An immunoproteomic approach to identifying immunoreactive proteins in *Leishmania infantum* amastigotes using sera of dogs infected with canine visceral leishmaniasis

Sajad Rashidi^a, Zahra Mojtahedi^b, Bahador Shahriari^c, Kurosh Kalantar^d, Ghasem Ghalamfarsa^e, Mehdi Mohebalif^f and Gholamreza Hatam^c

^aDepartment of Parasitology and Mycology, Shiraz University of Medical Sciences, Shiraz, Iran; ^bInstitute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran; ^cBasic Sciences in Infectious Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran; ^dDepartment of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran; ^eMedicinal Plants Research Center, Faculty of Medicine, Yasuj University of Medical Sciences, Yasuj, Iran; ^fDepartment of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Visceral leishmaniasis (VL), the most severe form of leishmaniasis, is caused by *Leishmania donovani* and *Leishmania infantum*. The infected dogs with canine visceral leishmaniasis (CVL) are important reservoirs for VL in humans, so the diagnosis, treatment and vaccination of the infected dogs will ultimately decrease the rate of human VL. Proteomics and immunoproteomics techniques have facilitated the introduction of novel drug, vaccine and diagnostic targets. Our immunoproteomic study was conducted to identify new immunoreactive proteins in amastigote form of *L. infantum*. The strain of *L. infantum* (MCAN/IR/07/Moheb-gh) was obtained from CVL-infected dogs. J774 macrophage cells were infected with the *L. infantum* promastigotes. The infected macrophages were ruptured, and pure amastigotes were extracted from the macrophages. After protein extraction, two-dimensional gel electrophoresis was employed for protein separation followed by Western blotting. Western blotting was performed, using symptomatic and asymptomatic sera of the infected dogs with CVL. Thirteen repeatable immunoreactive spots were identified by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Some, including prohibitin, ornithine aminotransferase, annexin A4, and apolipoprotein A-I, have been critically involved in metabolic pathways, survival, and pathogenicity of *Leishmania* parasites. Further investigations are required to confirm our identified immunoreactive proteins as a biomarker for CVL.

KEYWORDS

Leishmania infantum;
amastigote;
immunoproteomics

Introduction

Leishmania parasites cause a group of diseases known as leishmaniasis. According to clinical manifestations, leishmaniasis is classified into three groups, known as visceral leishmaniasis (VL), cutaneous and mucocutaneous leishmaniasis. VL is the most severe form that is caused by *Leishmania donovani* and *Leishmania*

infantum [1]. In developing countries, co-infection of VL with HIV also poses a serious threat and has become a major challenge to the control of VL [2,3]. The clinical presentations of VL are somehow vague, and the mortality rate remains high, particularly in patients with a late diagnosis [4].

Due to the lack of effective drugs and the side effects, new drugs and vaccines seem to be urgently

needed for VL [5]. Although few vaccines against canine visceral leishmaniasis (CVL) are commercially available, no approved vaccine has been produced against human VL so far [6]. The infected dogs with CVL are important reservoirs for VL in humans, especially in the Mediterranean regions. Due to this issue, discovering and designing new effective vaccine candidates and diagnostic markers against CVL would eventually lead to a decrease in VL infection in humans [7,8].

Sequencing of *Leishmania* genome has provided an opportunity for identification and development of novel targets for vaccines and drugs against leishmaniasis [9]. Also, identification of immunoreactive targets of *L. infantum* using hyperimmune sera from dogs naturally infected with this parasite has improved the diagnosis of VL and CVL. It seems that exploitation of a variety of technologies including proteomics and immunoproteomics will open a new avenue to reach these aims earlier [10–14].

Since amastigotes are the pathogenic form of *Leishmania* parasites in humans as well as animal hosts, they are more appropriate for investigations focusing on vaccines, drugs and diagnosis. Comparison of the axenic form of amastigotes and the form obtained from the macrophage cell lines has shown the differences in intracellular transportation, metabolic processes, and response to oxidative stress [15]. Due to the high similarity of the amastigotes extracted from macrophages *in vitro* and the amastigotes in the infected hosts, it seems that extracting amastigotes from macrophages (not amastigote-like) can mimic the *in vivo* situation in dogs.

Since obtaining pure amastigotes *in vitro* is challenging, most of the previous studies preferred to select promastigotes for proteomic studies in *Leishmania* parasites [16–18]. Due to the lack of immunoproteomic studies on the *Leishmania* amastigote form, this study was conducted for introducing new immunoreactive proteins in amastigotes of *L. infantum*, using two-dimensional gel electrophoresis (2-DE) followed by Western blotting with CVL-infected dogs' sera.

Materials and methods

Propagation of amastigotes in J774 macrophages

Macrophages and parasites

L. infantum strain (MCAN/IR/07/Moheb-gh) was provided by the Department of Parasitology and Mycology, Tehran University of Medical Sciences, Iran. This strain was obtained from the CVL-infected dogs. Briefly, *L. infantum* promastigotes were mass cultivated in the stationary growth phase in RPMI-1640 (Shelmax Company)

supplemented with 15% (v/v) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 25°C. Semi-adherent J774 macrophage cell line was provided by the Department of Immunology, Shiraz University of Medical Sciences, Iran. The macrophages were grown in DMEM containing 10% FCS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin.

Macrophages infectivity

J774 macrophages were cultivated in DMEM containing 10% FCS. They were then pelleted at $450 \times g$ for 5 min at room temperature (RT). After diluting the pellet with DMEM, 3×10^5 , macrophages were added per well.

In the next step, promastigotes that were cultivated in the stationary growth phase were used for infecting the J774 macrophages (20 parasites/1 macrophage). Promastigotes were pelleted with the centrifuge at $1250 \times g$ for 10 min and the obtained pellets were re-suspended in DMEM. Finally, 6×10^6 promastigotes were added to the macrophages in each well.

After incubating the cell culture plate at 37°C with 5% CO₂ for 24 h, un-phagocytized promastigotes were removed by substitution of the supernatant with fresh DMEM containing 10% FCS. Then, the cell culture plate was incubated for 8–10 days at 37°C in 5% CO₂, and fresh DMEM media (450 µl) were added to each well every day. By preparation of smear and Giemsa staining, the load of infectivity was checked [19].

Isolation of amastigotes from macrophages

DMEM media (100–200 µl) containing 0.05% (w/v) saponin was added to each well followed by turning up and down and passing the contents of each well from the needle (Gauge 27). The contents of all wells were gathered, shaken firmly, and centrifuged at $100 \times g$ for 5 min at 4°C. The supernatant was collected and centrifuged at $2000 \times g$ for 10 min at 4°C. The obtained pellet was washed 2 times with DMEM at $2000 \times g$ for 10 min at 4°C. Finally, the obtained pellet was re-suspended in 2 ml DMEM and frozen at –70°C. By smear preparation and Giemsa staining, the pure amastigotes were observed [19].

Protein extraction

The extracted amastigotes were washed 3 times, each time for 10 min at $5000 \times g$ at 4°C with cold Tris-HCL 40 Mm, pH 7.2. The obtained pellet was re-suspended in a lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, 40 Mm dithiothreitol (DTT), 2% IPG buffer pH [3–10], and a protease inhibitor cocktail; Roche, Penzberg, Germany) in equal volumes. After incubation for 1 h at RT with occasional vortexing, the suspension was centrifuged for 20 min at $13,800 \times g$ at 4°C. The supernatant (extracted proteins) was kept at –70°C, and protein concentration

was measured by the Bradford assay with bovine serum albumin (BSA) (Merck), as the standard [20].

SDS-PAGE and Western blotting of the extracted proteins from amastigotes

Approximately, 40 µg of the obtained proteins were electrophoresed using SDS-PAGE that was performed on 4% stacking gels over 12% separating gels (Roche Applied Science). Sample lysates were boiled at 100°C for 5 min in 6X SDS gel-loading buffer consisting of 375 mM Tris-HCl (pH 6.8), 60% glycerol, 12% SDS, 30% 2-mercaptoethanol, and 0.6% bromophenol blue. After running, using an electroblotting system (Bio-Rad, Hercules, California, USA), the gel was transferred (20 V, 1 h) to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The non-specific binding sites on the membranes were then blocked by BSA [21], and CVL-infected dogs' pooled sera (n = 5) (asymptomatic and symptomatic) and non-infected dogs' pooled sera (n = 5) were used to probe the membranes. Symptomatic and asymptomatic sera of the infected dogs with CVL were achieved from Meshginshahr city, Ardabil province, Iran. All sera were checked for CVL with direct agglutination test (DAT). The extracted proteins from non-infected J774 macrophages were used as a negative control. Antibody binding proceeding was performed with 1:1000 dilutions of primary anti-sera in skim milk (2 h at RT). After three washes, 15 min each, with PBS containing 0.5% Tween-20, HRP rabbit anti-dog (IgG) (1:4000 dilutions, Abcam, USA) was allowed to react (1.5 h at RT) with proteins. After washing, the immunoreactive bands were developed using diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma, Germany). For scanning the obtained bands, the ChemiDoc MP Imaging System (Bio-Rad, USA) was used. The experiment was repeated three times with different CVL-infected and non-infected dogs' pooled sera to confirm the results.

2-DE Western blotting

The Protean IEF cell system (Bio-Rad, Philadelphia, PA, USA) was employed to perform the first-dimensional isoelectric focusing (IEF) step. Approximately, 500 µg of the protein sample was added per immobilized pH gradient (IPG) strip (pH 3–10 nonlinear, 18 cm) by active rehydration (50 V, 20°C, 14 h) in rehydration buffer (2 M thiourea (Merck, Darmstadt, Germany), 8 M urea, 2% w/v CHAPS, 0.3% wt/vol DTT, 2% vol/vol IPG buffer (pH, 3–10) and bromophenol blue) followed by IEF for a total of 62,000 Vh.

After IEF, each strip was equilibrated in 10 mL equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 30% wt/vol glycerol, 2% wt/vol SDS) containing 65 mM DTT (in the first step) and 135 mM iodoacetamide (in the second

step) at RT for 15 min. The second dimension and Western blotting were the same as Section 2.3.

After immunodetection step, GS-800 Calibrated Densitometer (Bio-Rad) and Prodigy SameSpots software (version 1.0) (Nonlinear Dynamics, Newcastle, UK) were used for scanning the blots and analysis of the digital images, respectively. The reference image was defined as an image with the greatest number of spots. Each blot was analyzed in a semi-automated way, and background intensity was subtracted from each sample.

Isolation of immunoreactive spots from the gel

After mapping the immunoreactive spots on the membrane, 2-DE was performed again with the extracted proteins from amastigotes, similar to Section 2.4. After running the gel, Coomassie brilliant blue staining was used for visualization of proteins. Gels were incubated overnight in staining solution containing 8% ammonium sulfate (Fluka), 1.6% orthophosphoric acid (Fluka), 20% methanol (Merck) and 0.12% Coomassie blue G 250 (Merck). The gels were de-stained with distilled water and then using a pipette; the spots identified on the membrane were punched from the gel.

In-gel digestion of protein samples and mass spectrometry (MS)

The in-gel digestion of protein sample and MS analysis were performed by the metabolomics and proteomics lab technology facility, Department of Biology, the University of York. The punched gels were placed into the wells of a ZipPlate (Montage In-Gel-DigestZP Kit, Millipore). The proteins in the punched gels were de-stained, digested with trypsin (Promega), extracted, purified on a C18 reverse phase matrix, and eluted in 8 µl of 60% acetonitrile, 0.1% trifluoroacetic acid (TFA).

Tandem matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS) was done on a BrukerAutoflex Mass Spectrometer (BrukerDaltonics, Bremen). Measurements were performed in the reflection mode, using Ion source 1 voltage: 19 kV; ion source 2 voltages: 16.5 kV; reflector voltage 20 kV; lens voltage 8 kV; 40 ns pulse time; 120 ns pulse extraction time; and matrix suppression < 500 Da. Using the Xtof analysis software package, version 5.1.5 (BrukerDaltonics), we analyzed all the spectra. Using a mix of peptides (Sigma–Aldrich), we calibrated the mass spectra internally [22].

Database search

The obtained masses generated by MALDI TOF/TOF MS were searched against peptide masses of the eukaryotes in the MASCOT program, the UniProt database (555,594 sequences; 199,016,217 residues) (Table 1).

Table 1. Identification of immunodominant proteins in *L. infantum* amastigotes.

Sample name	The number of spots on the gel	Protein name	Matched peptides	Mr (theoretical)	pI (theoretical)	Score	Protein sequence coverage	Accession No.
Am 1	23	Prohibitin	9	29.8	5.57	1055	48%	P67779
Am 2	33	Voltage-dependent anion-selective channel protein 1	4	30.8	8.62	514	24%	Q9TT15
Am 4	40	Ornithine aminotransferase	3	48.7	6.19	227	7%	P29758
Am 5	7	Annexin A4	5	36.1	5.43	374	21%	P97429
Am 6	20	Protein disulfide-isomerase A3	4	57.1	5.88	377	11%	P27773
Am 7	15	A single peptide match to phosphoglycerate kinase 1	1	44.9	8.48	84	3%	Q3T0P6
Am 8	16	A single peptide match to mitochondrial import receptor subunit TOM40 homolog	1	38	6.79	56	3%	Q1LZB5
Am 9	19	Aldehyde dehydrogenase	3	57	6.63	215	6%	P11884
Am 11	22	Apolipoprotein A-I	3	30.2	5.71	163	10%	P15497
Am 12	12	A single peptide match to 60 kDa heat shock protein, mitochondrial	1	61.1	5.7	78	2%	P10809
Am 13	10	A single peptide match to complement component 1 Q subcomponent-binding protein (C1QBP)	1	31.3	4.82	73	4%	O35658

Results

Propagation and isolation of amastigotes from the J774 macrophages

The infectivity results indicated that most of the macrophages were infected with amastigotes, and the rate of infectivity was almost 95%. Using microscopic method, we clearly detected the amastigotes in macrophages with an acceptable load (more than 10 amastigotes per infected macrophage). Finally, amastigotes were completely isolated from the debris of the macrophages [19].

SDS-PAGE and Western blotting of the extracted proteins from amastigotes

Before 2-DE Western blotting, the ranges of molecular weights of immunoreactive bands in amastigotes were determined. Several bands were seen, including (Figure 1) bands ranging from 18.4 to 25 kDa, a band at 14.4 kDa, two bands between 35 and 45 kDa, and another band between 66.2 and 116 kDa. Also, at 66.2 kDa a common band between test (amastigotes) and negative control (un-infected macrophages) was observed.

2-DE Western blotting and identification of proteins by MS

The *L. infantum* amastigotes were extracted from the macrophages and lysed. The lysates were separated by 2-DE using isoelectric focusing (Figure 2(a–c)). Three gels were run for each test. Considering our findings, approximately 900 spots were seen on each of the 2-DE gels and approximately 150 immunoreactive spots were seen on the PVDF. Thirteen repeatable, intense immunoreactive spots were separated from the gel for MS analysis (Figure 2(d)) and finally, 11 immunoreactive proteins were identified by MALDI TOF/TOF MS. The identified protein spots were prohibitin (PHB), ornithine aminotransferase (OAT),

Figure 1. Western blotting of amastigote of *L. infantum*; (a) ladder, (b) amastigote antigens probed with CVL-infected dog's sera, (c) extracted antigens of uninfected J774 macrophage probed with CVL-infected dog's sera, (d) amastigote antigens probed with un-infected dog's sera, (e) extracted antigens of uninfected J774 macrophage probed with un-infected dog's sera (all infected and un-infected sera were checked with DAT for CVL).

annexin A4 (ANX-A4), apolipoprotein A-I (APO- A-I), voltage-dependent anion-selective channel protein 1 (VDAC-1), protein disulfide-isomerase A3 (PDI-A3), translocase of the outer mitochondrial membrane (TOM40), heat shock protein 60 (HSP60), aldehyde dehydrogenase (ALDH), complement component 1 Q subcomponent-binding protein (C1QBP), and phosphoglycerate kinase 1 (PGK-1). The description of the proteins is demonstrated in Table 1.

Using the *L. infantum* genome project database (www.genedb.org) and published literature, we retrieved the putative functions of the identified proteins. Several

Figure 2. (a) The immunoproteome of *L. infantum* amastigotes probed with CVL-infected dog's sera, (b) the immunoproteome of *L. infantum* amastigotes probed with non-infected dog's sera, (c) the immunoproteome of non-infected macrophage probed with CVL-infected dog's sera, (d) selected spots for MS analysis (all infected and un-infected sera were checked with DAT for CVL).

identified proteins had varying or multiple functions including metabolism, survival, and pathogenicity. Two of the spots that gave no matches had spectra dominated by trypsin and keratin. The scores of all obtained proteins were more than 62 and were significant. Most of the identified proteins had a high score of more than 150. The score of an obtained single peptide match to TOM40 was less than the significant range, but having appropriate protein sequence coverage, we were persuaded to consider it as a probable immunoreactive protein in *L. infantum* amastigotes.

Discussion

Here, an immunoproteomic approach was conducted on *L. infantum* amastigotes to identify the immunoreactive proteins and introduce new candidates for future applications, such as diagnosis and vaccine against CVL. PHB, OAT, ANX-A4, HSP60, PGK-1, APO A-I, ALDH, PDI-A3, C1QBP, VDAC-1, and TOM40 were identified in this study. The immunogenicity of the identified proteins in *Leishmania* amastigotes may also further explain the pathogenicity, survival, and metabolism of these parasites with a complex life cycle.

Different HSPs have been studied in some parasites including *Leishmania* [23–27]. HSP genes are conserved and important in the virulence of *Leishmania* parasites [26,28]. HSP60 is implicated in mitochondrial protein import and macromolecular assembly [29]. Up-regulation of HSP60 in amastigotes compared with promastigotes and in amphotericin B resistant *Leishmania* spp. was reported in previous studies [30,31].

Although some HSPs strongly elicit the immune responses, few studies were found with respect to HSP60 as an immunogenic protein [26,32–34]. The results of a study revealed that recombinant *L. major* HSP60 elicited humoral immune response in patients with tegumentary leishmaniasis (TL). High-level expression of *Leishmania* HSP60 isoforms in infected cells suggested that they might be involved in the pathogenesis and an attractive target for the immune response [35]. Future investigations will clarify the feasibility of HSP60 as a diagnostic marker and vaccine target.

C1QBP was the other identified immunoreactive protein. C1QBP is a multi-compartmental protein in the mitochondria that is involved in inflammation, regulation of complement activation, ribosome biogenesis, regulation of apoptosis, and transcriptional regulation [36–38]. To the best of our knowledge, no report exists with respect to C1QBP in *Leishmania* parasites. Checking the expression of this protein in *Leishmania* lesions and detecting anti-C1QBP antibody in leishmaniasis patients can reveal more details in immunogenicity and diagnostic properties of this protein.

ALDH is another protein which was found in amastigotes. It belongs to the mitochondrial enzymes in *Leishmania* parasites and is related to the metabolic functions and pathogenicity [39–42]. Down-regulation of ALDH is associated with the loss of parasites' infectivity, and up-regulation in promastigotes compared to amastigotes can further confirm the mentioned functions of this enzyme in *Leishmania* parasites [43,44]. Immunogenicity of ALDH has been shown in

humans, such as head and neck cancer patients [45]. Whether ALDH can be considered in *Leishmania* as a diagnostic marker needs more investigation.

In previous studies, the relationship of APOs in the mammalian lipophosphoglycans (LPGs) with a virulent and immunogenic protein (Kinetoplastid membrane protein-11 (KMP-11)) in *Leishmania* parasites was reported. These studies declare that the overall lipid bilayer pressure can be regulated with KMP-11, using collaboration with the APOs [46–51]. However, there is no information with respect to the function of the APOs in the structure of *Leishmania* parasites and it should be considered in future investigations.

The PGK obtained in our study matched with the PGK B due to its molecular weights and the high ratio of expression of PGK B in amastigote form of *Leishmania* parasites [52,53]. Generally, PGKs are essential enzymes involved in the survival and glycolytic pathway of some *Leishmania* spp [54,55]. Up-regulation of the PGK gene in antimony-resistant *Leishmania* spp [56], and the confirmation of the immunostimulant property of this enzyme in *Leishmania* parasites can further propose this protein as a critical marker in future research.

PDI, a protein with varied molecular weights (15, 40, 47, 52, and 57 kDa), is involved in virulence and survival in *Leishmania* parasites by means of toxification of macrophage environment [57–60]. It was postulated that PDI in *Leishmania* parasites elicit protective immunity through the pro-inflammatory cytokines of CD8+ and CD4+ (Th1 and Th17) cells [58,61–63]. Also, it was shown that the combination of PDI and two other proteins of *Leishmania* promastigotes had 75% prophylactic efficacy and provoked Th1-biased cytokine responses both in golden hamsters and human [64]. Thus, further research supports the idea that this protein could be considered as a vaccine candidate, diagnostic marker and a new target for anti-*Leishmania* chemotherapy with regard to leishmaniasis [12,65–67].

ANXs are soluble proteins with vesicular transport and extracellular functions. The genomic datasets did not reveal any ANX domains in *Leishmania* parasites; however, an experimental study introduced ANX-A5 as a marker involved in survival and pathogenicity of *Leishmania* parasites [68–73]. Although suppression of the inflammation and presentation of histopathological symptoms of lesions in patients with cutaneous leishmaniasis are related to the ANX-A1, no function has been reported for ANX-A4 in *Leishmania* parasites so far [72–75]. Detection of anti-ANX-A4 in the sera of dogs infected with CVL can further confirm this protein as a diagnostic marker. Also, some studies pointed out that the ANXs of the parasites can modulate the host-immune and host-parasite interactions, so they might be attractive targets for vaccine and treatment in leishmaniasis in future [73,76].

OAT, as an introduced enzyme in the structure of *L. infantum* amastigote in our study, has very narrow substrate specificity in protozoa. The information regarding OAT in *Leishmania* parasites is scarce, but some other aminotransferases were investigated in these parasites [77,78]. Metabolic functions of OAT may propose this enzyme as a drug target in leishmaniasis in the near future [78,79].

VDAC is the most abundant protein in the mitochondrial outer membrane (MOM). In *L. major*, up-regulation of VDAC gene was reported in metacyclic promastigotes compared to procyclic [80]. Adaptation of the *Leishmania* parasite with the pH and nutritional stresses is considered as an important function of VDAC in this parasite. Also, further critical functions including metabolic flux, apoptosis, intracellular signaling and blocking of VDAC with 4-aminopyridine and glibenclamide might introduce this protein as a therapeutic target in *Leishmania* spp [81–84].

PHBs with 95–100% similarity within the genus in *Leishmania* parasites have a role in cell survival, metabolism, and inflammation [85–88]. In *Leishmania*, the PHB is an actin-interacting protein and has high homology with phagosome proteins [89]. The results of a study showed that the infectivity of macrophage with promastigotes is reduced, using anti-prohibitin antibodies [87]. An immunological study conducted in recent years showed that the PHB was able to provoke the Th1 cellular response by producing high levels of IFN- γ , IL-12, and GM-CSF in the immunized animals and induced high level of IFN- γ in PBMCs from recovered leishmaniasis patients [90]. Provoking antibodies against PHB in VL patients and introducing it as an immunodominant protein in previous studies can further confirm our result [87,90,91].

The review of the literature revealed that most of the reported proteins in our study were of mitochondrial origin. It seems that more proteomic studies could provide further details about the *Leishmania* mitochondrial proteins contents with the lowest levels of cytosolic contamination. Based on the results of this study, we observed the primary immunostimulatory property of these introduced proteins. For proposing of them as vaccine candidates and diagnostic marker for CVL, the Th1 stimulatory property of these proteins should be checked using the assays which evaluate the activation of this cell in the presence of these proteins both *in vitro* and *in vivo*. Also, the prophylactic efficacy of these peptides in golden hamster against *L. infantum* challenge should be investigated.

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Disclosure statement

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