10	29.17	0.25	0.88
	1×10^1	10	33.27	1.18	3.57
	1	10		ND	

ND, no detection

Table 3: Demographic and clinical characteristics of cows with presence of *Waddlia chondrophila* DNA in twelve genital samples and results of the real-time PCR.

Animal no.	**Age**	**Sex**	**Specimen**	**Other information**	**recA real-time PCR mean Ct value (DNA copies/µl)**
1	2 years	Female	Vaginal swab	Abortion	32,47 (27 copies)
2	2 years	Female	Vaginal swab	Abortion, fatigue	33,39 (11,31copies)
3	2 years	Female	Vaginal swab	Abortion, fever, loss appetite, weakness, diarrhea	25,21 (1307 copies)
4	3 years	Female	Vaginal swab	Abortion	33,38 (12,44 copies)
5	3 years	Female	Vaginal swab	Abortion, respiratory diseases	32,5 (25,69 copies)
6	2 years	Female	Vaginal swab	Abortion, diarrhea	28,33 (190 copies)
7	3 years	Female	Vaginal swab	Abortion	32,8 (17 copies)
8	3 years	Female	Vaginal swab	Abortion	33,4 (9,4 copies)
9	4 years	Female	Vaginal swab	Abortion, fever, loss appetite	28,55 (127 copies)
10	2 years	Female	Vaginal swab	Abortion	33,39 (11,51 copies)
11	2 years	Female	Vaginal swab	Abortion	32,74 (19 copies)
12	4 years	Female	Vaginal swab	Abortion, fever, diarrhea	31,59 (48 copies)

Figure 1 Amplification, standard and melting curves of the real-time PCR assay.

(A) Amplification curves were generated by fluorescence data collected at each cycle during the final phase of the PCR. Values are duplicates of different dilutions of the control plasmid used as standard. Control plasmid copy numbers per sample were 10^1, 10^2, 10^3, 10^4, 10^5 and negative control (NC). The R^2 linearity value from linear regression is 0.989.

(B) Conventional melt curves of serial dilutions of control plasmid DNA, in green different signals obtained from the standard plasmid dilutions (copy numbers are 10^1 to 10^5).

(C) Real-time PCR reaction products checked on agarose gel 2 %: serial 10-fold dilutions of control plasmid. Specific bands of approximately 150 bp were visualized for all replicates of recombinant plasmid dilutions, except the concentration of 1 copie/μL. L : MassRuler Low-Range DNA Ladder (Fermentas, Burlington, Ontario, Canada). 1, 2, 3, 4, 5, and 6: control plasmid dilutions with concentrations of 10^5, 10^4, 10^3, 10^2, 10, 1 copies/µl, respectively. W: no template control (water).

Figure 2 Reproducibility of the real-time PCR.

Inter and intra-run reproducibility was evaluated among ten different runs representing 50 duplicates of a positive control. (A) Plot of cycle threshold (Ct) values of first duplicate versus second duplicate showing intra-run reproducibility. The solid line represents the linear regression whereas the dashed lines represent the 95% prediction interval. (B) Bland-Altman graph representing the Ct difference between both duplicates according to the average Ct of duplicates. The dashed line represents the 95% confidence interval (CI 95%). (C) Inter-run variability.

Figure 3 Real-Time PCR melt curve data and 2% agarose gel image for determining primer specificity and product size.

The Real-Time PCR melt curve data identifies one peak with a melting temperature of 83 °C for all reaction wells. (A) In green: signal obtained from the plasmid dilution 10^5; in black: signals obtained from different positives samples; derivative −dRFU/dT where RFU is relative fluorescence units and T is time; NC: negative control. This indicates the presence of only one PCR product. (B) Real-time PCR reaction products checked on agarose gel 2 %: specific bands of approximately 150 bp were visualized for all positives samples. L: MassRuler Low-Range DNA Ladder (Fermentas, Burlington, Ontario, Canada). Lane 1 to 12 : the twelve positive samples. W: no template control (water).