Comparative Immunology, Microbiology and Infectious Diseases 39 (2015) 39–45

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Development of a new PCR-based assay to detect *Anaplasmataceae* and the first report of *Anaplasma phagocytophilum* and *Anaplasma platys* in cattle from Algeria

Mustapha Dahmani[a], Bernard Davoust[a], Mohamed Seghir Benterki[b], Florence Fenollar[a,c], Didier Raoult[a,c], Oleg Mediannikov[a,c,*]

[a] *Research Unit of Emerging Infectious and Tropical Diseases (URMITE), UMR CNRS 7278 IRD 198 INSERM 1095, Aix-Marseille Université, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France*
[b] *Clinique vétérinaire Le Refuge, Batna, Algeria*
[c] *Research Unit of Emerging Infectious and Tropical Diseases (URMITE), UMR CNRS 7278 IRD 198 INSERM 1095, Institut de Recherche pour le Développement, Centre IRD Hann, BP 1386, Dakar, Senegal*

ARTICLE INFO

Article history:
Received 11 December 2014
Received in revised form 6 February 2015
Accepted 16 February 2015

Keywords:
Anaplasma phagocytophilum
Anaplasma platys
Anaplasmataceae
PCR assay
23S rRNA gene
Cattle
Algeria

ABSTRACT

Bovine anaplasmosis is a hemoparasitic disease considered as a major constraint to cattle production in many countries. This pathology is at least partially caused by *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Anaplasma centrale*, and *Anaplasma bovis*. The global threat and emergence of these species in animals require the reliable identification of these bacteria in animal samples. In this study, we developed a new qPCR tool targeting the 23S rRNA gene for the detection of *Anaplasmataceae* bacteria. The primers and probe for the qPCR reaction had 100% specificity and could identify at least *A. phagocytophilum*, *A. marginale*, *A. centrale*, *Anaplasma ovis*, *Anaplasma platys*, *Ehrlichia canis*, *Ehrlichia ruminantium*, *Neorickettisa sennetsu*, and *Neorickettsia risticii*. We used this tool to test samples of bovines from Batna (Algeria), an area from which bovine anaplasmosis have never been reported. We identified three genetic variants of *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. "variant 4". This finding should attract the attention of public authorities to assess the involvement of these pathogens in human and animal health.

1. Introduction

Bovine anaplasmosis (ehrlichiosis) is a hemoparasitic disease considered a major constraint to cattle production in many countries [1]. This infection can be caused by *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Anaplasma centrale*, or *Anaplasma bovis* [2]. *A. phagocytophilum* is the agent of human granulocytic ehrlichiosis [3]. This infection is reported worldwide and is considered the most widespread tick borne infection in animals in Europe [4], essentially in ruminants [5]. *A. marginale* is distributed worldwide and causes considerable economic loss in the dairy industry [6]. *A. centrale* is occasionally associated with clinical diseases and is used as a live vaccine in Israel, Australia, Africa, and South America [7]. These two bacteria infect erythrocytes and cluster together and apart from the granulocytic *A. phagocytophilum*, supporting the interpretation that they are within distinct genera [3]. Information about *A. bovis* is still limited compared

* Corresponding author at: Research Unit of Emerging Infectious and Tropical Diseases (URMITE), UMR CNRS 7278 IRD 198 INSERM 1095, Institut de Recherche pour le Développement, Centre IRD Hann, BP 1386 Dakar, Senegal. Tel.: +221 338493580.
E-mail address: olegusss1@gmail.com (O. Mediannikov).

http://dx.doi.org/10.1016/j.cimid.2015.02.002
0147-9571/

with other *Anaplasmataceae* with no type strain available. *A. bovis* was detected in Africa and Asia [8–10] causing bovine ehrlichiosis, and it is considered phylogenetically more closely related to *A. phagocytophilum* than to *A. marginale* or *A. centrale*. *Morulae* are found in the monocytes of infected cattle [2]. *Anaplasma platys* has been considered a pathogen that mostly infects dogs, but recently, a strain of *Anaplasma* sp. closely related to *A. platys* was detected in ruminants from Sicily; however, the author did not report any symptoms associated with this infection [11].

In Algeria, there is a lack of information about the *Anaplasmataceae* infection in ruminants. Furthermore, the species implicated in the pathology of bovine anaplasmosis in this country has not been reported. Consequently, in order to screen these bacteria we have developed new qPCR tool based on the 23S ribosomal RNA gene that have broad sensitivity (*Anaplasmataceae* family-specific) and can amplify bacteria belonging to the *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* genera. The following identification of the bacterial species was based on sequencing of the amplicons of a portion of 23S- and 16S rRNA genes. We have applied this new diagnostic tool to blood samples of bovines from Algeria that exhibited the characteristic symptoms of bovine anaplasmosis in order to identify and genetically characterize the *Anaplasmataceae* responsible for this infection.

2. Materials and methods

2.1. Design of the new system of diagnosis

2.1.1. Anaplasmataceae family-specific probe and primers design

In order to develop a qPCR-based tool for the screening of the samples for the presence of already known and potentially unknown obligatory intracellular bacteria from the *Anaplasmataceae* family, several genes were primarily tested to develop the *Anaplasmataceae* family-specific set of primers. Among those tested, the *rpoB* and *groEL* genes were considered too diverse, with few common patterns within the different species of *Anaplasmataceae* (data not shown). 16S rRNA (*rrs*) was determined to be not sufficiently diverse for discriminating among closely related species, such as *Anaplasma ovis*, *A. marginale*, and *A. centrale* [12]. The gene encoding the 23S subunit of ribosomal RNA (*rrl*) was selected for its large size and the presence of, on the one hand, highly conserved regions that enabled developing the family-specific oligonucleotide and, on the other hand, sufficient discrimination between closely related species of *Anaplasmataceae*.

The sequences of *A. phagocytophilum* (CP006616 and CP006618), *A. centrale* (CP001759), *A. marginale* (NR076579, CP000030 and CP006847), *Ehrlichia chaffeensis* (AF416765), *Ehrlichia canis* (NR076375), *Ehrlichia ruminantium* (NR077000), *Ehrlichia muris* (CP006917), and *Ochrobactrum anthropi* (NR076113) were aligned using the BioEdit softwar [13]. A conserved region specific for *Anaplasmataceae* bacteria was chosen to design a set of primers and probe for the real time PCR assay and three primers for the conventional PCR assay. Primers sequences for the qPCR assay were designed to generate a 169 bp fragment with the Taqman® probe. The primers of the two conventional PCR assays shared the same forward primers and generated a fragment of approximately 700 bp and 500 bp, respectively (Table 1). The overall objective was to design a tool capable to identify most bacteria belonging to the family of *Anaplasmataceae* and potentially, a new species. The approach was to use the 23S-based qPCR for the screening of the samples and to identify the species based on the separately amplified larger portions of 23S and 16S rRNA-coding genes.

Table 1
Primers and probes used in this study.

Primers and probe	Sequences 5′–3′
23S rRNA gene	
TtAna-f	TGACAGCGTACCTTTTGCAT
TtAna-r	TGGAGGACCGAACCTGTTAC
TtAna-s	FAM-GGATTAGACCCGAAACCAAG-TAMRA
Ana23S-212f	ATAAGCTGCGGGGAATTGTC
Ana23S-908r	GTAACAGGTTCGGTCCTCCA
Ana23S-753r	TGCAAAAGGTACGCTGTCAC
16S rRNA gene	
Ehr-16S-D	GGTACCYACAGAAGAAGTCC
Ehr-16S-R	TAGCACTCATCGTTTACAGC

2.1.2. Specificity and reproducibility of the new tools of diagnosis

The specificity of the Taqman® assay and the conventional PCR assay was tested using DNA extracted from the following organisms: *A. phagocytophilum*, *A. marginale*, *A. centrale*, *A. ovis* [12], *A. platys* (kindly supplied by Pr. L. Chabanne (VetAgro Sup Lyon, France) obtained from the blood of a dog from the Gard department, France), *E. canis*, *E. ruminantium*, *Neorickettsia sennetsu*, *Neorickettsia risticii*, *Rickettsia conorii*, *Rickettsia felis*, *Rickettsia typhi*, *Rickettsia massiliae*, *Rickettsia raoultii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Streptococcus agalactiae*, *Streptococcus oralis*, *Citrobacter koseri*, *Haemophilus influenza*, *Serratia marcescens*, *Klebsiella oxytoca*, *Gardnerella vaginalis*, *Stenotrophomonas maltophilia* and human DNA. A serial dilution was prepared for each sample of DNA for testing the sensibility of the Taqman® assay. At the first stage, all samples were tested by the 23S-based qPCR tool. Then those that were found positive (*Anaplasma* spp., *Ehrlichia* spp. and *Neorickettsia* spp.) were subjected to the two conventional PCRs. All amplified samples were sequenced to ensure there was no non-specific amplification and contamination. In all experiments, distilled water was included as negative control, and processed as described below. The purpose of this approach was to assess that 23S-based qPCR and conventional PCRs were able to amplify all available species of *Anaplasmataceae* family and not other bacteria.

The sequences of the 23S rRNA gene of *A. ovis* and *A. platys* were not available in the GenBank database. Subsequently, the sequences obtained from the positive controls were deposited in GenBank (GenBank access numbers for 23S rRNA genes: *A. ovis*: KM021411, *A. platys*: KM021412).