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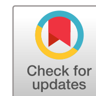
LETTER TO THE EDITOR



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

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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 *ikeda* 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 *ikeda*) 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 *ikeda* 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 *ikeda*. 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 *ikeda* 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 *ikeda* 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 *ikeda* 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

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                          10        20        30        40        50        60
                  ....|....|....|....|....|....|....|....|....|....|....|....|...
42Crev_buffeli    GCAACCAAGGCCGAACAGG-TCATAGCCGGTACTACCGAGGAG-GAGAAATCTGACAAGAAGA
KM504988_buffeli  ...................-...........C...........-...................
KM504987_buffeli  ...................-.......................-...................
KM504986_buffeli  ...................-.......................-...................
AB021223_buffeli  ...................-.......................-...................
AB491349_buffeli  ...................-.......................-...................
AB469178_buffeli  ...................-...........G...........-...................
AB491348_buffeli  ...................-.T.........G....T......-...................
67Brev_ikeda      .N.................-.T.........G..G.T......-...................
22Brev_ikeda      ...................-.TG........G..G.T.....AG.....G.............
7Drev_ikeda       ...................-.T.........G....T......-...................
37Brev_ikeda      ...................-.TG........G..........AG.....G.............
47Brev_ikeda      ...................-.TG.-......G....A.....A-.....G...........AG
57Crev_ikeda      ...................-.TG.T......G....T.....A-.....G.............
62Brev_ikeda      CA.................T.T.........G....T.....N-...................
D84447_ikeda      ...........T.......-.TG.T......G....T.....A-.....G.............
AB469180_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
AB491354_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
AB491353_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
AB491352_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
AB469181_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
AB469176_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
KM504993_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
KM504992_ikeda    ...........T.......-.TG.T......G....T.....A-.....G.............
D84446_chitose    .................AA-C.G.T....T.G.C........A-........C..........
AB469177_chitose  .................AA-C.G.T....T.G.C........A-........C..........
AB469179_chitose  .................AA-C.G.T....T.G.C........A-........C..........
AB491351_chitose  .................AA-C.G.T....T.G.C........A-........C..........
AB491350_chitose  .................AA-C.G.T....T.G.C........A-........C..........
KM504990_chitose  ..................A-C.G.T....T.G.C........A-........C..........
KM504989_chitose  ..................A-C.G.T....T.G.C........A-........C..........
KM504991_chitose  ..................A-C.G.T....T.G.C........A-........C..........
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FIG 1 Alignment of the *p23* nucleotide sequences of the genotypes *buffeli* and *ikeda* of *T. orientalis* determined here (bold and underlined) with the reference sequences of *buffeli*, *chitose*, and *ikeda*. 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 *ikeda* by MT-PCR also tested positive for *ikeda* 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 *ikeda* 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 *ikeda* of *T. orientalis*. Phylogenetic analyses revealed that *p23* sequences of genotypes *buffeli* (GenBank accession no. KY235360 and KY235361) and *ikeda* (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 *ikeda* (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 *ikeda*. 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 *ikeda*) 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

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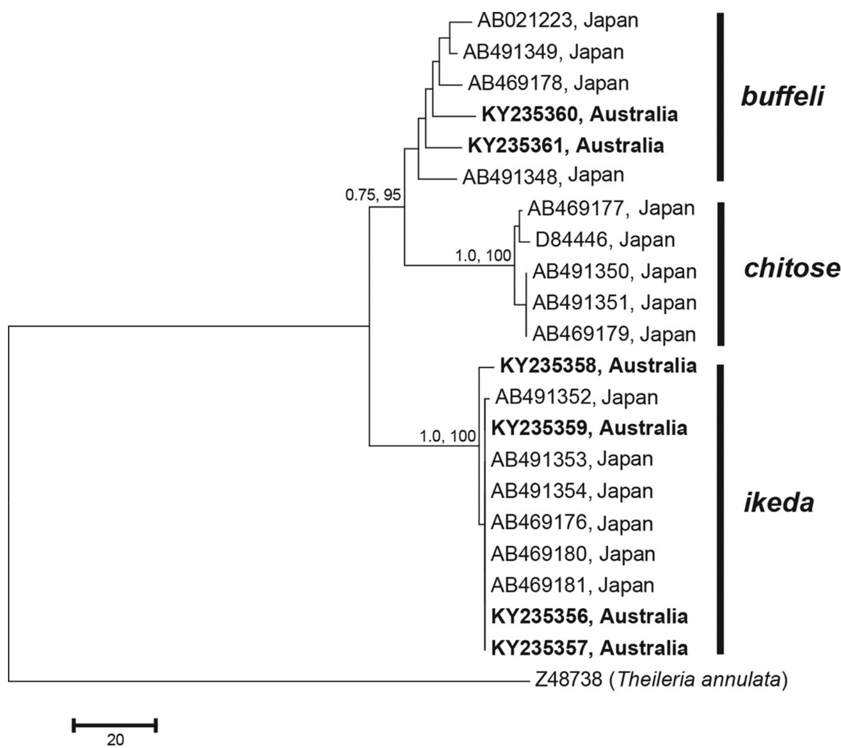

FIG 2 Genetic relationships of genotypes *buffeli* and *ikeda* of *T. orientalis* determined in this study. The relationships were inferred based on phylogenetic analyses of the *p23* sequences determined herein (bold) and reference sequences using Bayesian inference (BI) and distance-based neighbor-joining (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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