DOI: 10.2478/s11686-013-0151-9
© W. Stefański Institute of Parasitology, PAS
Acta Parasitologica, 2013, 58(3), 376–383; ISSN 1230-2821

VERSITA

Ancylostoma ceylanicum metalloprotease 6 DNA vaccination induces partial protection against hookworm challenge infection

Marcin Wiśniewski[1]*, Sławomir Jaros[1,2], Piotr Bąska[3], Michael Cappello[4] and Halina Wędrychowicz[1,5]

[1] Division of Parasitology and Parasitic Diseases, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Warsaw, Poland; [2] Laboratory of Molecular Biology, Mabion Ltd., Łódz, Poland; [3] Division of Pharmacology and Toxicology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Warsaw, Poland; [4]Program in International Child Health, Departments of Pediatrics, Microbial Pathogenesis, and Public Health, Yale University School of Medicine, New Haven, Connecticut 06520-8081, USA; [5] Laboratory of Molecular Parasitology, W. Stefański Institute of Parasitology PAS, Warsaw, Poland

Abstract

Hookworms are blood feeding intestinal nematodes that infect more than 500 million people and cause iron deficiency anemia. Infected children suffer from physical and cognitive growth retardation. Because of potential anthelminthic drug resistance, the need for vaccine development is urgent. Numerous antigens have been tested in animal models as vaccines against hookworm infection, but there is no effective human vaccine. We cloned a cDNA encoding *Ancylostoma ceylanicum* metalloprotease 6 (*Ace-mep-6*). Ace-MEP-6 is a protein with a predicted molecular mass of 101.87 kDa and based on computational analysis it is very likely to be engaged in food processing via hemoglobin digestion. Groups of hamsters were immunized with an *Ace-mep-6* cDNA vaccine, either once or three times. Animals that were administered one dose developed high resistance (80%, $p < 0.01$) against challenge infection, whereas triple immunization resulted in no worm burden reduction. These results suggest that DNA vaccines can be powerful tools in ancylostomiasis control, although the mechanisms through which protection is conferred remain unclear.

Keywords

Hookworm, *Ancylostoma*, metalloprotease, DNA vaccination

Introduction

Hookworms are half inch long blood feeding nematodes that infect more than 500 million people worldwide (Bethony *et al.* 2006), especially in the tropics and subtropical rural regions (Brooker *et al.* 2004; Bungiro and Capello 2004). Infective larvae enter humans by the oral route or via active skin penetration (Brooker *et al.* 2004). *Necator americanus* is the most prevalent species of hookworm, whereas *A. duodenale* occurs in more geographically restricted areas (Hotez *et al.* 2004). *Ancylostoma ceylanicum* is considered as a zoonotic species (Fujiwara *et al.* 2006), but can cause patent infection in humans (Ngui *et al.* 2012). Other species like *Ancylostoma caninum* and *Ancylostoma brasiliense* cause eosinophilic enteritis and cutaneous larva migrans syndrome (Hotez *et al.* 2004) and also cause zoonoses (Fujiwara *et al.* 2006). Blood loss caused by one worm has been estimated at 0.2 ml per day (Pawlowski *et al.* 1991), and heavy infection results in iron deficiency anemia. In children, hookworm infection may lead to physical, mental and cognitive growth retardation (Hotez *et al.* 2003). The recommended way of controlling ancylostomiasis is through administration of anthelmintic drugs, mainly benzimidazoles. However, given concerns about decreasing efficacy (Humphries *et al.* 2011, 2012; Albonico *et al.* 2003) and rapid reinfection after treatment (Albonico *et al.* 1995; 2003) there is an urgent need for development of an effective vaccine.

In 1978 Miller reported that irradiated *A. caninum* L3 larvae induce high protection among dogs (Miller 1978). But because of veterinarians' doubts and pet owners concerns that the 90% protection generated by administration of attenuated larvae was not sufficient, the vaccine was removed from the market (Fujiwara *et al.* 2006a). Similar experiments conducted by Fujiwara *et al.* (2006a) showed that vaccination with irra-

*Corresponding author: marcin_wisniewski@sggw.pl

diated L3 larvae induced 53% reduction of worm burden following challenge infection in dogs. Moreover, the worms recovered from vaccinated dogs were up to 13% shorter. But since vaccine comprised of attenuated larvae is unsuitable for humans because of potential toxicity and ethical concerns, there has been an extensive search for antigens crucial for inducing protection to accelerate development of a recombinant vaccine comprised of well characterized antigens.To date, numerous antigens combined with different adjuvants have been tested with different rates of success (Xiao *et al.* 2008)

Hookworms are thought to facilitate feeding by catalyzing hemoglobin degradation (Williamson *et al.* 2004; Ranjit *et al.* 2009) that is why hemoglobinases are in the interests of scientists (Wasyl *et al.* 2013; Person *et al.* 2010). Promising vaccination with aspartic proteases, the first enzyme in the cascade of hemoglobin proteolysis (Ranjit *et al.* 2009) has raised hopes for successful vaccine development (Loukas *et al.* 2005; Pearson *et al.* 2009). After digestion by protease, hemoglobin is subsequently cleaved by cysteine and metalloproteases (Ranjit *et al.* 2009). It is unknown whether a vaccine targeting aspartic proteases is the only way to interfere with parasite feeding, or if a vaccine aimed at enzymes engaged in subsequent food processing might also work. We chose to test Ace-MEP-6 as a vaccine antigen and asses the number of doses necessary for protection. Since IFNγ levels correlate with protection against adult worms (Quinnell *et al.* 2004), and this cytokine may be released following cDNA vaccination, we decided to use a vaccine comprised of Ace-MEP-6 cDNA with a lipid adjuvant, FuGENE 6 Transfection Reagent (Roche). Advantages of DNA vaccines include low cost, simple design/administration, insignificant construct immunogenicity and relatively fast production (Wędrychowicz and Wiśniewski 2003; Abdulhaqq and Weiner 2008). All this led us to assess the vaccine potential of *Ace-mep-6* administered as a DNA vaccine.

Materials and Methods

RACE-PCR

Total RNA was isolated form 100 adult *Ancylostoma ceylanicum* using Total RNA PLUS kit (A&A Biotechnology). RNA was reverse transcribed to cDNA using M-MuLV Reverse Transcriptase (MBI Fermentas) followed by RNaseH treatment and purification with Clean-Up kit (A&A Biotechnology). The poly(C) tail was added using Terminal Deoxynucleotidyl Transferase (MBI Fermentas) according to manufactures protocol followed by purification with Clean-Up kit (A&A Biotechnology). The total cDNA was amplified using pTBam primer (CCGCCACGCGTGGATCCGTTTTTTTTTTTTTTT) and polyG primer (GGGGGGGGGGGGGGG). The reaction was proceeded in total volume of 50 µl (1U *Taq*, 0.2 mM dNTPs each, 1.5 $MgCl_2$, 200 nM of each primer, 1 x buffer for *Taq* polymerase, 60 ng of matrix total cDNA): 94°C – 5 min, 10 x (94°C – 30 sec, 50°C – 30 sec, 72°C – 5 min), 72°C – 10 min. The PCR product was used as matrix for cloning of cDNA encoding Ace-MEP-6.

Based on sequences of *Ancylostoma caninum* and *Haemonchous contortus* metalloproteases primer for 3`RACE PCR AcemepL–(GATCAGACTGTAGATCCATGCGAGG) was designed. The 3` end of *Ace-mep-6* cDNA was amplified using AcemepL and pTBam primers. The reaction was carried in the total volume of 50 µl as follows: 94°C – 3 min, 35 x (94°C – 30 sec, 56°C – 30 sec, 72°C – 2 min), 72°C – 10 min (1 U *Taq*, 0.2 mM dNTPs each, 1.5 $MgCl_2$, 200 nM of each primer, 1 x buffer for *Taq* polymerase, 12 ng of matrix DNA). The amplified band was extracted from the gel, blunt ended using *Pfu* polymerase (72°C, 30 min; 1 U *Pfu*, 200 nM dNTPs mix, 1 x buffer for *Pfu* polymerase, 1 µg of PCR product), purified with Clean-Up kit (A&A Biotechnolgy), digested with *Bam*HI enzyme, purified with Clean-Up kit followed by ligation with pBluescript digested with *Bam*HI and *Eco*RV enzymes. The insert was cloned and sequenced. Based on the results of the sequencing the new primer – Acemep5`(ATCATGTTGTCAATCGGGGTCCTGG) was designed to amplify the 5` end of *Ace-mep-6* cDNA. The PCR with polyG and Acemep5` primers was proceeded in the total volume of 50 µl as follows: 94°C – 3 min, 35 x (94°C – 30 sec, 56°C – 30 sec, 72°C – 1 min), 72°C – 10 min (1 U *Taq*, 0,2 mM dNTPs each, 2 mM $MgCl_2$, 200 nM of each primer , 1 x buffer for *Taq* polymerase, 12 ng of matrix total cDNA).The product was extracted from the gel, ligated with pCR 2.1 vector (Invitrogen), cloned and sequenced.

Computational analysis

The protein parameters (molecular weight, pI, signal peptide, potential phosphorylation sites) were calculated using EXPASY server. To identify potential disulfide bridges 3D structure was constructed with LOMETS metaserver (Wu and Zhang 2007). The highest scored structure was chosen, visualized with PyMOL and the number and location of disulfide bridges were assessed by visual examination of the structure. Since Hc-MEP-1 (Gen Bank no CAA99352.1) (Redmond *et al.* 1997), Ac-MEP-1 (Gen Bank No AAG29103.2) (Jones and Hotez 2002) and Na-MEP-1 (ACB13196.1) (Ranjit *et al.* 2009) show homology to Ac-MEP-6 and are considered to be expressed on gut microvilli the structure of those proteins were modeled with LOMETS and we checked if there are similarities indicating that Ace-MEP-6 may be also associated with gut microvilli.

Vaccination trials

For cloning *Ace-mep-6* in pCDNA 3.1 vector the Acmep6 LpcDNA (AGCTAGCATGGTCTTCAATATCCTT) and Acmep6RpcDNA (TTCTAGACTCCTGAACCCAAACTGA) primers were designed. The primers Acmep6RpcDNA and Acmep6RpcDNA contained sequences recognized by restric-

tion enzymes *Nhe*I and *Xba*I respectively (underlined). Both, PCR product and vector were digested with *Nhe*I and *Xba*I, ligated and the recombinant pCDNA 3.1 vector containing *Ace-mep-6* cDNA fragment (nucleotides 77-2712) was cloned. The sequence integrity of the construct was confirmed by nucleotide sequence analysis.

The vaccine trials were conducted in 8–10 week old male Golden Syrian Hamsters. The animals were divided into 5 groups (5–6 animals/group): immunized x 1, immunized x 3, challenge and two adjuvant control groups. All immunizations were administered in a total volume of 200 µl. On day 1, 14, 28 of the experiment immunized x 3 group received 50 µg of pcDNA3.1+/*Ace-mep-6* combined with Fugene 6 (Roche) (8:1 *v/v*) applied intranasally. The appropriate adjuvant control animals received 50 µg pcDNA3.1+ combined with Fugene 6 (8:1 *v/v*) applied on day 1, 14, 28. On day 28 animals from immunized x 1 group received 50 µg of pcDNA3.1+/*Ace-mep-6* combined with Fugene 6 (8:1 *v/v*) and the animals from appropriate control group received pcDNA3.1+ combined with Fugene 6 (8:1 *v/v*). On 42 day of the experiment animals from all 5 groups were challenged with 60 L3 *A. ceylanicum* larvae and euthanized 21 days later. Directly after euthanasia the hematocrit value was determined and the number of worms in the gut of each hamster was counted. All animals were maintained under standard conditions according to local regulations, all experiments were approved by the Third Local Ethical Committee on Warsaw University of Life Sciences – SGGW. The study was conducted adhering to the institution's guidelines for animal husbandry.

Statistical evaluation

The data were analyzed for statistically significant differences between groups using the Kruskal-Wallis test.

Results

RACE-PCR

Sequencing of cloned product resulted in indentifying a novel 2815 bp cDNA sequence encoding *A. ceylanicum* zinc metalloprotease 6 (*Ace-mep-6*) which was deposited in GenBank (AY371701.1).

Computational analysis

All analyses were performed in July 2012. Ace-MEP-6 is a protein with a theoretical molecular mass of 101.87 kDa with 28 phosphorylation sites of serine (63, 68, 73, 74, 83, 85, 101, 108, 111, 116, 117, 128, 132, 163, 210, 212, 234, 257, 320, 441, 524, 525, 617, 658, 688, 803, 860, 897), 7 phosphorylation sites of threonine (57, 79, 88, 89, 97, 98, 109, 112, 121, 124, 129, 131, 133, 138, 139, 140, 141, 240, 256, 259, 364, 465, 616, 751, 789, 813, 846) and 14 phosphorylation sites of tyrosine (157, 164, 166, 172, 188, 338, 427, 500, 546, 622, 627, 638, 677, 695). Amino acids 181 – 897 build M13 conserved domain (cd08662). The structure is stabilized by 3 computationally defined disulfide bridges (184 – 882, 192 – 842, 764 – 896). Although analysis with signal 4.0 showed no signal peptide, TMHMM v 2.0 identified transmembrane region (7 – 29) and indicated that N term of Ace-MEP-6 is on the cytoplasmic side of the membrane with 0,98 probability. Molecular modeling showed the presence of 163 amino acid long peptide with undefined structure (Fig. 1). Similar results were obtained for Ac-MEP-1, Na-MEP-1 and Hc-MEP-1 that are associated with gut microvilli (Jones and Hotez 2002; Ranjit *et al.* 2009; Redmond *et. al.* 1997) (Fig. 1). Ac-MEP-1, Na-MEP-1 and Hc-MEP have computationally defined (respectively 132, 132 and 65 long) amino acid peptidewith undefined

Table I. Nematodes proteins deposited in GenBank identified as similar to Ace-MEP-6

Number of proteins similar to Ace-EP-6	Species	Protein name	Gen Bank Accession number	BLAST score	% of identity	% of similarity
1	*Ancylostoma ceylanicum*	Ace-MEP-7	AAQ75757.1	1036	55	69
1	*Necator americanus*	MEP	ACB13196.1	1192	62	78
3	*Ancylostoma caninum*	MEP-1	AAG29103.2	1014	54	69
		MENP-1	AAK67057.1	493	45	62
		MEP-2	AAG29105.1	257	32	51
5	*Haemonchus contortus*	MEP-3	AAC31568.1	834	50	67
		MEP	AAC03561.1	766	48	68
		MEP	CAA99352.1	763	48	68
		MEP2	AAC28740.1	577	44	62
		MEP4	AAD41474.1	445	44	65
3	*Ascaris suum*	Neprilysin-1	ADY44517.1	526	47	63
		Neprilysin-1	ADY39823.1	272	28	46
		Neprilysin-1	ADY40242.1	266	29	44
2	*Trichinella spiralis*	Pept. fam. M13	XP_003372438.1	263	29	46
29	*Caenorhabditisspp.*	Pept. fam. M13	EFV51629.1	263	29	46

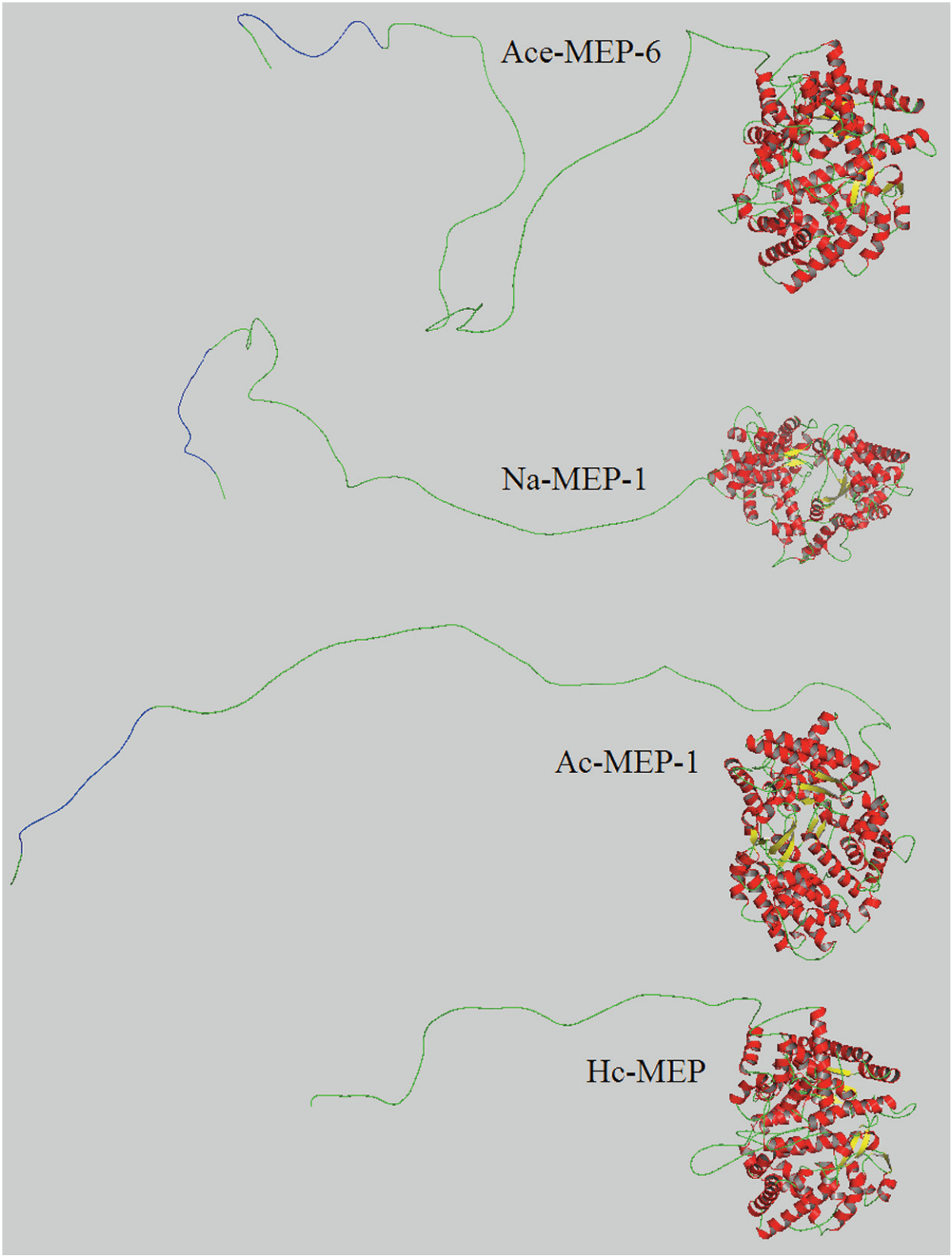

Figure 1. Computationally predicted teritary structure of Ace-MEP-6 and proteins showing high level of similarity. Ace-MEP-6 – *Ancylostoma ceylanicum* metalloprotease 6 (GenBank No. AAQ75756.1), Ac-MEP-1 – *Ancylostoma caninum* metalloprotease 1 (GenBank No AAG29103.2), Na-MEP-1 – *Necator americanus* metalloprotease 1 (GenBank No.. ACB13196.1), Hc-MEP-1 – *Haemonchus contortus* metalloprotease (Gen Bank No. CAA99352.1) Helices are colored with red, sheets are colored with yellow. Green is undefined structure. Potential transmembrane region is showed in blue

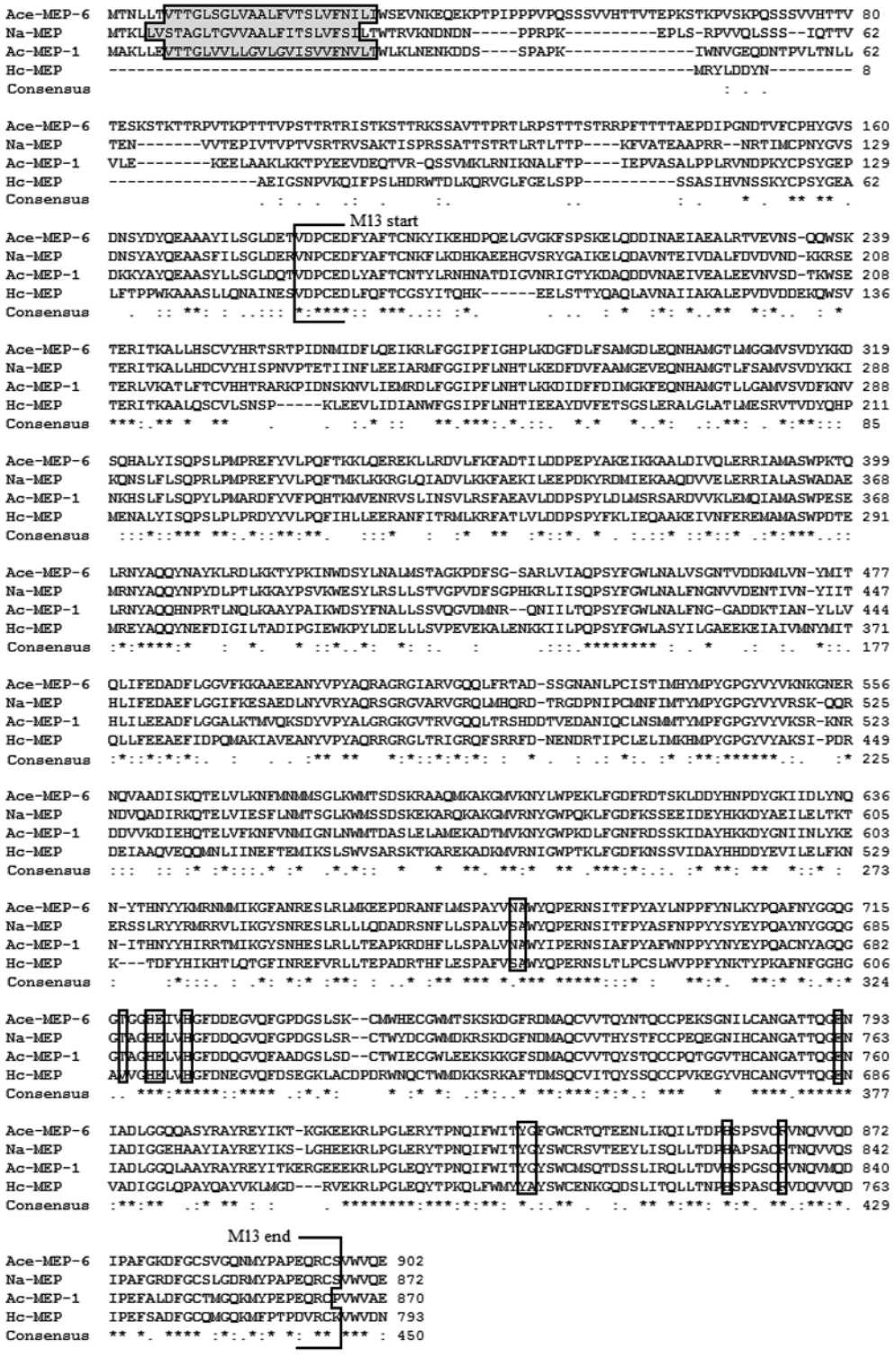

Figure 2. Alignment of of Ace-MEP-6 and proteins showing a high level of similarity. Ace-MEP-6 – *Ancylostoma ceylanicum* metalloprotease 6 (GenBank No. AAQ75756.1), Ac-MEP-1 – *Ancylostoma caninum* metalloprotease 1 (GenBank No. AAG29103.2), Na-MEP-1 – *Necator americanus* metalloprotease 1 (GenBank No. ACB13196.1), Hc-MEP-1 – *Haemonchus contortus* metalloprotease (Gen Bank No CAA99352.1). Potential transmembrane region is shaded. Amino acids of active site are showed in block. The frame shows the beginning and the end of M13 domain. Identical amino acids are showed in consensus as "*", conserved substitutions are showed as ":", semi-conserved substitution are showed as ".". Vertical lines in the sequences are gaps

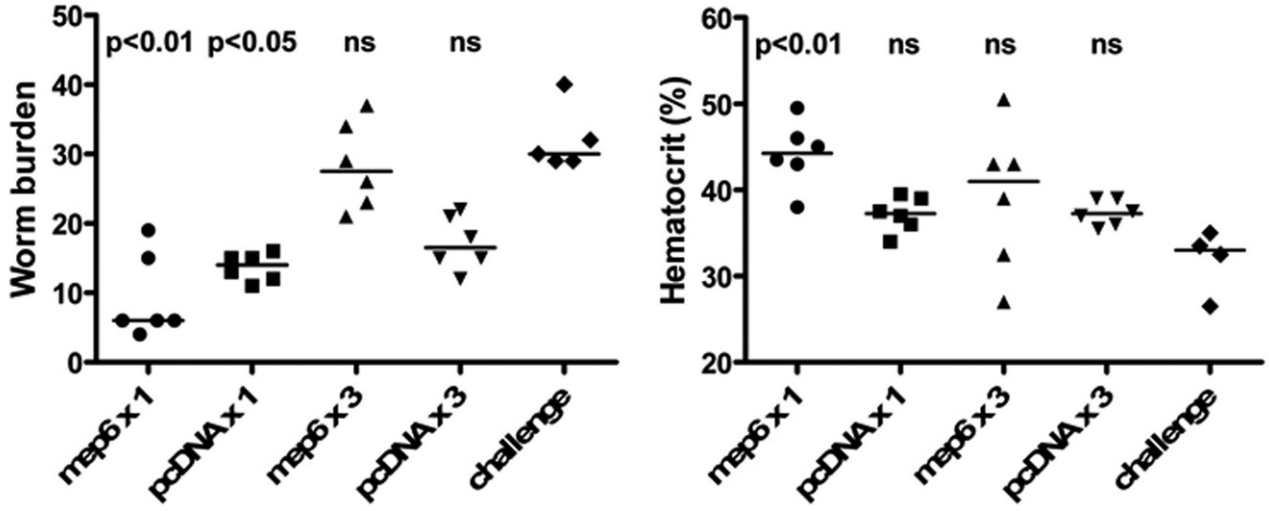

Fig. 3. Worm burden and hematocrit after vaccination with *Ace-mep-6*/pcDNA3.1. The vertical line among groups shows median. The animals were immunized on day 28 (mep6 x 1) or on day 1, 14, 28 (mep6 x 3) using pcDNA3.1+/*Ace-mep-6* combined with Fugene 6. The animals were also immunized with pcDNA3.1+ combined with Fugene 6 on day 28 (pcDNA x 1), on day 1, 14, 28 (pcDNA x 3). The number of worms in the groups was compared to animals not immunized and infected with *A. ceylanicum* (challenge). The p value for each comparison is shown on the figure. The statistically not significant differences are marked as "ns"

structure. Neither of them contained signal peptide but Ac-MEP-1 and Na-MEP-1 contain transmembrane region (7 – 29, 5 – 27 respectively) (Fig.1, Fig. 2).

BlastP identified 150 proteins (of verbates and inverbates) which of 44 were nematode proteins, showing homology to Ace-MEP-6 (Table I). Three of parasitic proteins (Na-MEP, Ace-MEP-1 and Hc-MEP), well characterized and showing homology to Ace-MEP-6 were chosen and aligned to Ace-MEP-6 (Fig. 2). The amino acid in active site shows some degree variety. The first amino acid of active site of Ace-MEP-6 is asparagine and in other proteases it may also be serine. The second amino acid is identical for Ace-MEP-6, Na-MEP-1 and Ac-MEP-1 (threonine) and different for Hc-MEP-1 (valine). The similar pattern occurs at 9th amino acid of active site which is glicine for Ace-MEP-6, Na-MEP-1, Ac-MEP-1 and alanine for Hc-MEP-1. Other 8 amino acid of active sites are identical for Ace-MEP-6, Na-MEP-1, Ac-MEP-1, Hc-MEP-1.

Vaccination trials

Single immunization resulted in an 80% ($p < 0,01$) reduction in worm burden and a 33% increase in hematocrit ($p < 0,01$). Although vaccination with the pcDNA3.1+ vector alone induced a 53% ($p < 0,05$) worm burden reduction, it was not associated with an improvement in hematocrit (Fig. 3).

Discussion

Ace-MEP-6 is potentially highly phosphorylated, and similar to orthologus metalloproteases from parasitic nematodes (Na-MEP-1, Ac-MEP-1, Hc-MEP) contains the M13 domain with an 11 amino acid active site. Results of Jones and Hotez (2002), Redmond *et. al.* (1997), Ranjit *et al.* (2009) showed that Ac-MEP-1, Hc-MEP and Na-MEP-1 are associated with gut microvilli. Here we computationally identified transmembrane peptide in Ace-MEP-6, Ac-MEP-1, and Na-MEP-1, which may indicate that Ace-MEP-6 is anchored in the cell membrane like Na-MEP-1 (Ranjit *et. al.* 2009) and is engaged in hemoglobin digestion. The important role of this protein in parasite physiology may be confirmed by the results of presented vaccine trials. However, gut associated metalloproteases are not the only class of hookworms metalloproteases. Hookworms express astacin like metalloproteases (Zhan *et al.* 2002; Mendez *et al.* 2005; Feng *et al.* 2007; Bąska *et al.* 2013). Immunization with Ace-MTP-1 (Mendez *et al.* 2005) and Ac-MTP-1 (Hotez *et al.* 2003) resulted in no statistical significant worm burden reduction but diminished fecal egg count was observed after immunization with Ace-MTP-1 (Mendez *et al.* 2005). But since belonging to different group of metalloproteases and different antigen delivery those results cannot be directly compared to ours.

Here we show that targeting other enzymes (metalloproteases) likely to be engaged in hemoglobin digestion is also effective, and should be explored further. The significant worm burden reduction and increasing hematocrit values observed in immunized hamsters after a single vaccination suggests that administration of cDNA encoding a protein antigen is very effective. Moreover, the stability of DNA is an advantage of this approach compared to protein vaccines. Purification of recombinant plasmid is less time consuming than expressing and purification a recombinant protein from eukaryotic expressing systems, whereas expressing in *E. coli*

may result in inclusion bodies formation and the need for protein refolding (Pearson *et. al.* 2009).

Hiszczyńska-Sawicka *et al.* (2010) reported that sheep immunized with a cDNA vaccine against toxoplasmosis secreted different amounts of IFNγ, depending on time after immunization and transfection agent administered. Our experiments additionally showed that differences depend on the number of immunizations. Single immunization in our case resulted in a greater worm burden reduction than three doses, which is desirable due to easier vaccine administration. The mechanism of lower efficacy after 3 immunizations is unknown, but may be caused by induction of immune tolerance or immunosuppression induced by the vaccine construct. Current human hookworm vaccine development has been hampered by allergic complication (Zhan *et al.* 2012). The vaccine trials of leading vaccine candidate for ancylostomiasis were held due to unacceptable toxicities, including generalized urticarial reactions in several volunteers (Bethony *et al.* 2011). The risk of an allergic reaction seems to be lower in the case of DNA immunization.

In conclusion: based on our computational predictions we hypothesize that Ace-MEP-6 is an M13 domain containing protein engaged in food processing via hemoglobin digestion. Our experiments indicate that Ace-MEP-6 is a promising vaccine antigen working with highly efficient manner when administered as a single cDNA dose.

This research was supported by a grant from the Polish Ministry of Science and Higher Education (Project No. 3 P06K 024 24) and a Clinical Research Grant from the March of Dimes Birth Defects Foundation (to MC).

References

Abdulhaqq S.A., Weiner D.B. 2008. DNA vaccines: developing new strategies to enhance immune responses. *Immunologic Research*, 42, 219–232. DOI: 10.1007/s12026-008-8076-3.

Albonico M., Bickle Q., Ramsan M., Montresor A., Savioli L., Taylor M. 2003. Efficiency of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar. *Bulletin of the World Health Organization*, 81, 343–352.

Albonico M., Smith P.G., Ercole E., Hall A., Chwaya H.M., Alawi K.S., Savioli L. 1995. Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 89, 538–541.

Bąska P., Wiśniewski M., Krzyżowska M., Długosz E., Zygner W., Górski P., Wędrychowicz H. 2013. Molecular cloning and characterisation of in vitro immune response against astacin-like metalloprotease Ace-MTP-2 from *Ancylostoma ceylanicum*. *Experimental Parasitology*, 133, 472–482. DOI: 10.1016/j.exppara.2013.01.006. Epub 2013 Jan 29.

Bethony J., Brooker S., Albonico M., Geiger S.M., Loukas A., Diemert D., Hotez P.J. 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet*, 367, 1521–1532. DOI: 10.1016/S0140-6736(06)68653-4.

Bethony J.M., Cole R.N., Guo X., Kamhawi S., Lightowlers M.W., Loukas A., Petri W., Reed S., Valenzuela J.G, Hotez P.J. 2011. Vaccines to combat the neglected tropical diseases. *Immunological Reviews*, 239, 237–270. DOI: 10.1111/j.1600-065X.2010.00976.x.

Brooker S., Bethony J., Hotez P.J. 2004. Human hookworm infection in the 21st century. *Advances in Parasitology*, 58, 197–288. DOI: 10.1016/S0065-308X(04)58004-1.

Bungiro R., Cappello M. 2004. Hookworm infection: new developments and prospects for control. *Current Opinion in Infectious Diseases*, 17, 421–426.

Feng J., Zhan B., Liu Y., Liu S., Williamson A., Goud G., Loukas A., Hotez P. 2007. Molecular cloning and characterization of Ac-MTP-2, an astacin-like metalloprotease released by adult *Ancylostoma caninum*. *Molecular and Biochemical Parasitology*, 152,132–138.

Fujiwara R.T., Geiger S.M., Bethony J., Mendez S. 2006. Comparative immunology of human and animal models of hookworm infection. *Parasite Immunology*, 28, 285–293. DOI: 10.1111/j.1365-3024.2006.00821.x.

Fujiwara R.T., Loukas A., Mendez S., Williamson A.L., Bueno L.L., Wang Y., Samuel A., Zhan B., Bottazzi M.E., Hotez P.J., Bethony J.M. 2006a. Vaccination with irradiated *Ancylostoma caninum* third stage larvae induces a Th2 protective response in dogs. *Vaccine*, 24, 501–509. DOI: 10.1016/j.vaccine.2005.07.091.

Hiszczyńska-Sawicka E., Li H., Xu J.B., Oledzka G., Kur J., Bickerstaffe R., Stankiewicz M. 2010. Comparison of immune response in sheep immunized with DNA vaccine encoding *Toxoplasma gondii* GRA7 antigen in different adjuvant formulations. *Experimental Parasitology*, 124, 365–372. DOI: 10.1016/j.exppara.2009.11.015.

Hotez P.J., Ashcom J., Zhan B., Bethony J., Loukas A., Hawdon J., Wang Y., Jin Q., Jones K.C., Dobardzic A., Dobardzic R., Bolden J., Essiet I., Brandt W., Russell P.K., Zook B.C., Howard B., Chacon M. 2003. Effect of vaccination with a recombinant fusion protein encoding an astacin like metalloprotease (MTP-1) secreted by host-stimulated *Ancylostoma caninum* third-stage infective larvae. *Journal of Parasitology*, 89, 853–855.

Hotez P.J., Brooker S., Bethony J.M., Bottazzi M.E., Loukas A., Xiao S. 2004. Hookworm infection. *New England Journal of Medicine*, 351, 799–807.

Hotez P.J., Zhan B., Bethony J.M., Loukas A., Williamson A., Goud G.N., Hawdon J.M., Dobardzic A., Dobardzic R., Ghosh K., Bottazzi M.E., Mendez S., Zook B., Wang Y., Liu S., Essiet-Gibson I., Chung-Debose S., Xiao S., Knox D., Meagher M., Inan M., Correa-Oliveira R., Vilk P., Shepherd H.R., Brandt W., Russell P.K. 2003. Progress in the development of a recombinant vaccine for human hookworm disease: the Human Hookworm Vaccine Initiative. *International Journal for Parasitology*, 33, 1245–1258. DOI: 10.1016/S0020-7519(03)00158–9.

Humphries D., Mosites E., Otchere J., Twum W.A., Woo L., Jones-Sanpei H., Harrison L.M., Bungiro R.D., Benham-Pyle B., Bimi L., Edoh D., Bosompem K., Wilson M., Cappello M. Epidemiology of hookworm infection in Kintampo North Municipality, Ghana: patterns of malaria coinfection, anemia, and albendazole treatment failure. *American Journal of Tropical Medicine and Hygiene*, 84, 792–800. DOI: 10.4269/ajtmh.2011.11-0003.

Humphries D, Nguyen S, Boakye D, Wilson M, Cappello M. 2012. The promise and pitfalls of mass drug administration to control intestinal helminth infections. *Current Opinion in Infectious Diseases,* 25, 584–589. DOI: 10.1097/QCO.0b013e328357e4cf.

Jones B.F., Hotez P.J. 2002. Molecular cloning and characterization of Ac-mep-1, a developmentally regulated gut luminal metal-

loendopeptidase from adult *Ancylostoma caninum* hookworms. *Molecular and Biochemical Parasitology*, 119, 107–116. DOI: 10.1016/S0166-6851(01)00409-1.

Loukas A., Bethony J.M., Mendez S., Fujiwara R.T., Goud G.N., Ranjit N., Zhan B., Jones K., Bottazzi M.E., Hotez P.J. 2005. Vaccination with Recombinant Aspartic Hemoglobinase Reduces Parasite Load and Blood Loss after Hookworm Infection in Dogs. *PLoS Medicine*, 2(10), e295. DOI:10.1371/journal.pmed.0020295.

Mendez S., Zhan B., Goud G., Ghosh K., Dobardzic A., Wu W., Liu S., Deumic V., Dobardzic R., Liu Y., Bethony J., Hotez P.J. 2005 Effect of combining the larval antigens *Ancylostoma* secreted protein 2 (ASP-2) and metalloprotease 1 (MTP-1) in protecting hamsters against hookworm infection and disease caused by *Ancylostoma ceylanicum*. *Vaccine*, 2, 23, (24), 3123–3130.

Miller T.A. 1978. Industrial development and field use of the canine hookworm vaccine. *Advances in Parasitology*, 16, 333–342.

Ngui R., Lim Y.A., Traub R., Mahmud R., Mistam M.S. 2012. Epidemiological and genetic data supporting the transmission of *Ancylostoma ceylanicum* among human and domestic animals. *PLOS Neglected Tropical Diseases*, 6, e1522. DOI: 10.1371/journal.pntd.0001522.

Pawlowski Z.S., Schad G.A, Stott G.J. 1991. *Hookworm Infection and Anemia. Approaches to Prevention and Control.* Geneva, World Health Organization.

Pearson M.S., Bethony J.M., Pickering D.A., de Oliveira L.M., Jariwala A., Santiago H., Miles A.P., Zhan B., Jiang D., Ranjit N., Mulvenna J., Tribolet L., Plieskatt J., Smith T., Bottazzi M.E., Jones K., Keegan B., Hotez P.J., Loukas A. 2009. An enzymatically inactivated hemoglobinase from *Necator americanus* induces neutralizing antibodies against multiple hookworm species and protects dogs against heterologous hookworm infection. *FASEB Journal*, 23, 3007–3019. DOI: 10.1096/fj.09-131433.

Pearson M.S, Pickering D.A, Tribolet L., Cooper L., Mulvenna J., Oliveira L.M., Bethony J.M., Hotez P.J., Loukas A. 2010. Neutralizing antibodies to the hookworm hemoglobinase Na-APR-1: implications for a multivalent vaccine against hookworm infection and schistosomiasis. *Journal of Infectious Diseases*, 15; 201, 1561–1569. DOI: 10.1086/651953.

Quinnell R.J., Pritchard D.I., Raiko A., Brown A.P., Shaw M.A. 2004. Immune responses in human necatoriasis: association between interleukin-5 responses and resistance to reinfection. *Journal of Infectious Diseases*, 190, 430–438. DOI: 10.1086/422256.

Ranjit N., Zhan B., Hamilton B., Stenzel D., Lowther J., Pearson M., Gorman J., Hotez P., Loukas A. 2009. Proteolytic degradation of hemoglobin in the intestine of the human hookworm *Necator americanus. Journal of Infectious Diseases*, 15, 199(6), 904–912. DOI:10.1086/597048.

Redmond D.L., Knox D.P., Newlands G., Smith W.D. 1997. Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*. *Molecular and Biochemical Parasitology*, 85, 77–87. DOI: 10.1016/S0166-6851(96)02812-5.

Wasyl K., Zawistowska-Deniziak A., Bąska P., Wędrychowicz H., Wiśniewski M. 2013. Molecular cloning and expression of the cDNA sequence encoding a novel aspartic protease from *Uncinaria stenocephala*. *Experimental Parasitology,* 134, 220–227. DOI: 10.1016/j.exppara.2013.03.

Wędrychowicz H., Wisniewski M. 2003. Progress in development of vaccines against most important gastrointestinal helminth parasites of humans and animals. *Acta Parasitologica*, 48, 239–245.

Williamson A.L., Lecchi P., Turk B.E., Choe Y., Hotez P.J., McKerrow J.H., Cantley L.C., Sajid M., Craik C.S., Loukas A. 2004. A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. *Journal of Biological Chemistry*, 20; 279(34), 35950–7. DOI: 10.1074/jbc.M405842200.

Wu S., Zhang Y. LOMETS: A local meta-threading-server for protein structure prediction. 2007. *Nucleic Acids Research*, 35, 3375–3382. DOI: 10.1093/nar/gkm251.

Xiao S, Zhan B, Xue J, Goud GN, Loukas A, Liu Y, Williamson A, Liu S, Deumic V, Hotez P. 2008. The evaluation of recombinant hookworm antigens as vaccines in hamsters (*Mesocricetus auratus*) challenged with human hookworm, *Necator americanus*. *Experimental Parasitology,* 118, 32–40. DOI: 10.1016/j.bbr.2011.03.031.

Zhan B., Hotez P.J., Wang Y., Hawdon J.M. 2002. A developmentally regulated metalloprotease secreted by host-stimulated *Ancylostoma caninum* third-stage infective larvae is a member of the astacin family of proteases. *Molecular and Biochemical Parasitology*, 9, 120(2), 291–296.

Zhan B., Santiago H., Keegan B., Gillespie P., Xue J., Bethony J., de Oliveira L.M., Jiang D., Diemert D., Xiao S.H., Jones K., Feng X., Hotez P.J., Bottazzi M.E. 2012. Fusion of Na-ASP-2 with human immunoglobulin Fcγ abrogates histamine release from basophils sensitized with anti-Na-ASP-2 IgE. *Parasite Immunology*, 34, 404–411. DOI: 10.1111/j.1365-3024.2012.01371.x.

Accepted June 07, 2013