Published in final edited form as:

Mol Cell Probes. 2004 April ; 18(2): 111–116. doi:10.1016/j.mcp.2003.10.002.

Development of a *p28*-based PCR assay for *Ehrlichia chaffeensis*

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Abstract

Detection of *Ehrlichia chaffeensis* is necessary to study interactions between the parasite and its vertebrate and invertebrate hosts. The purpose of this study was to develop a sensitive, specific PCR assay for *E. chaffeensis* based on the outer membrane protein gene, *p28*. Candidate primer sets were identified and ranked based on annealing scores, similarities to three major *p28* sequence clusters, dissimilarity to *E. canis p30*, an ortholog of *p28*, and the proximities of flanking primer sequences for nested PCR. The relative sensitivities of five optimized single-step and two nested PCR assays were determined, and the most sensitive assay was found to be a single-step PCR that was as much as 1000-fold more sensitive than a standard 16S rDNA-based nested PCR assay. This *p28*-based PCR assay amplified the target amplicon from isolates representative of all three major clusters of known *p28* sequences, and this assay did not amplify template prepared from either of the two species most closely related to *E. chaffeensis*, *E. canis* and *E. muris*. These results indicate that this sensitive, specific and isolate-universal single-step PCR assay will be a useful tool in characterizing the transmission of this important zoonotic pathogen.

Keywords

Ehrlichia chaffeensis; Human monocytic ehrlichiosis; PCR assay

1. Introduction

Ehrlichia chaffeensis is the agent of human monocytic ehrlichiosis (HME), a zoonotic tick-borne disease found in the United States and possibly worldwide [1,2]. This organism is classified in the order Rickettsiales, family Anaplasmataceae, which includes vector-borne, obligate intracellular bacteria that reside within parasitophorous vacuoles [3]. *Ehrlichia* species described to date are reported to utilize ticks of the family Ixodidae as biological vectors, and *E. chaffeensis*, like *E. canis* and *E. muris*, infects monocytes of its mammalian hosts. An accurate prevalence of HME is not known at this time because of the range of mild to serious symptoms, because HME is not reportable in many states and because HME is often misdiagnosed as Rocky Mountain spotted fever (RMSF). Misdiagnosis of HME is

even more troubling when RMSF is also discounted due to symptomatic differences between the diseases, and an ineffective or potentially harmful treatment of affected patients is prescribed [4]. A rapid, sensitive test for *E. chaffeensis* infection is needed.

E. chaffeensis is difficult to detect in its hosts, due in large part to its low levels in vertebrate blood. Therefore, the most widely used assays for *E. chaffeensis* in mammals are serological, which are limited to vertebrate hosts and only indicative of exposure rather than active infection. Direct detection of this pathogen in tick and mammalian hosts would be useful for experimental and epidemiological studies, diagnosis and monitoring of infections.

A sensitive, specific PCR assay is the most feasible approach to detection of *E. chaffeensis* in both mammals and ticks. Most *E. chaffeensis*-specific PCR assays reported to date are based on nested primer sets that anneal to the 16S rRNA gene (16S rDNA) [5-11]. These nested PCR assays have been used extensively to detect the pathogen in mammals and pools of naturally exposed ticks. More recently, single-step reverse transcription (RT)-PCR has also been utilized to amplify an outer membrane protein-1 (*omp-1*) paralog, *p28-10*, from pools of experimentally infected ticks [12], indicating that an outer membrane protein gene sequence could provide an even more sensitive PCR assay for this organism. Indeed, an assay based on *p30*, which is the *E. canis* homolog of *p28*, indicated that this assay was significantly more sensitive than the respective 16S rDNA-based assay for that organism [13]. This finding could be because species-specific nucleic acid sequences, such as those often encoding outer membrane proteins, can provide greater ranges of unique targets for design of primers for PCR assays [14,15]. Several such PCR assays have been used to directly detect other anaplasma pathogens within both vertebrate and invertebrate hosts [13,16,17].

The purpose of this study was to develop and evaluate a PCR assay with primers based on the *E. chaffeensis* outer membrane protein, *p28*. This paralog from the *E. chaffeensis omp-1* multiple gene cluster was chosen because it has been characterized from several *E. chaffeensis* and *E. canis* isolates, allowing the design of primers predicted to be both common and restricted to the target species [13-15]. Several candidate primer sets were considered, resulting in a single-step PCR assay that was species-specific for *E. chaffeensis*, to amplify DNA from isolates representative of the three major *p28* sequence clusters, and to be more sensitive than a conventional nested 16S rDNA-based PCR assay.

2. Materials and methods

Ehrlichia template DNA

E. chaffeensis (Arkansas, St. Vincent and 91HE17 isolates) and *E. canis* (Ebony isolate) were cultivated in a DH82 cell line as previously reported [18]. The *E. chaffeensis* 91HE17 isolate was kindly provided by Dr. David Walker at the University of Texas Medical Branch at Galveston, and the *E. chaffeensis* St. Vincent isolate was purchased from ATCC (Manassas, VA). DNA was isolated from infected host cells by protein digestion (0.1 mg/ml proteinase K, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.45% (v/v) Tween-20, 0.45% (v/v) NP40) at 55 °C for 1 h) followed by phenol/chloroform extraction and ethanol precipitation as previously described for *E. canis* [13]. DNA from *E. muris* was provided as reported elsewhere [19].

PCR assays

The *E. chaffeensis p28* and *E. canis p30* sequences used for primer design are reported elsewhere [14,15]. Candidate primer sequences were identified with Prime software (Wisconsin Package V. 10.2; Genetic Computer Group [GCG], Madison, WI) and further evaluated with Pileup (GCG) as previously described with a few modifications [13]. Briefly,

primer sets were identified from the *p28* open reading frame (ORF) of the *E. chaffeensis* Arkansas isolate or from a consensus of *p28* ORF sequences representing the Arkansas, 91HE17 and St. Vincent isolates. These primer sets were ranked based upon annealing score, identity to *p28* ORFs representative of other major clusters and divergence from the homologous *E. cains p30* ORF. Five of the 45 primer sets were selected for optimization and evaluation based upon rank and relative positions flanking or internal to other primer sets for nested PCR. The nested 16S rDNA-based PCR assay was performed as previously described [11].

Optimization of PCR assays

E. chaffeensis (Arkansas isolate) DNA isolated from infected DH82 cells was used throughout the optimization process. Each PCR assay was performed in either an Applied Biosystems 2400 or 9600 thermal cycler and each reaction consisted of 1X PCR buffer and 0.2 mM deoxynucleoside triphosphate mix (dNTP). The primers, dNTP mix and platinum *Taq* polymerase were purchased from Invitrogen (Carlsbad, CA). The reaction profile, unless stated otherwise, consisted of 94 °C for 2.0 min; followed by 25 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 1 min; followed by a final extension at 72 °C for 7.0 min. The five single-step assays were progressively optimized for concentrations of MgCl₂ (0.0 to 5.0 mM in increments of 0.5 mM), primers (0 to 1.0 μM in increments of 0.1 μM), platinum *Taq* polymerase (0 to 90 units/ml in increments of 5 units/ml), followed by annealing temperature (45 to 70 °C in increments of 5 °C) and cycle number (20 to 65 cycles in increments of 5 cycles).

Each PCR product (20 μl) was added to 5 μl loading buffer (40% glycerol, 1X TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA), and separated by electrophoresis on a 1.5% agarose gel with 0.5 μg of ethidium bromide per ml in 1X TBE at 80 V for 1.5 h. A 100 bp ladder (Invitrogen, Carlsbad, CA) was used as the molecular weight standard. Ethidium bromide staining intensities of target amplicons were compared to identify optimum parameters.

Sensitivity and specificity

The relative sensitivities of PCR assays were compared by amplification of the same tenfold dilution series of *E. chaffeensis* (Arkansas isolate) DNA template. Specificity of the most sensitive *p28*-based PCR assay was tested by attempted amplification of 1 ng of *E. canis* and *E. muris* DNA preparations.

DNA sequencing

Amplicons were purified with a QIAquick PCR purification kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's directions, and submitted to The Plant-Microbe Genomics Facility at The Ohio State University for cycle sequencing in the presence of both the forward and reverse primers of the selected primer set.

3. Results

Primer design

Of 45 primer sets identified, forward primers, ranging 18 to 25 nucleotides in length, were found between nucleotides 15 and 101 of the *p28* ORF. The reverse primers, ranging 18 to 26 nucleotides in length, were found between nucleotides 293 and 674 of the *p28* ORF. Five primer sets were selected for further analysis (Table 1). Primer sets 1U and 3U were predicted to be universal to all three isolate clusters, while primer sets 11A, 13A and 20A appeared to be specific to the Arkansas isolate sequence. Each primer set tested amplified a single amplicon of the expected size from 50 ng of DNA from *E. chaffeensis*-infected cells under non-optimized conditions (Fig. 1).

Optimization of selected primer sets

Optimum PCR conditions for each primer set were determined to ensure the greatest sensitivity and specificity possible for each assay. The optimized reaction parameters of each primer set are shown in Table 2.

Characterization of the *p28*-based PCR assay

Development of a sensitive assay for detection of *E. chaffeensis* was the primary objective of this work. Thus, once optimized, the relative sensitivities of five single-step and two nested PCR assays were compared to identify the most appropriate assay for detection of *E. chaffeensis*. The single-step PCR assay with primer set 1U was found to be the most sensitive of the *p28*-based assays. The sensitivity of this assay was then compared to a previously described 16S rDNA-based assay to determine if the new *p28*-based assay would be more suitable for detection of *E. chaffeensis* under experimental conditions. This question was addressed by amplification of the same tenfold template dilution series with both assays (Fig. 2). The assay with *p28* primer set 1U amplified the 277 bp target amplicon from each template up to the 1×10^{-5} dilution, while the 389 bp target was amplified from dilutions up to 1×10^{-2} with the nested 16S rDNA-based assay.

Generation of the appropriate amplicon from divergent isolates of the target species is another desirable trait that was predicted for this PCR assay. Analysis of *p28* ORF sequences indicated that primer set 1U should be universal for different isolates of *E. chaffeensis*. This prediction was verified by testing primer set 1U with representatives of the three major *p28* ORF sequence clusters: Arkansas, St. Vincent and 91HE17 [15]. Identities of the three *E. chaffeensis* isolate templates were confirmed by comparison of 620 bp amplicons generated from each template with primer set 3U (Table 1) to the published sequences for each isolate (data not shown). The 277 bp target amplicon generated by primer set 1U was observed for all three *E. chaffeensis* isolates tested (Fig. 3).

Specificity of the assay is also important for diagnosis of natural infections and for experimental studies involving mixed infections with different pathogen species. Identity to the corresponding *E. canis p30* sequence was a criterion in the selection of *p28*-based primers, because it was expected that primers that fail to amplify DNA from a closely related species such as *E. canis* would be specific for *E. chaffeensis*. To confirm this expectation, the ability of primer set 1U to amplify DNA from *E. canis* or *E. muris* was tested. The 1U-based PCR assay failed to amplify DNA template from either *E. canis* or *E. muris*, while the 277 bp target amplicon was produced from the same amount of *E. chaffeensis* DNA (Fig. 4).

4. Discussion

Direct detection of pathogens in vertebrate and invertebrate hosts is important for experimental studies and ecological investigations of naturally occurring disease. This capability is particularly important for organisms, like *E. chaffeensis*, that produce a wide range of mild to severe symptoms in human beings, but appear to produce mild or subclinical signs in potential reservoir hosts [2,20-22]. Serologic tests for documenting exposure to pathogens can be useful for routine diagnosis and identification of potential vertebrate reservoirs, but such tests are not indicative of active infection and are not easily adapted to detecting invertebrate exposure to these organisms. Conversely, molecular diagnostic techniques have contributed extensively toward detection of active infections in vertebrate and invertebrate hosts, primarily due to their high sensitivity and adjustable specificity to organisms of interest. For *E. chaffeensis*, the development of a nested 16S rDNA-based PCR assay has resulted in accumulation of considerable information, much of

which has involved identification of infected mammalian and tick hosts in nature. However, PCR assays designed with species-specific sequences, rather than sequences that are highly conserved among bacteria, are likely to provide a broad selection of both optimally sensitive and specific primer sequences.

The approach to primer design for *p28*-based PCR was similar to that employed for a *p30*-based PCR assay for *E. canis* [13]. In contrast to *p30*, which is identical among *E. canis* isolates reported to date [14], *p28* sequences fall into three different clusters representing different *E. chaffeensis* isolates [15]. Thus, this approach required some modification, and two strategies were used for identification of optimal *p28* primer sequences. One strategy was to identify primer sets for *p28* of the most extensively experimentally characterized *E. chaffeensis* isolate, Arkansas. This approach produced numerous candidate primer sets with better overall annealing scores under more stringent criteria for primer selection. Although these primer sets were predicted to provide suitable PCR assays, these assays might not have been able to detect isolates within the two remaining *p28* sequence clusters. This potential frailty was acceptable for experimental transmission studies with that isolate, but primers expected to amplify target from all reported *p28* ORFs would provide a more versatile assay. Thus, the second strategy for primer design utilized a consensus of the three major *p28* sequences described for different isolates of *E. chaffeensis*. As expected, the latter strategy yielded fewer optimal candidate primer sets, but, interestingly, this approach also produced the most sensitive primer set tested in this report.

Repeated comparison of the relative sensitivities of the five single-step and two nested assays revealed that the most sensitive assay developed was a single-step PCR. This observation was encouraging because single-step PCR assays offer several advantages over nested PCR, including reduced labor, reduced cost, reduced likelihood of carryover contamination (a particularly important concern when transferring template from a primary to a nested PCR) and adaptability to quantitative techniques such as competitive or real time PCR. Another fortuitous property of our most sensitive assay was that these primers were predicted to be universal for all three *p28* sequence clusters, so this assay was predicted to be useful for detection of different isolates of *E. chaffeensis*. Although these primers were shown to amplify template from three isolates representing different *p28* sequence clusters, it is unlikely that the full scope of *p28* gene diversity among *E. chaffeensis* isolates is fully known and more studies will be required to confirm the universality of these primers for future isolates.

The relative sensitivity of this assay to the nested 16S rDNA-based PCR 'standard' was required to estimate the potential utility of this assay for experimental investigations. In our hands, this single-step *p28*-based PCR assay was 1000-fold more sensitive than the nested 16S rDNA-based PCR assay for *E. chaffeensis*. Previous work has shown that RT-PCR of 16S rRNA is 100-fold more sensitive than the same nested 16S rDNA-based assay [11]. Although application of RT-PCR to a *p28*-based PCR assay could also be worthwhile, there are some important considerations prior to application of such an assay. One concern would be the different mRNA levels of different *p28* paralogs during the course of *E. chaffeensis* infections, which could result in varying sensitivity of the assay and would preclude any potential quantification of infection levels based on original template copy numbers. Indeed, it has been reported that the *omp-1* paralog used for this assay, *p28*, is not transcribed in the tick host [12]; thus adaptation of RT-PCR with *p28* primers would only be expected to detect *E. chaffeensis* in vertebrate host cells. Another concern with RT-PCR is the inherent difficulty in isolating and preserving prokaryotic RNA template, which could make this technique minimally suitable as a diagnostic test. Another promising single-step 16S rDNA-based assay that uses real time PCR to detect an 84 bp *E. chaffeensis* DNA target has been reported [23]. The latter assay was reported to detect the equivalent of 10 ehrlichial

genomes, and demonstrated to amplify *Anaplasma phagocytophilum* and all *Ehrlichia* spp. tested with SYBR Green detection or to specifically detect *E. chaffeensis* when TaqMan detection chemistry was employed.

Specificity to *E. chaffeensis* is also an important consideration for applications that involve mixed experimental infections with different *Ehrlichia* spp. or natural exposure to the pathogen. Although the primers for this *p28*-based assay were expected to be specific for *E. chaffeensis* due to their divergence from corresponding sequences of *E. canis*, the potential amplification of other copies in the *omp-1* gene family of other *Ehrlichia* spp. should not be ignored. In this study, we demonstrated the failure of primer set 1U to amplify the target amplicon from DNA templates isolated from *E. canis* and *E. muris*, the two known species most closely related to *E. chaffeensis* as determined by 16S rDNA homology [3], indicating that these primers are not likely to amplify this 277 bp amplicon from more distantly related pathogen species that parasitize ticks and mammals. Species-specific amplification of a product that is readily distinguished from smaller primer artifacts, without a hybridization step, allows the versatility to detect the *E. chaffeensis*-specific amplicon in agarose gels and, potentially, with various real time detection chemistries.

This sensitive, species-specific and potentially isolate-universal single-step PCR assay is expected to be useful for detection of experimental and natural infections of *E. chaffeensis* in vertebrate and invertebrate hosts in which the pathogen load can be low. Development of procedures to utilize this assay to detect *E. chaffeensis* in experimentally infected mammalian and ticks hosts is under way, and further evaluation of this assay for application as a diagnostic test is warranted.

Acknowledgments

This work was supported by National Institutes of Health Grant R01 AI 47932 (R.W.S.). We thank Drs David Walker and Donald Bouyer of the University of Texas Medical Branch at Galveston for the *E. chaffeensis* 91HE17 isolate, and we acknowledge the technical assistance of D.L. Grover and N. Theus.

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Fig. 1.

Non-optimized amplification of *E. chaffeensis* DNA with different primer sets. All five primer sets were tested in duplicate reactions with 50 ng of *E. chaffeensis* DNA as described in the text. Lanes 1 and 2 represent reagents and template without primers as a negative control, lanes 3 and 4 represent primer set 20A, lanes 5 and 6 represent primer set 1U, lanes 7 and 8 represent primer set 13A, lanes 9 and 10 represent primer set 3U and lanes 11 and 12 represent primer set 11A. The molecular weight standard (M) is a 100 base pair ladder.

Fig. 2.

Relative sensitivity of the *p28*-based PCR assay with primer set 1U. PCR was performed with the 16S rDNA nested primer sets (A) and the *p28* primer set 1U (B) with the same tenfold dilution series of *E. chaffeensis* DNA. A 100 base pair ladder (M) served as the size standard. Lanes 1-6 represent assays of 1×10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} or 10^{-5} dilutions, respectively, of 1 ng of DNA template prepared from *E. chaffeensis*-infected DH82 cells as described in the text.

Fig. 3.

Detection of *E. chaffeensis* isolates representative of three major *p28* clusters. Lanes 3, 4 and 5 are assays of 50, 5 and 0.5 ng, respectively, of DNA extracted from Arkansas isolate-infected cells. Lanes 6, 7 and 8 are assays of 50, 5 and 0.5 ng, respectively, of DNA extracted from St. Vincent isolate-infected cells. Lanes 9, 10 and 11 are assays of 50, 5 and 0.5 ng, respectively, of DNA extracted from 91HE17 isolate-infected cells. Lane 1 is a template-free control and a 100 base pair ladder (M) served as the size standard.

Fig. 4. Specificity of the *p28*-based PCR assay with primer set 1U. Lanes 1 and 2, 3 and 4 and 5 and 6 represent duplicate PCR assays of equivalent amounts of *E. chaffeensis* (Arkansas isolate), *E. canis* (Ebony isolate) and *E. muris* DNA, respectively. The molecular weight standard (M) is a 100 bp ladder.

Table 1Oligonucleotide sequences of *E. chaffeensis* p28 primer sets

Primer set	Forward	Reverse	Amplicon size (bp)
1U	ECH84-101 (aggtagtgggtattaacgg)	ECH360-341 (agatacttcaagctctattc)	277
3U	ECH50-68 (cttctctacgtggagtac)	ECH669-649 (gcttatagtagcttaaac)	620
11A	ECH58-78 (cctggagtatcattttccgac)	ECH341-320 (cttgaccatccattgagtaac)	284
13A	ECH78-99 (cccagcaggtagtggtattaac)	ECH314-294 (gctcctgcaaacctaaaaac)	237
20A	ECH78-99	ECH352-331 (caagctctattcttggaccatc)	275

Table 2

Optimized parameters for different primer sets

Primer set	MgCl ₂ (mM)	Primer (μM)	Enzyme (U/μl)	Annealing temperature (°C)	Cycle number
1U	2.0	0.9	0.07	50	60
3U	5.0	0.8	0.05	50	50
11A	4.0	0.7	0.06	65	55
13A	3.5	0.6	0.05	65	50
20A	3.0	0.8	0.01	65	50