Inadequate Differentiation of *Theileria orientalis* Genotypes *buffeli* and *ikedai* in a Multiplexed Tandem PCR (MT-PCR) Assay Using the *p23* Gene as a Marker

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KEYWORDS *p23* gene, *buffeli*, cross amplification, diagnosis, *ikedai*, *Theileria orientalis*

Oriental theileriosis is a tick-borne disease of bovids caused by hemoprotozoan parasites within the *Theileria orientalis* complex. In the last 5 years, hundreds of outbreaks of oriental theileriosis were recorded in cattle from Australia and New Zealand (1–4), mainly ascribed to *ikedai* and *chitose*, pathogenic genotypes of all 11 currently known genotypes of *T. orientalis* (5). In 2015, we established and validated a multiplexed tandem PCR (MT-PCR) assay for the simultaneous detection, differentiation, and quantitation of four commonly prevalent pathogenic (*chitose* and *ikedai*) and nonpathogenic (*buffeli* and *type 5*) genotypes of *T. orientalis* in Australasia (6). This assay was designed by AusDiagnostics Pty. Ltd., Australia; in this assay, two primer pairs target the sequences of the first internal transcribed spacer and the *p23* gene of *T. orientalis* for genotypes *ikedai* and *buffeli*, respectively, and two pairs target the major piroplasm surface protein (*MPSP*) gene for both *chitose* and *type 5* to amplify regions of 70 to 115 bp (6).

Following its design, the MT-PCR was assessed to detect *T. orientalis* genotypes in blood samples from bovines in Australasia, and it performed well, achieving diagnostic specificities and sensitivities of 94.0 to 98.9% and 97.1 to 98.9%, respectively (6–9). However, when tested using samples from a number of locations in Ethiopia (10), Pakistan (11), and Vietnam (12), results revealed marked variations among peak melting temperatures and single-strand conformation polymorphism (SSCP) profiles for the genotype *buffeli*. Therefore, MT-PCR amplicons were sequenced and compared with previously published *p23* sequences of *T. orientalis* (13–15).

Of 20 *buffeli* MT-PCR amplicons sequenced, 40% (8/20) represented genotype *buffeli*, whereas 60% (12/20) represented *ikedai*. Comparison of the *p23* sequences characterized in this study with those published previously from Australia (13) and Japan (14, 15) revealed one *buffeli* sequence (42Crev) and seven *ikedai* sequences (67Brev, 22Brev, 7Drev, 37Brev, 47Brev, 57Crev, 62Brev) of *T. orientalis* (Fig. 1). Furthermore, the comparison showed that the sequence representing *buffeli* (42Crev) was identical to those reported previously from Australia (GenBank accession no. [KM504986](#) and [KM504987](#)) and Japan ([AB491349](#) and [AB021223](#)) (Fig. 1). However, it was different from those of accession no. [KM504988](#) (one transversion, A↔C) (Australia), [AB469178](#) (one transition, A↔C) (Japan), and [AB491348](#) (three transitions, A↔G or C↔T) (Japan) (Fig. 1). Of the seven *p23* sequences representing *ikedai* determined here (67Brev, 22Brev, 7Drev, 37Brev, 47Brev, 57Crev, 62Brev), nucleotide variability of 3.2 to 20% was linked mainly to the transitions C↔T and A↔G, and these sequences were more closely related to previously published *p23* sequences for *ikedai* than for *buffeli* (Fig. 1).

Furthermore, in order to verify the cross-reactivity exhibited by the *p23* primer pair in MT-PCR, we tested all 20 samples using conventional PCR to amplify a longer region

Accepted manuscript posted online 7
December 2016

Citation Gebrekidan H, Gasser RB, Jabbar A. 2017. Inadequate differentiation of *Theileria orientalis* genotypes *buffeli* and *ikedai* in a multiplexed tandem PCR (MT-PCR) assay using the *p23* gene as a marker. J Clin Microbiol 55:641–644. <https://doi.org/10.1128/JCM.02017-16>.

Editor Brad Fenwick, University of Tennessee

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FIG 1 Alignment of the *p23* nucleotide sequences of the genotypes *buffeli* and *ikedai* of *T. orientalis* determined here (bold and underlined) with the reference sequences of *buffeli*, *chitose*, and *ikedai*. The unique number at the beginning of each reference sequence represents the GenBank accession number.

of the *p23* gene (~601 bp) using primers designed by Ota et al. (14). Results revealed that the samples that tested positive for genotype *ikedai* by MT-PCR also tested positive for *ikedai* using an amplification of a longer region of the *p23* gene by conventional PCR, and the same was true for genotype *buffeli*. Hence, there was a concordance between the results of MT-PCR and conventional PCR in assigning genotypes *buffeli* and *ikedai* of *T. orientalis* using the *p23* gene. To assess the relationship of *p23* sequences obtained by sequencing of amplicons by conventional PCR, phylogenetic analyses were undertaken by compiling *p23* sequences generated in this study and those available in GenBank representing genotypes *buffeli*, *chitose*, and *ikedai* of *T. orientalis*. Phylogenetic analyses revealed that *p23* sequences of genotypes *buffeli* (GenBank accession no. KY235360 and KY235361) and *ikedai* (KY235356 to KY235359) grouped with previously published sequences of the respective genotypes (Fig. 2).

The results indicate that the primer pair targeting the *p23* gene region to amplify genotype *buffeli* of *T. orientalis* using MT-PCR is nonspecific, leading to a cross-amplification from genotype *ikedai* (Fig. 1). Currently, the assay specificity for genotype *buffeli* is linked to only a single nucleotide in the forward primer designed by AusDiagnostics Pty. Ltd., being insufficient to achieve selectivity from *ikedai*. Previously, when we tested DNA samples using the *MPSP* gene, we did not observe any significant variation in peak melting temperatures or SSCP profiles among MT-PCR amplicons from genotype *buffeli* (6). Additionally, we did not find any discrepancy in the assignments of three genotypes (*buffeli*, *chitose*, and *ikedai*) of *T. orientalis* using the *MPSP* gene or the *p23* gene (6, 16). However, sequence variation in the *p23* gene region identified in this study is likely due to population variation within *T. orientalis* (*buffeli*) in blood samples from cattle and buffaloes from locations outside Victoria, Australia (New South Wales and South Australia within Australia, and Ethiopia, New Zealand, and

FIG 2 Genetic relationships of genotypes *buffeli* and *ikeda* of *T. orientalis* determined in this study. The relationships were inferred based on phylogenetic analyses of the *p23* sequences determined herein (bold) and reference sequences using Bayesian inference (BI) and distance-based neighbor-joining (NJ) methods. *Theileria annulata* was used as the outgroup. There was a concordance in the topology between this NJ tree and that produced using BI (not shown). Nodal support (from left to right) is given as a posterior probability for BI and as a bootstrap value for NJ analyses. The scale bar indicates the number of inferred substitutions per nucleotide site.

Pakistan) (7–12); at the time, the *p23* gene had not been assessed for variation within *T. orientalis* outside Victoria, Australia (16).

The findings of the conventional PCR followed by DNA sequencing revealed that the *p23* gene is a good molecular marker to differentiate genotypes of *T. orientalis* when larger fragments of the DNA are targeted. Therefore, based on the current evidence, it is concluded that the primers designed for the *p23* gene by AusDiagnostics Pty. Ltd. do not allow the accurate differentiation of genotypes *buffeli* and *ikeda* within the *T. orientalis* complex.

Accession number(s). *p23* nucleotide sequences of genotypes *buffeli* and *ikeda* of *T. orientalis* determined here are 42Crev_Buffeli, 67Brev_Ikeda, 22Brev_Ikeda, 7Drev_Ikeda, 37Brev_Ikeda, 47Brev_Ikeda, 57Brev_Ikeda, and 62Brev_Ikeda. The *p23* sequences of genotype *buffeli* are GenBank accession no. KY235360 and KY235361, and those of genotype *ikeda* are KY235356 to KY235359.

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