Identification of a novel β-adrenergic octopamine receptor-like gene (βAOR-like) and increased ATP-binding cassette B10 (ABCB10) expression in a *Rhipicephalus microplus* cell line derived from acaricide-resistant ticks

H. H. Caline Koh-Tan¹, Erin Strachan², Katherine Cooper², Lesley Bell-Sakyi³ and Nicholas N. Jonsson¹*

**Abstract**

**Background:** The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is an economically important parasite of livestock. Effective control of ticks using acaricides is threatened by the emergence of resistance to many existing compounds. Several continuous *R. microplus* cell lines have been established and provide an under-utilised resource for studies into acaricide targets and potential genetic mutations associated with resistance. As a first step to genetic studies using these resources, this study aimed to determine the presence or absence of two genes and their transcripts that have been linked with acaricide function in cattle ticks: β-adrenergic octopamine receptor (βAOR, associated with amitraz resistance) and ATP-binding cassette B10 (ABCB10, associated with macrocyclic lactone resistance) in six *R. microplus* cell lines, five other *Rhipicephalus* spp. cell lines and three cell lines representing other tick genera (*Amblyomma variegatum*, *Ixodes ricinus* and *Hyalomma anatolicum*).

**Methods:** End-point polymerase chain reaction (PCR) was used for detection of the βAOR gene and transcripts in DNA and RNA extracted from the tick cell lines, followed by capillary sequencing of amplicons. Quantitative real-time PCR (qPCR) was performed to determine the levels of expression of ABCB10.

**Results:** βAOR gene expression was detected in all *Rhipicephalus* spp. cell lines. We observed a second amplicon of approximately 220 bp for the βAOR gene in the *R. microplus* cell line BME/CTVM6, derived from acaricide-resistant ticks. Sequencing of this transcript variant identified a 36 bp insertion in the βAOR gene, leading to a 12-amino acid insertion (LLKTLALVTIIS) in the first transmembrane domain of the protein. In addition, nine synonymous SNPs were also discovered in *R. appendiculatus*, *R. evertsi* and *R. sanguineus* cell lines. Some of these SNPs appear to be unique to each species, providing potential tools for differentiating the tick species. The BME/CTVM6 cell line had significantly higher ABCB10 (*P* = 0.002) expression than the other *R. microplus* cell lines.

**Conclusions:** The present study has identified a new βAOR gene and demonstrated a higher ABCB10 expression level in the BME/CTVM6 cell line, indicating that tick cell lines provide a useful experimental tool for acaricide resistance studies and further elucidation of tick genetics.

**Keywords:** Acaricide resistance, Amitraz, Synthetic pyrethroid, Ivermectin, Dieldrin, Tick cell line
Background

Rhipicephalus (Boophilus) microplus, also known as the cattle tick or southern cattle tick, is a parasite of major global economic importance. Sales-Junior et al. [1] estimated that more than 75% of the cattle population worldwide was infested with R. microplus, making it one of the most prevalent of bovine parasites. Rhipicephalus microplus causes significant economic losses; direct losses include damage to the hide from tick attachment, anaemia and in severe cases death [2]. More importantly, cattle ticks act as vectors of the protozoan and bacterial pathogens Babesia bigemina, Babesia bovis and Anaplasma marginale, all of which cause production losses and can be fatal [3]. Rhipicephalus appendiculatus is an important tick of cattle in eastern Africa, where it transmits Theileria parva [4]. Rhipicephalus sanguineus is a globally widespread tick that is commonly found on dogs [5]. Amblyomma variegatum infests cattle throughout sub-Saharan Africa and transmits Ehrlichia ruminantium, the causal agent of heartwater [6]. Ixodes ricinus is the most widely distributed tick species in northern, western and central Europe and is of human significance as the vector of Lyme disease (Borrelia burgdorferi) as well as being an important vector of several diseases of cattle and sheep [4, 7]. Hyalomma anatolicum infests cattle in North Africa, the Middle East and Asia and transmits Theileria annulata, of economic significance in the region [4].

The short, one-host life-cycle of R. microplus acts together with high fecundity to result in rapid selection for development of acaricide resistance. There are several mechanisms by which acaricide resistance has evolved; those most commonly noted are target site modification and metabolic resistance [8–10]. Target site modification has been most clearly demonstrated in the para sodium channel, the target of synthetic pyrethroids (SP) [11, 12]. Metabolic resistance occurs as a result of modified ability to detoxify and/or sequester pesticides, mediated by families of enzymes known for detoxification of xenobiotics, such as the cytochrome P450s, esterases, glutathione S-transferases and ATP-binding cassette (ABC) transporters [10]. Penetration resistance is a little-studied mechanism which might also occur in ticks [9].

Octopamine receptors have been shown from studies in insects to be the target for formamidines such as amitraz, leading to the hypothesis that mutations in octopamine receptors would confer resistance to formamidines [13]. There are three main types of octopamine receptors in insects: α-adrenergic-like, β-adrenergic-like and tyramine/octopamine (tyraminergic) [14]. Our previous work reported an association between polymorphism in the β-adrenergic octopamine receptor (βAOR) and resistance to amitraz in R. microplus ticks in Australia [15]. A non-synonymous A→T at position 181 resulted in substitution of isoleucine to phenylalanine (I61F) in the first trans-membrane domain. It is therefore expected that any resistance conferred by mutation in βAOR would be restricted to compounds that would bind to this receptor type.

ATP-binding cassette (ABC) transporters are a superfamily of transmembrane proteins, consisting of up to 8 subfamilies [16] found in all organisms, including R. microplus [17]. They transport substrates across cell membranes as a monomeric or dimeric channel, often against concentration gradients [16]. An association between the ABC transporter and ivermectin (macrocyclic lactone) resistance amongst cattle ticks was first reported by Pohl et al. [17]. These authors detected a 3–6-fold increase in gene expression of R. microplus ABC subtype B10 (RmABCB10) in two different ivermectin-resistant strains. The importance of increased ABCB10 expression through metabolic processes was subsequently demonstrated in an ivermectin resistance-induced R. microplus cell line [18] but it is uncertain if ABCB10 plays a role in resistance to other classes of acaricides.

Over 40 continuous tick cell lines derived from species of worldwide economic importance are maintained in the Tick Cell Biobank at The Pirbright Institute [19]. The establishment of tick cell lines is slow and challenging, and the cultures are composed of cells obtained from hundreds or thousands of embryos or multiple developing nymphs or adults. Further, the cultures are composed of multiple cell types and the cells can be of variable ploidy [20], with proportions of the different cell populations in a cell line varying over time. Therefore, although a small number of acaricide resistance selection studies have been successfully conducted using continuous tick cell lines [21, 22], because they are non-clonal and phenotypically and genotypically heterogeneous it cannot be assumed that any continuous tick cell line is a reliable and consistent model for examining acaricide resistance.

The purpose of the present study was to assess the suitability of six R. microplus continuous cell lines for future genomic and transcriptomic studies on resistance to acaricides. In this study we wished to confirm that two genes that have been strongly associated with resistance (one that operates via target site modification and one that operates via metabolic resistance mechanisms) are expressed by tick cells in vitro, to identify new variants of these genes if any were present, and to determine whether there is variation in their expression level. These two genes were selected for this study because (i) amitraz and macrocyclic lactones (including ivermectin) are globally the most widely used acaricides at present, (ii) we have well-established protocols for working with these genes, and (iii) they represent two of the main mechanisms of resistance-detoxification (ABCB10) and target-site insensitivity (βAOR). The first objective was to partially sequence the βAOR gene for single nucleotide polymorphisms (SNPs) that were reported previously by Corley et al. [15]. The second objective was to determine...
the expression level of \textit{ABCB10} in the \textit{Rhipicephalus} spp., in particular the \textit{R. microplus}, cell lines. The study design did not allow a robust determination of the relationship between genotype or expression level and the resistance status of the population of ticks from which the continuous cell lines were developed.

\textbf{Methods}

\textbf{Cell lines}

Fourteen tick cell lines, derived from \textit{R. microplus}, three other \textit{Rhipicephalus} spp., \textit{A. variegatum}, \textit{H. anatolicum} and \textit{I. ricinus}, were provided by the Tick Cell Biobank at The Pirbright Institute. The cell lines, passage level(s) tested, species and instar of origin, geographic origin and acaricide resistance status of the parent ticks (if known) are listed in Table 1. All tick cell lines were maintained in 2 ml complete culture medium supplemented with foetal bovine serum in flat-sided culture tubes (Nunc) [23]. Prior to nucleic acid extraction, the tubes were completely filled with culture medium and transferred to the University of Glasgow at room temperature. On arrival, the entire contents of each tube of cells was centrifuged at 300 \textit{rcf} for 5 min, most of the supernatant medium was discarded, leaving only 3 ml of medium to re-suspend the cells. The cells were then divided into 3 aliquots in 1.5 ml microtubes and centrifuged at 900 \textit{rcf} for 5 min. The supernatants were then discarded and the cell pellets frozen at −80 °C for nucleic acid extraction.

\textbf{Extraction of nucleic acids}

Total RNA was extracted from cell pellets using the Qiagen miRNeasy Mini Kit (#217004) with on-column DNase digestion following the manufacturer’s protocol for extraction from cells. Genomic DNA (gDNA) was extracted from cell pellets using the Qiagen QiaAMP Mini Kit (#51304) following the manufacturer’s protocol. The RNA and DNA concentrations were measured spectrophotometrically using a Nanodrop ND-1000 and quality was confirmed at absorbance 260/280 nm to be ≥1.95.

\textbf{Reverse transcription}

First strand complementary DNA (cDNA) was synthesised using 800 ng total RNA with TaqMan Reverse

\begin{table}[h]
\centering
\caption{Tick cell lines used in the study}
\begin{tabular}{llcccccc}
\hline
Species & Cell line & Passage level tested & Instar of origin & Year initiated & Geographical origin (strain) & Reference & Acaricide resistance status of parent ticks if known \\
\hline
\textit{Rhipicephalus (Boophilus)} microplus & BmVIII-SCC & 41 & Embryo & 1979 & Mexico & [32] & Susceptible \\
& BME/CTVM2 & 140 & Embryo & 1983 & Costa Rica (Paquera) & [33] & Susceptible \\
& BME/CTVM5 & 67; 8 & Embryo & 1983 & Colombia (Paso Ancho) & [20] & Resistant to organophosphates, organochlorines, Amitraz \\
& BME/CTVM6 & 211; 233; 32; 221; 243 & Embryo & 1983 & Colombia (Paso Ancho) & [33] & Resistant to organophosphates, organochlorines, Amitraz \\
& BME/CTVM23 & 54 & Embryo & 2005 & Mozambique (Mozambique) & [34] & Not known \\
& BME/CTVM30 & 17 & Embryo & 2005 & Mozambique (Mozambique) & [34, 35] & Not known \\
\textit{Rhipicephalus appendiculatus} & RAN/CTVM3 & 66 & Developing adult & 1978 & Kenya (Muguga) & [36] & Not known \\
& RA243 & 344 & Developing adult & 1971 & East Africa & [37] & Not known \\
\textit{Rhipicephalus evertsi} & REN/CTVM32 & 16 & Developing adult & 2010 & South Africa & [23] & Not known \\
\textit{Rhipicephalus sanguineus} & RML-RSE & 83 & Embryo & 1980s & United States of America & [38, 39] & Not known \\
& RSE/PILS3S & 4 & Embryo & 2012 & France & This study & Not known \\
\textit{Amblyomma variegatum} & AVL/CTVM13 & 127 & Developing nymph & 1989 & Southern Africa & [40] & Not known \\
\textit{Hyalomma anatolicum} & HAE/CTVM9 & 195 & Embryo & 1986 & India (Ludhiana) & [41] & Not known \\
\textit{Ixodes ricinus} & IRE/CTVM19 & 222 & Embryo & 1999 & United Kingdom & [20] & Not known \\
\hline
\end{tabular}
\end{table}

All cell lines were grown in culture media and at incubation temperatures as described by Bell-Sakyi et al. [23] except RSE/PILS3S, which was established in L-15 medium at 28 °C from eggs laid by a single incompletely-engorged female \textit{R. sanguineus} tick kindly provided by Dr Cristina Socolovschi and Dr Oleg Mediannikov, URMITE, Marseille, France
Transcription Reagents (Life Technologies #N8080234) in a 20 µl reaction volume according to the manufacturer’s instructions. This was performed in a Life Technologies SimpliAmp™ Thermal Cycler using the recommended cycling conditions. The cDNA was then diluted with 30 µl of RNase-free water, before any subsequent experiments.

Polymerase chain reaction (PCR)
Each reaction was set up using a HotStar Taq Plus DNA Polymerase kit (Qiagen #203603) in a 20 µl volume containing final concentrations of 1× buffer (with 1.5 mM MgCl₂), dNTPs (0.2 mM), forward primer (0.5 µM), reverse primer (0.5 µM), Taq polymerase (5 U/µl), RNase-free H₂O and 2 µl of cDNA or 20 ng of gDNA. The primers and PCR conditions used are shown in Table 2. A non-template control (NTC) was included in every PCR. End-point PCR for ABCBI0 and the housekeeping gene β-actin (ActB) using cDNA of all cell lines was done to check for presence of transcript before proceeding to quantitative PCR (qPCR). Thermal cycling conditions were initial activation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, annealing temperature (Table 2) for 1 min at 72 °C; and final extension at 60 °C for 30 min.

Sequencing of βAOR
All detected amplicons from gDNA and cDNA, with the exception of gDNA from BME/CTVM5, were cleaned for sequencing using Agencourt AMPure XP (Beckman Coulter #A63880) according to the manufacturer’s instructions and reconstituted in 40 µl of RNase-free water. For cell line BME/CTVM5, only the amplicon from cDNA was sequenced because the other two genomic fragments that were amplified were not transcribed. Cleaned amplicons were checked by 1.5 % agarose gel electrophoresis that were amplified were not transcribed. Cleaned amplicons from gDNA and cDNA of BME/CTVM2, BME/CTVM5 (passage 67), BME/CTVM23, BME/CTVM30 and BmVIII-SCC were sequenced by PCR in gDNA and cDNA respectively (Fig. 1a). An alternative amplicon of the expected size, 183 bp, was detected in gDNA and cDNA of BME/CTVM2, BME/CTVM5 (passage 67), BME/CTVM23, BME/CTVM30 and BmVIII-SCC. An alternative amplicon

Real-time quantitative PCR (qPCR)
Real-time quantitative measurements of ABCBI0 and ActB expression were determined in a 10 µl reaction volume containing 1X Brilliant III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent Technologies #600882), 0.03 µM of ROX reference dye, 0.5 µM forward and reverse primers (Table 2) and 2 µl of cDNA. A non-template control (NTC) was included in every run. Each sample was set up in technical duplicates. The qPCR was performed using the real-time system Stratagene MX3000p and cycling conditions were initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min; final extension at 60 °C for 30 min; and finally a melting curve for confirmation of single amplicon. The cycle threshold (Ct) values were exported for analysis in Excel. The experiment was replicated twice with all nine cell lines investigated and twice more with only R. microplus cell lines.

Statistical analysis
Expression of the ABCBI0 gene was normalised to ActB expression and the mean ratio for technical duplicates calculated based on the Pfaffl method [24], using BmVIII-SCC as the calibrator cell line. Differences between the cell lines were tested using one-way analysis of variance (ANOVA) with Tukey’s post-hoc test on Minitab v17.

Results
Detection of the βAOR gene and transcripts in tick cell lines
The βAOR gene and transcripts from the six R. microplus cell lines were detected by PCR in gDNA and cDNA respectively (Fig. 1a). An amplicon of the expected size, 183 bp, was detected in gDNA and cDNA of BME/CTVM2, BME/CTVM5 (passage 67), BME/CTVM23, BME/CTVM30 and BmVIII-SCC. An alternative amplicon

Table 2 Primers and PCR conditions

| Oligo name | Sequence (5’-3’) | AT | Amplicon | Accession No. | Positions | Reference |
|------------|-----------------|----|----------|--------------|----------|----------|
| βAOR-For   | GAAATCTGACGGAGACGAGGAA | 61 °C (Rm) | 183 bp | JN974909 | 95–277 | [15] |
| βAOR-Rev   | GGCGACAGATGAGTAAGATTG | 58 °C (Rhipi) | 189 bp | | | |
| ABCBI0-For | GCCGCAGTTGTCACCTTGTGTTT | 61 °C | 96 bp | JN098446 | 887–982 | [17] |
| ABCBI0-Rev | AGCTCGCTGCTGACCTTGCTC | | | | | |
| ActBm1-For | GAGAAGATACTCCGGTGTGATCGG | 61 °C | 203 bp | AY255624 | 1095–1297 | [42] |
| ActBm1-Rev | CGTACGGTGGGCGGTGGCCGG | | | | | |

Abbreviations: AT annealing temperature, bp base pair, Rm Rhipicephalus microplus, Rhipi other Rhipicephalus species
of ~220 bp was detected in gDNA and cDNA of BME/CTVM6 (mixture of cells from two sublines at passages 211 and 233, separate since passage 148). There were also two additional amplicons of ~245 bp and ~220 bp in gDNA of BME/CTVM5 (passage 67); these were not detected in cDNA from this cell line (Fig. 1a). To determine whether or not the alternative/additional amplicons detected in BME/CTVM5 and BME/CTVM6 cells were consistently present in these cell lines at different passage levels, the earliest available passages of BME/CTVM5 (passage 8) and BME/CTVM6 (passage 32) and from the two high-passage sublines of BME/CTVM6 (passages 221 and 243 extracted separately) were tested. In all cases the respective amplicons detected in gDNA from the first high passages tested were also present in the respective low passages and the two sublines of BME/CTVM6 (Additional file 1: Figure S1).

Amplicons of the expected 183 bp size were detected in gDNA and cDNA of RAN/CTVM3, RA243, REN/CTVM32 and RSE/PILS35, but only in gDNA of RML-RSE (RML). No βAOR gene or transcript was detected in cell lines AVL/CTVM13 (AVL), HAE/CTVM9 (HAE) or IRE/CTVM19 (IRE). Abbreviations: M, Marker; NTC, Non-template control; −ve, reverse transcription negative control.

**Fig. 1** βAOR PCR in *Rhipicephalus* cell lines. 

**a** Detection of βAOR in genomic DNA (gDNA) and complementary DNA (cDNA) of *Rhipicephalus microplus* cell lines. The expected amplicon of 183 bp was detected in gDNA and cDNA of cell lines BME/CTVM2 (2), BME/CTVM5 (5), BME/CTVM23 (23), BME/CTVM30 (30) and BrmIII-SCC (SCC). BME/CTVM6 (6) gave an alternative amplicon of ~220 bp from both gDNA and cDNA. Additional amplicons of ~245 bp and ~220 bp were also detected in gDNA of BME/CTVM5. 

**b** Detection of βAOR in gDNA and cDNA of other tick cell lines. The expected amplicon was detected in gDNA and cDNA of cell lines RAN/CTVM3 (RAN), REN/CTVM32 (REN), RSE/PILS35 (RSE) and RA243, and only in gDNA of RML-RSE (RML). No βAOR gene or transcript was detected in cell lines AVL/CTVM13 (AVL), HAE/CTVM9 (HAE) or IRE/CTVM19 (IRE). Abbreviations: M, Marker; NTC, Non-template control; −ve, reverse transcription negative control.
RSE (Fig. 1b). The βAOR gene and transcripts were not detected in cell lines AVL/CTVM13, HAE/CTVM9 and IRE/CTVM19 (Fig. 1b) using the primers in Table 2 in two attempts. Consequently, these three cell lines were excluded from further investigation.

**βAOR gene variations**

Sequencing and comparison with the published sequence (GenBank JN974909) of the βAOR gene of *R. microplus* (strain NRFS) revealed that the transcript variant in BME/CTVM6 and the 220 bp gene variant in BME/CTVM5 contained a 36 bp insertion at position 190 from the start codon (Fig. 2a), not present in any of the other tick cell lines. This resulted in a 12-aa insertion LLKTLALVTIIS (Fig. 2b), which appeared to be an additional leucine (L) followed by a duplicated LKTLALVTIIS. Using the TMHMM prediction and TMRPres2D diagram representation, this insertion was predicted to occur within the first transmembrane domain leaving an extracellular domain comprising 66-aa instead of 54-aa (Fig. 2c). A discontinuous megablast search did not find any match for this insertion in any other species.

Attempts to sequence the additional 245 bp amplicon from BME/CTVM5 were unsuccessful despite successful gel extraction of the product (Additional file 2: Figure S2). There were two regions of ambiguity within the amplicon between regions of homology of the sequences, suggesting that there was more than one binding site for the primers. PCR amplification of the purified 245 bp amplicon produced the same three amplicons seen in Fig. 1a and Additional file 1: Figure S1. This confirms that there was more than one binding site for the primers.

Nine synonymous SNPs were also discovered in *R. appendiculatus*, *R. evertsi* and *R. sanguineus* cell lines (Table 3). Three SNPs (C141T, A225G and A231C) were present in all five *R. appendiculatus*, *R. evertsi* and *R. sanguineus* cell lines, while C189G was present only in the *R. evertsi* and *R. sanguineus* lines. A195C and G204C were unique to *R. appendiculatus* cell lines while G204A, C216G, G222A and C228G were unique to *R. sanguineus* cell lines.

These sequences have been submitted to GenBank under the following accession numbers: KU836738 (BmVIII-SCC), KU836739 (BME/CTVM2), KU836740 (BME/CTVM5), KU836741 (BME/CTVM6), KU836742 (BME/CTVM23), KU836743 (BME/CTVM30), KU836744 (RAN/CTVM3), KU836745 (RA243), KU836746 (REN/CTVM32), KU836747 (RML-RSE) and KU836748 (RSE/PILS35).

**Expression of ABCB10**

After normalisation to *ActB*, expression of the *ABCB10* gene in BME/CTVM6 was significantly higher (ANOVA $F_{(9,10)} = 8.1, P = 0.002$), by approximately seven-fold, compared to expression in all the other cell lines (Fig. 3). There was no significant difference in the *ABCB10* expression level between any of the other cell lines.

**Discussion**

Acaricide resistance in *R. microplus* has been associated with mutations in several genes including the βAOR gene [15]. We demonstrated expression of this gene in six tick cell lines derived from four strains of *R. microplus* with different acaricide resistance status. Corley et al. [15] identified two non-synonymous SNPs for two highly conserved adjacent isoleucine residues of the first transmembrane domain of the β-adrenergic-like octopamine receptor (βAOR) in *R. microplus*. The first identified SNP resulting in an amino acid substitution of I61F was found in the Ultimo tick strain from Australia, which was resistant to amitraz, synthetic pyrethroids and organophosphates. Our study used the same primers and detected the βAOR gene and transcripts in all the *R. microplus* cell lines that retain the highly conserved isoleucine at amino acids 61 and 62. However, we discovered an alternative βAOR gene and transcript with a 36 bp insertion in the cell line BME/CTVM6, derived from *R. microplus* (Paso Ancho strain) resistant to multiple acaricides. This insertion introduced 12 aa into the first transmembrane domain of the seven-transmembrane protein structure, resulting in a longer extracellular domain. The βAOR protein is the predicted target for formamidines [25]; hence, we hypothesise that the longer extracellular domain could lead to a pore site that is less accessible to formamidines and might confer resistance to them.

The BME/CTVM5 cell line, derived from the same *R. microplus* strain as BME/CTVM6, has potentially three different βAOR genes, only one of which was transcribed. The transcribed gene is completely homologous to the published βAOR gene JN974909 [25]. One of the other two gene variants appears to give the same fragment size as that of BME/CTVM6 but was not transcribed. Sequencing of this 220 bp amplicon yielded the same sequence as the alternative gene and transcript detected in BME/CTVM6. Multiple attempts to sequence the 245 bp amplicon that was detected only in BME/CTVM5 were unsuccessful, often producing very noisy signals. This suggests multiple primer binding sites within the 245 bp fragment. A repeat PCR performed on the gel-extracted and cleaned 245 bp PCR amplicon from the initial PCR produced the same three amplicons, confirming the existence of multiple binding sites for the primers, most likely due to an evolutionary process of gene duplication. It is notable that despite BME/CTVM5 cells having potentially three variants of the βAOR gene, present at both low and high passage levels, only one seems to have been transcribed. As the two cell lines were each derived from different pooled egg batches laid by over 50 female
**Fig. 2** (See legend on next page.)

### a)

| Sequence | |  |
|----------|---|---|
| T160C (HVT) | |  |
| JN974490  | GC | TCC AAT TCT CAA TGG GCA GGC CAA GAC ACC  |
| JN974490 | | TCC AAT TCT CAA TGG GCA GGC CAA GAC ACC  |

### b)

| Sequence | |  |
|----------|---|---|
| BNF1G028 | CTT | TTA TTA |
| BNF1G028 | CTT | TTA TTA |

### c)

| Domain |  |
|--------|---|
| CYTOPLASMIC |  |
| EXTRACELLULAR |  |

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**Legend:**
- **Fig. 2:** (See legend on next page.)
- **JN974490:** MVAAMWNIWRE MRSGGRQQOQS MSNNAAANAS LGNLTDDEAG DDESEWELVLK LVLKTLALV1189 ...  
- **BNE/CVM:** MVAAMWNIWRE MRSGGRQQOQS MSNNAAANAS LGNLTDDEAG DDESEWELVLK LVLKTLALV1189 ...  

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ticks, a possible explanation for this phenomenon is that BME/CTVM5 and BME/CTVM6 were derived from a population of ticks in which resistance was developing but in which the resistance-conferring allelic variants were not fixed at 100% in the population. The establishment of the two cell lines would likely have been subject to normal stochastic effects on founder populations and subsequent genetic drift, leading to fixation of the initially more frequent allele over time after establishment of the culture. If this is the case, it highlights the potential value of the heterogeneous cell culture model for studies on the evolution of acaricide resistance, and draws attention to aspects of the system that must be taken into consideration in the design of experiments. We hypothesise that the \( \beta_{AOR} \) gene was first duplicated in the base population. Gene duplication or multiple gene copy number has been linked to evolutionary adaptation in the parasitic trematode \textit{Fasciola hepatica} [26], the mosquito \textit{Anopheles gambiae} [27] and \textit{R. microplus} [28, 29].

Pohl et al. [18] reported an association between increased expression of ABC transporter genes, \( ABCB10 \) in particular, and ivermectin resistance. BME/CTVM6 demonstrated approximately seven-fold higher \( ABCB10 \) expression when compared to BmVIII-SCC, a cell line derived from acaricide-susceptible ticks (Patricia Holman, personal communication). The importance of \( ABCB10 \) expression level in ivermectin resistance was demonstrated in a \textit{R. microplus} cell line while in an \textit{I. scapularis} cell line, another ABC transporter gene, \( ABCB8 \), was upregulated [30]. BME/CTVM5 demonstrated a trend towards higher \( ABCB10 \) expression compared to BmVIII-SCC. In order to verify if these increased gene expression levels translate to protein expression levels, further work will be necessary.

It is interesting that the BME/CTVM6 cell line derived from an acaricide-resistant strain has both a different \( \beta_{AOR} \) gene and a higher level of \( ABCB10 \) expression compared to the other \textit{R. microplus} cell lines, whereas the BME/CTVM5 cell line, derived from the same strain, did not show these characteristics. It is also notable that both BME/CTVM5 and BME/CTVM6 were established over 30 years ago, prior to the recent heavy and widespread use

| Position | Allele | Consequence | Species – Strain/Cell line | Reference |
|----------|--------|-------------|-----------------------------|-----------|
| 181      | A / T  | I / F       | \( R. microplus \) – Ultimo strain | [15]      |
| 185      | T / C  | I / T       | \( R. microplus \) – southeast Queensland, Australia | [15]      |
| 141      | C / T  | synonymous  | \( R. appendiculatus \) – RAN/CTVM3, RA243; \( R. evertsi \) – REN/CTVM32; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 189      | C / G  | synonymous  | \( R. evertsi \) – REN/CTVM32; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 190      | 36 bp insertion | 12 amino acid insertion (LLKTLALVTIIIS) | \( R. microplus \) – BME/CTVM6 |          |
| 195      | A / C  | synonymous  | \( R. appendiculatus \) – RAN/CTVM3, RA243; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 204      | G / C / A | synonymous | \( R. appendiculatus \) – RAN/CTVM3, RA243; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 216      | C / G  | synonymous  | \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 222      | G / A  | synonymous  | \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 225      | A / G  | synonymous  | \( R. appendiculatus \) – RAN/CTVM3, RA243; \( R. evertsi \) – REN/CTVM32; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 228      | C / G  | synonymous  | \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
| 231      | A / C  | synonymous  | \( R. appendiculatus \) – RAN/CTVM3, RA243; \( R. evertsi \) – REN/CTVM32; \( R. sanguineus \) – RML-RSE, RSE/PILS35 |          |
of macrocyclic lactone products and the documented emergence of ivermectin resistance. It is possible that ABCB10 played a role in resistance to acaricides other than ivermectins, but it is unlikely that any mutation in βAOR would lead to resistance to macrocyclic lactones, as the macrocyclic lactones are not known to bind to βAOR.

The fact that the same tick strain has returned quite different results in terms of the expression of a detoxifying gene and the genotype of a target gene clearly demonstrates the importance of initial stochastic effects at the time of the establishment of the cell culture.

Nine synonymous SNPs were found in *R. appendiculatus*, *R. evertsi* and *R. sanguineus* cell lines. Some of these SNPs appear to be unique to individual species. These SNPs provide a potential tool for identifying the tick species; however, they need to be verified with *R. appendiculatus*, *R. evertsi* and *R. sanguineus* ticks from different regions of the world and with known acaricide resistance status.

Failure to detect the βAOR gene in gDNA extracted from the *Amblyomma*, *Hyalomma* and *Ixodes* spp. cell lines could be due to insufficient sequence similarity between the tick genera, such that the primers designed for *R. microplus* did not bind to the gene in DNA or RNA extracted from these cell lines. A search for adrenergic octopamine receptor genes in *Ixodes scapularis* (https://www.vectorbase.org/organisms/ixodes-scapularis and http://www.ncbi.nlm.nih.gov/nuccore) found nothing, but there were numerous putative G-protein coupled octopamine receptors. Alternatively, the βAOR gene may be absent from these species or genera. It is more likely that the annotation for β-adrenergic-like octopamine receptor is incomplete.

The *R. microplus* cell lines have proven to be useful tools for investigating tick genetics in relation to acaricide resistance as evidenced from the findings of this study and other reports [18, 21, 22]. This study has also shown that cultures established from field populations have the potential to include and fix different variants of the same genes, such that some care is needed in interpretation of the results.

**Conclusions**

The present study has identified a new βAOR gene and demonstrated a higher ABCB10 expression level in the BME/CTVM6 cell line, indicating that tick cell lines provide a useful experimental tool for acaricide resistance studies and further elucidation of tick genetics.

**Additional files**

**Additional file 1: Figure S1.** βAOR gene in earlier passages of *Rhipicephalus* cell lines BME/CTVM5 and BME/CTVM6. Detection of βAOR gene in *Rhipicephalus microplus* cell lines BME/CTVM5 passage 8 (5p8) and BME/CTVM6 passages 32, 221 and 243 (6p32, 6p221 and 6p243, respectively). Amplicons of 183 bp, 220 bp and 245 bp were detected in the gDNA of BME/CTVM5 and amplicon of only 220 bp was detected in BME/CTVM6 passages. M = Marker. (TIF 2846 kb)

**Additional file 2: Figure S2.** βAOR sequences of the 245 bp amplicon from BME/CTVM5 genomic DNA. There were two regions of heterogeneity from the sequencing traces of the alternative 245 bp amplicon detected in BME/CTVM5, indicating the existence of multiple primer binding sites for the sequencing reactions. (TIF 9962 kb)
Abbreviations
aa, amino acid; ABCB10, ATP-binding cassette B10; ANOVA, analysis of variance; bp, base-pair; cDNA, complementary deoxyribonucleic acid; gDNA, genomic deoxyribonucleic acid; NTC, non-template control; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; BAOR, β-adrenergic octopamine receptor; SNP, single nucleotide polymorphism.

Acknowledgements
The authors would like to thank Dr Patricia Holman of Texas A&M University for provision of the BmVIII-SCC cell line.

Funding
This project was funded through ES’s honours project and BBSRC research experience placement (REP) awarded to KC (through allocations of REPs direct to University of Glasgow, School of Veterinary Medicine 2015). LBS is supported by the United Kingdom Biotechnology and Biological Sciences Research Council’s Institute Strategic Programme Grant (BBS/E/I/00001741) to the Pirbright Institute.

Availability of data and material
All data generated or analysed during this study are included in this published article and its supplementary information files. Sequences are submitted in the GenBank database under accession numbers KU836738–KU836748.

Authors’ contributions
CKT contributed to experimental design, completed the sequencing and statistical analysis, interpreted the data and prepared the manuscript. ES and KC performed the majority of the experiments for this study. LBS provided the tick cell lines and contributed to the preparation of this manuscript. NJ conceived and designed the study, interpreted the data and contributed to the preparation of this manuscript. All authors read and approved the final version of the manuscript.

Authors’ information
CKT is research associate for principal investigator NJ; ES was an MSc Honours project student; KC is a BVMS student; and LBS is a collaborator and the curator of Tick Cell Biobank at The Pirbright Institute.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Author details
1Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, McCall Building, Bearsden Road, Glasgow G61 1QH, UK. School of Veterinary Medicine, University of Glasgow, Glasgow G61 1QH, UK. 2The Pirbright Institute, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UK.

Received: 1 March 2016 Accepted: 17 July 2016

Published online: 02 August 2016

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