

# Redundancy and recombination in the *Echinococcus* AgB multigene family: is there any similarity with protozoan contingency genes?

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(Received 23 March 2006; revised 24 April 2006; accepted 25 April 2006; first published online 4 July 2006)

## SUMMARY

Numerous genetic variants of the *Echinococcus* antigen B (AgB) are encountered within a single metacestode. This could be a reflection of gene redundancy or the result of a somatic hypermutation process. We evaluate the complexity of the AgB multigene family by characterizing the upstream promoter regions of the 4 already known genes (*EgAgB1-EgAgB4*) and evaluating their redundancy in the genome of 3 *Echinococcus* species (*E. granulosus*, *E. ortleppi* and *E. multilocularis*) using PCR-based approaches. We have ascertained that the number of AgB gene copies is quite variable, both within and between species. The most repetitive gene seems to be *AgB3*, of which there are more than 110 copies in *E. ortleppi*. For *E. granulosus*, we have cloned and characterized 10 distinct upstream promoter regions of *AgB3* from a single metacestode. Our sequences suggest that *AgB1* and *AgB3* are involved in gene conversion. These results are discussed in light of the role of gene redundancy and recombination in parasite evasion mechanisms of host immunity, which at present are known for protozoan organisms, but virtually unknown for multicellular parasites.

Key words: hydatid disease, antigen B, promoter, recombination, antigen diversity, real-time PCR.

## INTRODUCTION

Protozoan parasites escape the host's immunological defences by switching their expressed antigens. Antigenic variation mediated by genetic recombination is well documented for protozoan parasites, but analogous mechanisms have never been characterized in multicellular parasites. In African trypanosomes, VSG (Variant Surface Glycoprotein) switching results either from homologous DNA recombination that is targeted to an active expression site, which is mainly gene conversion, or from transcriptional *in situ* switching between different VSG expression sites (Pays, 2005). The active VSG expression site is considered as a recombination hot-spot that generates genes for new VSGs and for novel VSG-based adaptive molecules. Following the genome sequencing, it was shown that trypanosomes have a high potential to generate novelties in this evolutionary process, since most VSGs are pseudogenes (Berriman *et al.* 2005).

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We have recently uncovered extensive genetic diversity underlying one of the major *Echinococcus* immunodiagnostic antigens, antigen B (AgB) (Chemale *et al.* 2001; Arend *et al.* 2004; Haag *et al.* 2004). The polymeric antigen is a complex 160 kDa thermo-stable lipoprotein built by 8 kDa subunits, encoded by at least 4 distinct, already characterized, genes *AgB1*, *AgB2*, *AgB3* and *AgB4* (Shepherd *et al.* 1991; Fernández *et al.* 1996; Chemale *et al.* 2001; Arend *et al.* 2004). Although several AgB gene variants have been isolated and sequenced, their redundancy and genomic organization is still unknown.

Several studies suggest that AgB has an important role in the parasite-host interaction. For instance, AgB is the first antigen recognized by specific antibodies in sera from mice infected with activated oncospheres (Zhang *et al.* 2003). Furthermore, AgB seems to be involved in the evasion of the host immune response, as indicated by its ability to inhibit polymorphonuclear cell recruitment (Shepherd *et al.* 1991; Riganò *et al.* 2001). Studying the immunological profile of human patients with different stages of hydatid disease, it has also been shown that parasite survival is predominantly associated with a Th2 lymphocyte polarization in its host (Riganò *et al.* 2001, 2004). Chemale *et al.* (2005) have

Table 1. *Echinococcus granulosus* AgB specific primers used in the LA-PCR (5'-3')

Gene	First step	Second step
<i>AgB1</i>	TCAGCAATCAACCCTCTGAGGTGGGA	TTTTTCATCACACACCTCGACGTGCGAG
<i>AgB2</i>	GTGTCCCGACGCATGACTTA	AACATACTTCTTCAGCACCTCACGAAT
<i>AgB3</i>	ACATACTCCTTCAGTGCCARGCGTGC	AGAAGTGCTTAATCTCGCTAATGGCC
<i>AgB4</i>	GTGTCCCGACGCATGACTTA	GACATATTTCTTCAACACTTCGTGAAC

suggested that AgB could also be involved in some kind of detoxifying mechanism, sequestering and buffering xenobiotics, since it does not release lipids in competition assays, and therefore does not seem to direct fatty acid metabolic pathways. Recent studies of AgB expression in *E. multilocularis* show that *AgB3* is constitutively expressed in all parasite stages, but the transcription of *AgB1*, *AgB2* and *AgB4* appears to be restricted to the larval stage (Mamuti *et al.* 2006).

Intriguingly, a single *E. granulosus* metacestode (cyst), originating from 1 single parasite egg, and thus to be considered as a clone, has been found to express a large number of slightly different variants of *AgB2* and *AgB4* (Arend *et al.* 2004). Moreover, cloned PCR fragments of *AgB1*-*AgB4* genes from individual protoscoleces, have shown that inter-protoscoleces genetic variation does not account for the total genetic variability encountered within a single hydatid cyst. A single protoscolex harbours several variants for each gene, although one of them is usually the most frequent (Haag *et al.* 2004). In the same study, theoretical analyses suggest that the concomitant presence of slightly different AgB variants within a single cyst may be adaptive. As parts of a highly immunogenic protein, AgB subunits clearly evolve by positive selection, but the heterogeneous distribution of the positively selected epitopes among subunits indicate distinct biological roles (Haag *et al.* 2006).

However, the meaning and origin of AgB diversity is incomplete because all genetic studies have included only the short coding sequences, which are no longer than 450 bp. It remains unclear if AgB diversity is a static reflection of high gene redundancy, in which different gene copies originated by duplication slowly diverge over time, or if genetic variability is generated by some particular mechanism, such as somatic hypermutation, recombination, or both, during the progression of the infection. Here we present an extensive cloning and sequencing study of the upstream flanking regions from the 4 already known *E. granulosus* genes (*EgAgB1*-*EgAgB4*), using long and accurate PCR (LA PCR) (Mukai and Nakagawa, 1996). Additionally, we evaluate the redundancy of each gene in 3 *Echinococcus* species (*E. granulosus*, *E. multilocularis*, and *E. ortleppi*) using a real-time PCR-based approach.

## MATERIALS AND METHODS

### Parasite materials

Total genomic DNA was phenol/chloroform-extracted from *E. granulosus* and *E. ortleppi* protoscoleces collected from bovine hydatid cysts using standard procedures. The respective species was identified by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit I gene as previously described (Bowles *et al.* 1992). Additionally, we used some previously isolated DNA from *E. multilocularis* metacestodes for comparison. One metacestode/cyst was considered as a single individual/isolate.

### LA-PCR

Approximately 10 µg of total genomic DNA derived from a single *E. granulosus* sheep strain metacestode (cyst) was digested with 50 U *Spe* I (New England) and 2.5 µg of the digestion was used for ligation to a *Xba* I cassette (Takara) at 16 °C for 30 min. One µl of ligation was used in 4 independent PCR reactions, each using a forward primer annealing to the adaptor (C1, Takara) and a reverse primer designed to anneal within the CDS of each of the 4 known *E. granulosus* AgB genes (Tab. 1 – first step), with the LA Taq polymerase (Takara) for reduced incorporation errors and long templates, following the manufacturer's instructions. Subsequently, 1 µl dilutions (1 : 10, 1 : 100 and 1 : 1000) of the first PCR amplicons were used as templates for a second enrichment-PCR step, using the C2 forward primer annealing to the adaptor and a second reverse primer specific for each AgB gene (Table 1).

The second PCR products were run in an agarose gel containing ethidium bromide, and stained bands were excised for purification using the Qiagen Gel Extraction Kit (Qiagen). One µl from the agarose-eluted DNA was cloned into the TOPO TA Vector for sequencing (Invitrogen). The recombinant plasmids were purified with columns (Qiagen) and sequenced using M13 (-20) forward and M13 reverse primers (Invitrogen) with the Big Dye Version 2.0 kit on an ABI 377 sequencer (Applied Biosystems).

### Real-time PCR

Four primer pairs were designed to specifically amplify the 4 *E. granulosus* or *E. multilocularis* AgB

Table 2. Primers used in the real-time PCR (5'-3')

Gene	Forward		Reverse
	<i>E. granulosus</i>	<i>E. multilocularis</i>	
<i>AgB1</i>	AAATGTTTGGCCGAAGTGAAGT	CGAGGAGTATGATGAAAATGC	GTAGATGGTTTATTGAGCAA
<i>AgB3</i>	AAAGCACACATGGGCAAGTG	GTTGATGAACTGACTAAGACGAA	
<i>AgB2</i>	GAAGGGTGTGATGAAGGCCAT	CAAAAGCACACAGGGGCAAG	GTGTCCCGACGCATGACTTA
<i>AgB4</i>	CGAGAGATGCAAGTGCCTCAT	CGAAAATTGAGCGAAAATCCGG	
<i>Mdh</i>	CGTTCCCTTCCATTTCCGAAAG		TTGGTGACAACGGCGTGAGAC

genes. We used a specific forward primer annealing to the 5' region inside the second exon of each gene and species, and a reverse primer annealing to the 3' flanking region of *AgB1* and *AgB3* or *AgB2* and *AgB4* in both species (respective primer sequences are shown in Table 2). For comparison, we also amplified a segment of the single copy cytosolic malate dehydrogenase gene (*mdh*) from each isolate.

The amplification was performed on a LightCycler (Roche Diagnostics) using the SYBR Green I as a dsDNA-specific fluorescent dye and a continuous monitoring. PCR was initiated by a 'hot start' of 15 min at 95 °C followed by 40 cycles of denaturing (94 °C, 15 sec), annealing (50 °C, 30 sec) and extension (72 °C, 30 sec). Fluorescence was measured each cycle at 72 °C. Amplifications of DNA molecules derived from either different *AgB* genes or the single copy *mdh* gene were performed in triplicates. Mean values from triplicate determinations for each sample were taken for the calculation of relative *AgB* copy numbers, using the ratio of *AgB* to *mdh* amplification. The relative quantifications were performed taking into account the efficiency of the PCR reactions with templates derived from different primer pairs/species. Efficiency was determined by the slope of the regression curve between the fluorescence crossing points and the values of a dilution series of the respective genomic DNA. A dilution series of the corresponding PCR products was also used to calculate the slope (efficiency), and the results were similar to those obtained with dilutions of genomic DNA.

## RESULTS

We have used 4 sets of specific primers complementary to the downstream CDS region of each *AgB* gene in combination with adaptor primers C1 and C2 from the LA PCR kit (Takara) to amplify the unknown upstream *AgB* flanking regions. After the second enrichment-PCR step, a clear band of about 0.9–1.0 kb appeared for *AgB2* and *AgB4*, while a complex banding pattern appeared for *AgB1* and *AgB3* (data not shown), the last target showing several amplicons ranging from 0.2 to more than 1.0 kb in size. All 47 clones sequenced in both directions showed a clear relation to one of the previously characterized *AgB* genes in their downstream portion, corresponding to the CDS, as shown by the scores of blast search hits (Table 3). Flanking regions of different clones from the same *AgB* gene are highly divergent in sequence, except for clones B4-A and B4-B, which differ mainly by the absence of the upstream *Spe* I restriction site in B4-A, leading to a larger fragment. The most divergent clones could not even be properly aligned, and the similarity among them was usually decreasing in the upstream direction. Figure 1 shows the alignment of the similar regions of our clones and a

Table 3. Blast and promoter search results obtained for the AgB clones sequenced in the present study

Clone	<i>n</i>	Accession	Size (bp)	Blast best hits			Promoter location (bp)	Score
				Region (bp)	e-value	Gene (accession)		
B1-A	8	<u>DQ148515</u>	1627	358–1627	0·0	<i>EmAgB1</i> ( <u>AB112079</u> )	1070–1020 1379–1429	0·96 0·98
B1-B	2	<u>DQ148516</u>	796	629–796	5e <sup>-88</sup>	<i>EgAgB1</i> ( <u>AY773091</u> )	474–524*	0·94
B1-C	1	<u>DQ148517</u>	701	351–701	6e <sup>-155</sup>	<i>EmAgB1</i> ( <u>AB112079</u> )	105–155 438–488	0·98 0·98
B3-A	2	<u>DQ148519</u>	793	68–324 558–636	2e <sup>-93</sup> 3e <sup>-30</sup>	<i>EmAgB1</i> ( <u>AB112079</u> ) <i>EmAgB3</i> ( <u>AB202117</u> )	497–547	0·97
B3-B	1	<u>DQ148520</u>	779	639–745 747–777	3e <sup>-49</sup> 6e <sup>-4</sup>	<i>EgAgB3</i> ( <u>AY871026</u> ) <i>EgAgB3</i> ( <u>AY871026</u> )	534–584	0·97
B3-C	3	<u>DQ148521</u>	803	633–769	3e <sup>-49</sup>	<i>EgAgB3</i> ( <u>AY871026</u> )	558–608	0·97
B3-D	1	<u>DQ152002</u>	650	582–650	4e <sup>-29</sup>	<i>EgAgB3</i> ( <u>AF362443</u> )	309–359	0·88
B3-E	1	<u>DQ152003</u>	309	202–232 254–309	2e <sup>-4</sup> 1e <sup>-21</sup>	<i>EgAgB3</i> ( <u>AF362443</u> ) <i>EgAgB3</i> ( <u>AF362443</u> )	n.i.	—
B3-F	1	<u>DQ152004</u>	241	138–241	5e <sup>-48</sup>	<i>EgAgB3</i> ( <u>AF362443</u> )	n.i.	—
B3-G	1	<u>DQ152005</u>	312	246–312	8e <sup>-26</sup>	<i>EgAgB3</i> ( <u>AF362443</u> )	n.i.	—
B3-H	1	<u>DQ152006</u>	291	97–203 236–291	1e <sup>-49</sup> 1e <sup>-21</sup>	<i>EgAgB3</i> ( <u>AY871026</u> ) <i>EgAgB3</i> ( <u>AY871026</u> )	n.i.	—
B3-I	1	<u>DQ152007</u>	241	186–241	9e <sup>-22</sup>	<i>EgAgB3</i> ( <u>AF362443</u> )	n.i.	—
B3-J	1	<u>DQ148523</u>	423	344–421	3e <sup>-32</sup>	<i>EgAgB5</i> ( <u>AY871025</u> )	79–129	0·88
B2-A	8	<u>DQ148518</u>	823	528–823	4e <sup>-150</sup>	<i>EgAgB2</i> ( <u>AY569355</u> )	461–511 703–753**	0·98 0·97
B4-A	1	<u>DQ148522</u>	1051	759–1051	8e <sup>-146</sup>	<i>EgAgB4</i> ( <u>AY569357</u> )	673–723	0·85
B4-B	8	<u>DQ152008</u>	865	573–865	2e <sup>-143</sup>	<i>EgAgB4</i> ( <u>AY569357</u> )	485–535	0·86

\* Not functional (no ATG in the neighbourhood).

\*\* Located inside the CDS.

n.i. = not identified.

comparison with the reference *AgB1-AgB4* CDS sequences used to design the primers for the LA-PCR strategy. Note that these alignments do not contain the complete clone sequences, but only the regions which could be compared through a multiple alignment.

Searching for consensus promoter motifs within our sequences using a neural network-based tool available at [http://www.fruitfly.org/seq\\_tools/promoter.html](http://www.fruitfly.org/seq_tools/promoter.html) (Roese, 2001), we found that the typical *AgB* promoter-like element includes a TATAA string 28 bp upstream from a stretch of (GT)<sub>n</sub> localized just after the putative transcription start point. The number of GT repeats is polymorphic both within and between genes, ranging from 3 in *AgB1* to 12–13 in *AgB4* (see Fig. 1). Several other putative promoter sequences appear isolated in distinct *AgB1* and *AgB3* clones, or accompanying a typical element from *AgB1* and *AgB2* genes (data not shown).

Clone B3-A is a clear recombinant. Its upstream non-coding region is highly similar to an *E. multilocularis AgB1* sequence, which is also the best hit for clones B1-A and B1-C, but its downstream CDS region is more similar to an *E. multilocularis AgB3* sequence (Table 3). This can be more clearly verified through a dot plot, as presented in Fig. 2.

The downstream region of B3-A is more similar to clone B3-C, whereas its upstream region has its counterpart in clone B1-A. To evaluate if this could be the result of a gene conversion event, we aligned the 8 B1-A and the 2 B3-A sequences using the BioEdit software version 5.0.9 (Hall, 1999) and searched for fragments involved in gene conversion with GENECONV version 1.8.1 (Sawyer, 1989). We found that between sites 88 and 203 the aligned region from the two B3-A clones has a greater similarity with the corresponding region in 3 sequences from the B1-A group, than would be expected by chance (based upon 10 000 permutations;  $P < 0\cdot001$  for both global inner and global outer fragments).

The heterogeneity of *AgB1* and *AgB3* flanking region leads to a question about the degree of redundancy of these genes in the *E. granulosus* genome. Although our previous work based on genomic Southern blots suggested redundancy (Chemale *et al.* 2001; Haag *et al.* 2004), the exact number of gene copies was never determined. For this, we decided to use another approach to access the question, using real-time PCR. We compared the number of copies of the 4 AgB genes in relation to a single copy *mdh* gene in 3 *Echinococcus* species. Our results show that the genes can be quite variable in numbers,

not only between, but also within species (Fig. 3). *AgB2* showed the lowest number of copies in the 3 taxa, whereas *AgB3* showed the highest in *E. granulosus* and *E. ortleppi*. In this species, the number of *AgB3* copies varied extensively, from 7 to 117. The most redundant AgB gene of *E. multilocularis* is *AgB4*, but the numbers also vary considerably in this species, from 10 to 69 copies.

#### DISCUSSION

In the present paper we show that the number of *Echinococcus* AgB genes is higher than previously thought. Furthermore, our results demonstrate that AgB numbers are quite variable between metacestodes from the same species, indicating that DNA rearrangements might be involved in some sort of gene amplification mechanism. The LA PCR experiments followed by sequencing of the *E. granulosus* AgB genes showed that about half of the repeated *AgB3* sequences do not seem to be functional, since they lack a consensus promoter motif. As suggested in a previous study (Haag *et al.* 2004), *AgB3*-related genes appear to be involved in gene conversion; some of these apparently non-functional copies could be intermediates or derivatives of recombination processes.

Furthermore, we sequenced an *AgB3* clone where the upstream region has a higher than expected similarity with an *AgB1* clone. This is a more reliable indication of gene conversion than that obtained in our previous study (Haag *et al.* 2004), where the sequences were derived from the same PCR reaction, and some could have been generated during fragment amplification. In the present case, clones B1-A and B3-A were obtained in independent PCR reactions, suggesting that gene conversion must have taken place in the parasite genomic DNA; clearly, they are not an experimental artifact.

The selective advantage/functional role of maintaining this kind of diversifying mechanism in the *Echinococcus* genome is a challenging issue. Since clones B1-A and B1-C contain 2 types of consensus promoter motifs upstream to the coding region, we think that gene conversion could up- or down-regulate a particular gene by introducing a new promoter motif within its upstream flanking region. We found that the first promoter motif within clone B1-A (sites 1070–1120 in clone B1-A, corresponding to sites 177–227 in clone B3-A), would generate an alternative protein, unrelated to AgB. Indeed, part of this motif is included within the fragment previously identified to result from gene conversion (sites 88 to 203). Further experimental approaches, such as comparing the functionality and relative strength of both putative promoters, should be performed to understand the relevance of gene conversion in this context.

Not only B1-A, B1-C, but also clone B2-A contain 2 consensus promoters in the same orientation as the AgB CDS. Furthermore, most *AgB1* and *AgB3* putative promoters overlap to consensus promoter motifs with a search score higher than 0.9 in the opposite orientation (data not shown). The additional promoter consensus motif of B2-A occurs within the second exon inside the *AgB2* CDS. In a previous study (Arend *et al.* 2004) we cloned an *AgB2* PCR fragment lacking the region corresponding to this particular motif; because the excised region showed conserved splicing signals, we hypothesized that this PCR product was derived from a retrotransposed sequence. *EgAgB2*-derived PCR fragments lacking the intron were found in other cloning experiments (Haag *et al.* 2004), indicating that this gene is prone to retrotransposition.

In *Plasmodium falciparum var* genes, it has been shown that the type of promoter associated with a particular gene strongly correlates with its chromosomal localization (in telomeric or internal domains), and that transcriptional control of genes located in different chromosomal regions occurs by distinct mechanisms (Voss *et al.* 2003). In *Trypanosoma brucei*, the expression of specific variant surface glycoprotein isoforms also relies upon gene location. Having a large repository of redundant *vsg* genes, DNA rearrangements are used to replace one isoform by another in the active expression site. However, the 'on' and 'off' states of a particular expression site promoter do not seem to be associated with DNA alterations (Borst and Ulbert, 2001). *Trypanosoma cruzi*, on the other hand, does not show antigen switching, but counts on the high genetic heterogeneity of its populations to evade immunity of a wide variety of hosts (Machado *et al.* 2006).

There remains much to be learned about how *E. granulosus* metacestodes confront the host immune system. It is clear that this interaction must be distinct from the well-known immunity escape mechanisms found in protozoans, essentially mediated by antigenic switches. *E. granulosus* metacestodes do not directly interact with the host cellular immune response, because they are physically protected by the laminated layer. However, antigenic variability might be critical during early intermediate host invasion, when oncospheres must reach the target organ from the digestive tract through the vascular system and establish a successful infection. At later stages, metacestode metabolites released into the environment could orchestrate the periparasitic inflammatory reaction in its own favour, and antigenic variability may represent one way to favour its survival potential. Although there is still no link between AgB diversity, gene conversion processes, and their adaptive significance in terms of immunity evasion, the





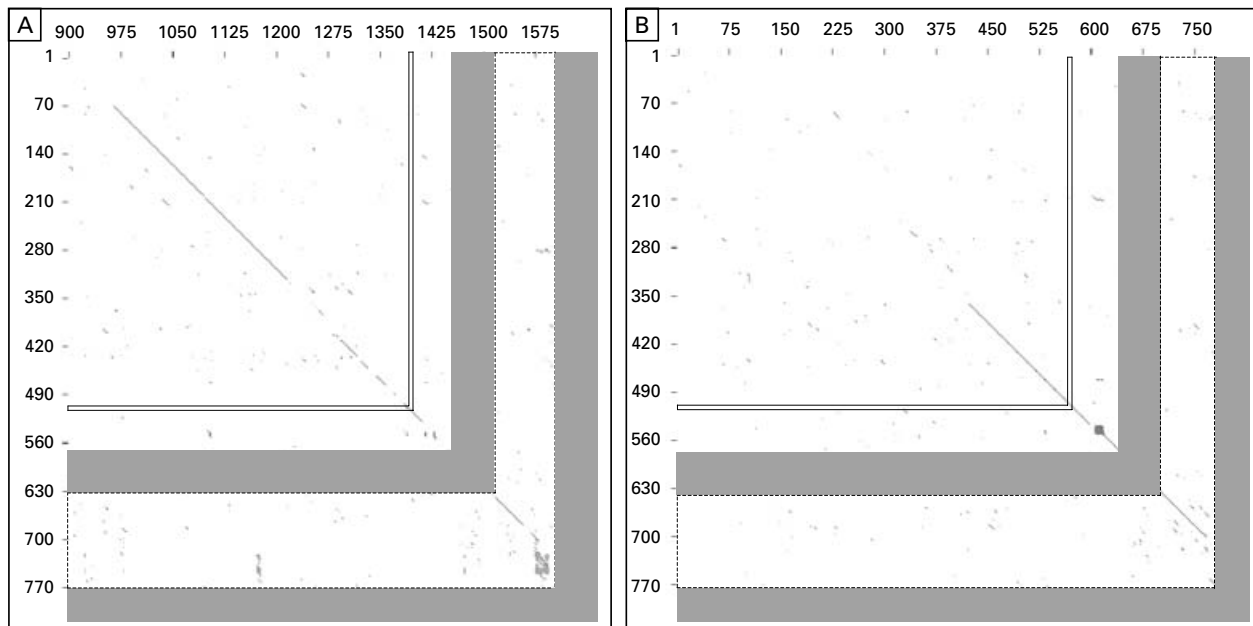


Fig. 2. Dot plot between clones B3A (vertical axis) and B1A (horizontal axis, A) and B3C (horizontal axis, B). Shaded areas correspond to the *EgAgB* exons, introns are included in the dashed box and the TATA box is displayed within a continuous box.

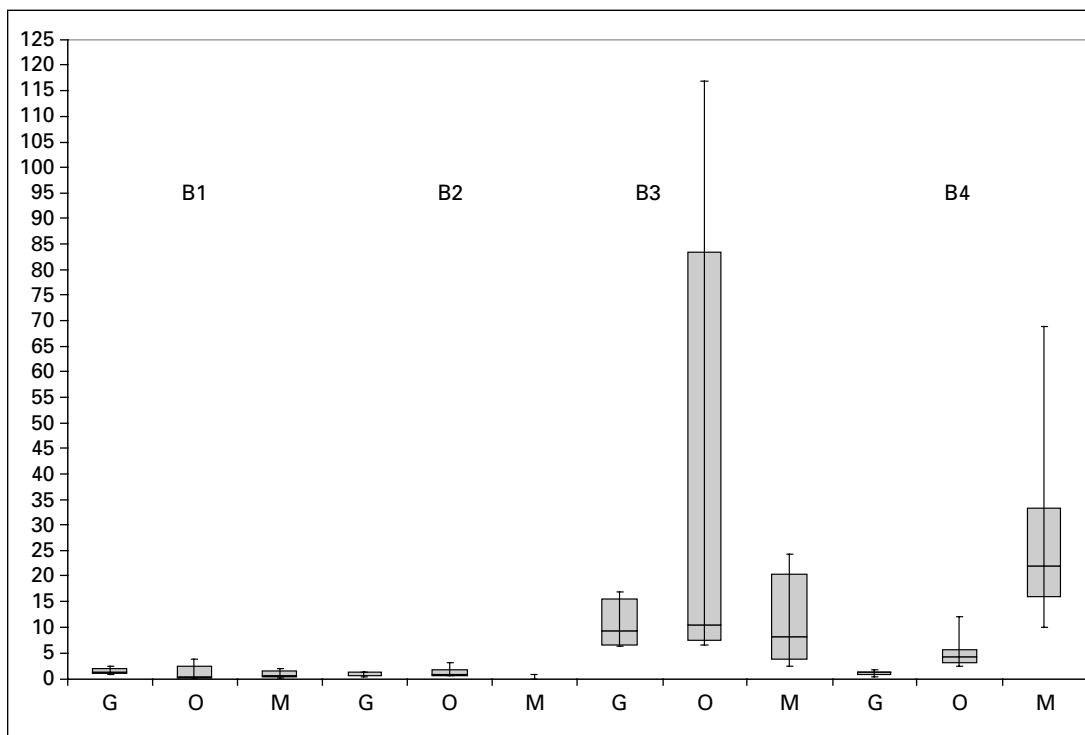


Fig. 3. Distribution of *AgB1–AgB4* copy numbers estimates in relation to the single copy *mdh* gene in 3 *Echinococcus* species: *E. granulosus* (sheep strain, G,  $n=5$ ), *E. ortleppi* (or *E. granulosus* cattle strain, O,  $n=6$ ) and *E. multilocularis* (M,  $n=9$ ). The middle horizontal line within the bars shows the median value, bars include the first superior and inferior quartiles and the vertical lines indicate the maximum and minimum values.

analogy with the protozoan parasite escape strategies is tempting.

We thank CNPq, CAPES and FAPERGS for supporting our work and Robin Gasser for the critical reading of the manuscript.

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