No ALAD Polymorphism in Bank Vole Populations from Unpolluted and Lead-Zinc Polluted Areas in Poland*

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Although a variety of biomarkers has been developed to assess the adverse effect of lead on human and animal populations, studies show that the most important factor conditioning the response may be the genetic make-up of an individual. ALAD (delta aminolevulinic acid dehydratase) gene polymorphism found in human populations results in the existence of two alleles: ALAD1 and ALAD2. The difference between them is the result of one nucleotide change (G177C, rs1800435) which implicates different enzyme affinity for lead ions. As a result, carriers of these alleles respond differently to lead exposure in terms of tissue lead content, as well as neurobehavioral response. The aim of our study was to determine if such a phenomenon is present in wild animal populations. Two hypotheses were tested: (i) does the same ALAD polymorphism occur in lead exposed rodent species and is the frequency of different alleles similar to that of humans, (ii) if polymorphism exists, is the tissue lead content higher in individuals having ALAD2 alleles. We used bank voles *(Myodes glareolus)* inhabiting different lead contaminated sites for this purpose. The results obtained show no polymorphism in the bank vole populations that were studied. Contrary to the results obtained for humans, our study shows that all animals were found to have the C nucleotide. In human populations this nucleotide determines the ALAD2 allele and is much less frequent.

Key words: metals, lead, bank vole, ALAD, ALAD polymorphism

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Great amounts of lead have been used and emitted by humans over thousands of years and lead deposits in different environments still pose danger of exposure and poisoning. At risk are not only people directly exposed to lead (i.e. smelter workers), but also human and animal populations inhabiting areas near different sources of contamination. Many biomarkers have been developed to determine lead exposure and assess its toxicity to individuals including analyses of blood lead levels and enzyme activity (ALA dehydratase and FECH, protoheme ferrolyase). As recent studies have shown, this information may be insufficient to assess lead behaviour in an organism and its later potential toxicity. A strong argument which supported the undertaking of our study on ALAD polymorphism was that ALAD enzyme activity was and remains to be an important biomarker of lead exposure in many human and animal studies, as well as a diagnostic tool (ONALAJA & CLAUDIO 2000). It is known that its activity is negatively correlated with the lead level in blood (VANPARYS *et al.* 2008) but the strength of the relationship depends on the species (COMPANY *et al.* 2011). This pattern was also observed in bird blood samples in which lead concentration was below 5 μg/dl (MARTÍNEZ-LÓPEZ *et al.* 2004). HOLLADAY *et al.* (2012) showed that even an 8 week period after dosing of lead pellets to birds was not sufficient to restore previous levels of ALAD. Studies on the DBA strain of mice with a duplication of the ALAD gene showed increased accumulation of lead in blood, kidney and liver when compared to mice with a single copy of this gene (CLAUDIO *et al.* 1996). According to some studies, the inhibition of ALAD may even reach 85% in highly polluted

populations (Vanparys *et al.* 2008). It is not clear how the existence of two ALAD alleles and different genotypes contribute to the response of the organism to lead intoxication. This has been studied so far in lead exposed human populations in connection with different physiological parameters (Pawlas *et al.* 2012; Montenegro *et al.* 2006; Gao *et al.* 2010). However, the results are not always straightforward or clearly concluded by authors.

The phenomena found in lead exposed human populations, delta aminolevulinic acid dehydratase gene polymorphism, strongly support the hypothesis that the genomic difference may be more important than other factors contributing to the distribution of lead in the organism (Chia *et al.* 2004). ALAD polymorphism in humans results in the existence of two alleles: ALAD1 and ALAD2 (Wetmur *et al.* 1991). The difference between the two amounts to a single nucleotide substitution (one SNP, G to C), which causes an amino acid substitution (lysine for asparagine) (Zheng *et al.* 2011) and the presence of three possible isozymes (ALAD 1-1, ALAD 1-2, and ALAD 2-2) which differ in electric charge. It is generally thought that ALAD2 increases the enzyme affinity for lead ions because of its higher electronegativity and ability to bind lead ions more tightly. Following this, the data present in the literature demonstrate that ALAD2 carriers have a higher blood lead level than homozygotes carrying ALAD1. However, this was observed only among individuals under high lead exposure, such as smelter workers (Scinicariello *et al.* 2006). Such polymorphism is important from a toxicological point of view because studies of different biomarkers indicated that ALAD2 carriers are more susceptible to renal injury, whereas ALAD1 carriers are at risk due to a decrease in neurobehavioral functions (Gao *et al.* 2010). The differences between ALAD1 and ALAD2 carriers are very complex (Scinicariello *et al.* 2006), and are still not comprehensively known. This may be the consequence of limitations on research carried out on humans and a lack of such studies conducted on animals, apart from the study of Ferrand and Amorim (1990) on domestic rabbit. It is currently unknown if such a phenomenon is also present in animal populations exposed to lead contamination or if it is only a characteristic of humans.

Such a hypothesis was put forward in our research on bank vole (*Myodes=Clethrionomys glareolus*) populations which have inhabited unpolluted and lead/zinc polluted sites for many generations. We chose a variety of study sites to assess whether or not a higher frequency of a certain allele is associated with higher lead exposure. In our investigation we focused on MspI polymorphism (G177C, rs1800435), the most widely studied polymorphism in human populations (Schwartz *et al.* 1997; Montenegro *et al.* 2006). Two hypotheses were tested in our study: (i) does the same ALAD polymorphism occur in lead exposed rodent species and is the frequency of different alleles similar to that of humans, (ii) if polymorphism exists, is the tissue lead content higher in individuals that possess ALAD2 alleles.

Material and Methods

Study sites and sample preparation

For the purpose of this study, we used animals from six different study sites: three forests designated as non polluted sites (Mikołajki, Niepołomice, Teleśnica Oszwarowa) and three sites from areas located near lead/zinc smelters in southern Poland (Katowice, Olkusz, Miasteczko Śląskie) caught from August-November 2009. The study was based on metal contents in the liver and kidneys of animals inhabiting these sites which were collected for a genetic and physiological project (FRISC, Factors of Population Extinction Risk, Nr PL0419) (Mikowska *et al.* 2014).

The animals were live-trapped and transported to the laboratory, where they were killed by cervical dislocation and dissected. The number of examined individuals is summarized in Table 1. We do not have information about age of individuals, however, using body mass of individuals at the day of dissection (varying between 14.47 g and 30.98 g) we can roughly estimate the age of animals. It seems that age structure of studied populations was balanced, including both young and adult individuals, except for the Niepołomice population, where individuals had lower body masses. All research complied with ethical standards concerning the treatment of animals. The authors obtained the agreement of the I Local Ethic Committee (Kraków, Poland, Decision no 48/2007 from 10.05.2007).

Tissue samples were collected and frozen at -80°C. DNA was isolated from the liver tissue using the DNeasy Blood and Tissue Kit (Qiagen) according to standard protocol. All PCR reactions were performed with Taq PCR Master Mix Kit (Qiagen) and an Eppendorf thermocycler.

Primer design and product amplification

Bank vole ALAD sequence was not available from GenBank, which is why we developed our ALAD primers based on mouse and rat ALAD sequences. The primers (F:GGATGTTCCTGATGATGTCCAG, R: TTGGGAACTCTGCTGGGGACG) were syn-

Table 1

Number and body mass (g) of individuals sampled in the study

Study sites		Number of males	Body mass (g)			Number of females	Body mass (g)			Total
			Median	First quartile	Third quartile		Median	First quartile	Third quartile	
Unpolluted sites	Mikołajki	9	24.33	22.50	24.42	7	24.55	17.82	26.90	16
	Niepołomice	6	14.99	14.59	15.93	6	16.07	15.88	21.06	12
	Teleśnica Oszwarowa	6	24.36	20.19	27.03	6	23.39	18.91	26.13	12
Polluted sites	Katowice	6	19.68	18.82	20.93	4	22.70	21.58	23.12	10
	Olkusz	9	20.17	16.86	20.44	5	18.60	18.06	18.79	14
	Miasteczko Śląskie	6	22.72	21.84	24.96	3	17.60	17.27	17.95	9
										73

thesized by Genomed, Poland. The obtained product, via use of this set of primes, was around 710 bp long, with the studied SNP located around 90 bp. We made attempts to design other primers which would help to amplify a fragment giving more distinct restriction products on agarose gels. Unfortunately, they did not work.

The amplified ALAD fragment was isolated from the agarose gel and sequenced to check for specificity. The obtained sequence was aligned with human ALAD, which allowed us to determine the location of the nucleotide of concern, similar to the human G177C. All nucleotide and protein sequences in this study were aligned using BLAST (http://blast.ncbi.nlm.nih.gov/) and ClustalW (http://www.genome.jp/tools/clustalw/) software.

Enzyme digestion

We used the Hpy166II enzyme (New England Biolabs) for the restriction cut. Virtual digestion (NEB Cutter) using the ALAD sequence of individuals studied and the Hpy166II confirmed that each sequence of the C nucleotide carrier would be cut into two fragments: around 620 and 90 bp. All digestions were performed according to standard protocol (4 μl H$_2$0, 1 μl of buffer, 1 μl of enzyme, 4 μl of DNA). After that, fragments from each individual were run on a 2% agarose gel together with a 100 bp *ladder* and undigested fragment (710 bp). This procedure helped to better distinguish between undigested and digested products (620 bp), as their size differs only in 90 base pairs. However, the results of digestion showed that there was no problem with recognizing them on 2% agarose gels.

Results

Results were obtained for 73 individuals from the six study sites (Tab. 1). All individuals from our study showed no polymorphism in the studied location. Amplified ALAD fragments from all individuals were successfully digested into two small fragments and showed no products of 710 bp size. This result means that all animals were homozygotes for the studied polymorphism. The important result produced is that all individuals had a C nucleotide in the location of G, whereas G is much more frequent in human populations. The form analogous to that obtained in our study is called ALAD2 in the human population. Our previous study (MIKOWSKA *et al.* 2014) showed significant differences of lead levels in the tissues of animals from unpolluted and polluted sites, however, these levels were rather low, up to 5.08 mg/kg dry weight in the liver and 2.25 mg/kg dry weight in the kidney. Since our results of genetic analysis showed no polymorphism, we cannot state if these tissue levels depend on genotype. Because our sampling included both young and adult individuals, and there was no polymorphism, we cannot state if other genotypes were eliminated from the population due to higher sensitivity and shorter lifespan.

Discussion

ALAD gene sequences and protein alignment show great similarity between different species from which sequences are available in GenBank (such as human, mouse and rat). We also noticed this similarity in the region where the human SNP is located (rs1800435), responsible for the existence of ALAD1 and ALAD2 alleles. This encour-

aged us to study this gene polymorphism in the bank vole, despite the fact that its genome is not yet fully sequenced. Although we expected the bank vole ALAD gene to be similar to that of the rat or mouse, at the beginning we had some difficulties in finding properly working primers. Despite existing differences, we were able to find a location which could have been responsible for the occurrence of polymorphism similar to that found in human. To check for possible allelic diversity we had to find a restriction enzyme (Hpy166II) that was suitable for our purposes. Because of some differences in human and rodent ALAD sequences we were unable to use the enzyme which was implemented in studies carried out on human beings (SCHWARTZ *et al.* 1997).

Our investigation did not show polymorphism analogous to human ALAD in the bank voles studied, thus, we did not find differences between the studied populations. The nucleotide present in the location that aligned with G177C of the human sequence was nucleotide C for the bank vole. In the human sequence, the C nucleotide is present in the ALAD2 allele and represents a less frequent genotype among human populations. Contrary to human, where ALAD1 is predominant, in our six studied bank vole populations only ALAD2 was present. In European human populations ALAD1 frequency ranges between 84% and 94% (SUZEN *et al.* 2003). In Asian human populations ALAD1 frequency may be even greater. Moreover, data available in the literature show no polymorphism in African human populations, resulting in the existence of only ALAD1 homozygotes (FUJIHARA *et al.* 2009).

So far, the only animal-based research performed on laboratory rabbit (FERRAND & AMORIM 1990) exhibited a less predominant distribution of one type of ALAD allele but still displayed the existence of both. The authors showed that in a random breeding of unrelated animals, the frequency was 0.31 and 0.69, respectively for ALAD1 and ALAD2. This allele frequency ratio may confirm that ALAD2 is more abundant among animals when compared to humans. There is, however, a huge gap in research concerning ALAD allele frequencies in wild animal species which causes problems for comparison and discussion. Our research on the bank vole is the first contribution to fill this gap.

Our results concerning bank vole populations show that ALAD polymorphism is not as common as we expected it to be, and even under different exposure to environmental lead, ALAD differences between populations were not observed. One of the explanations for this may be that polymorphism is a species characteristic or the effects of environmental lead levels have not caused genomic adaptation in the case of this SNP. The occurrence of the second allele might be conditioned by microevolution, however, some life history traits and short life expectancy can alter this process. There is also a possibility that polymorphism exists in general, but was not detected due to a low number of samples or low number of representative populations. In the studied populations both young and adult individuals were present and all were characterised by the same genotype, meaning that the lifespan of bank voles is not correlated with a specific genotype. Assuming that one genotype produces more vulnerable individuals to lead exposure than others, the age structure of populations should be unbalanced, in the direction of a majority of young individuals. That was not the case in our study.

Finally, the occurrence and subsequent selection of a second allele might be connected to other environmental factors or a lead exposure pattern that we are unaware of.

These conclusions show that more studies need to be carried out concerning wild animal populations (for example populations originating in other geographical regions) and more data on the subject needs to be obtained. Research on animals is even more necessary because human research imposes many constraints concerning ethical rules. We cannot state which of the genotypes, ALAD1 or ALAD2, is more favourable for individuals living in the polluted sites based on contemporary results of studies carried out on human populations. Most human research discusses people with an occupational exposure to lead. SCHWARTZ *et al.* (1995) showed that ALAD2 is more frequent among workers exposed for over 6 years compared to those exposed for less than six years. This may suggest that ALAD2 is favourable in coping with lead intoxication. Also, research has proven that individuals with the ALAD1-2 genotype have better results in neuropsychological tests compared to those with ALAD1 (BELLINGER *et al.* 1994). However, it has been shown that ALAD2 carriers have higher blood lead levels under high lead exposure which can lead to more drastic toxic effects (FLEMING *et al.* 1998). LI *et al.* (2011) showed that downregulation of ALAD transcription caused by lead exposure may originate from ALAD methylation.

By using an animal model for both genetic and physiological studies, not only can the frequency of alleles be assessed in relation to a specific population and environment conditions, but also some other parameters, for example, metal levels in different types of tissue, enzyme activity and histological changes. This multidisciplinary approach will help to answer several questions and clear up uncertainties which have emerged in research performed on human populations.

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