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SHORT COMMUNICATION

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Missense single nucleotide variants affecting CYP3A catalytic activity are present in Limousine cattle

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ABSTRACT

Cytochrome P450 (CYP) 3A is one of the most important subfamily of drug metabolising enzymes. Genetic factors such as breed and allelic variants might modify CYP3A expression and enzyme activity and lead to inter- and intra-individual variability in clinical response or toxicity. Cattle *CYP3A* gene cluster has been recently re-sequenced in 300 Piedmontese cattle by deep targeted sequencing. Thirteen missense single nucleotide variants (SNVs) were identified, and for five of them an impact on CYP3A activity was demonstrated *in vitro*. In the present work, we assessed the genetic frequency of these five SNVs in Limousine, a cattle breed widely raised for meat production in Veneto Region. A total of 215 cows were genotyped using specific melting curve assays. Only two missense SNVs out of five, both located on *CYP3A28* coding sequence, were detected: *rs*384467435 and *rs*454167819. The former mutant allele was present in homozygosis in the 4% of tested cows, while in heterozygosis in the 24% of animals. The second SNV was detected only in heterozygosis in 11 cows out of 215 (i.e. 4.9%). These findings suggest the presence of missense SNVs, proved to halve CYP3A28 catalytic activity, in both Limousine and Piedmontese meat cattle breeds. As a consequence, these SNVs might impact on the kinetics of xenobiotics metabolised by CYP3A, including drugs and natural toxins like ivermectin and aflatoxins, thereby resulting in toxicity or accumulation of harmful residues in foodstuffs. This result paves the way for new considerations on the fate of xenobiotics in cattle farming.

HIGHLIGHTS

- Missense single nucleotide variants affecting CYP3A catalytic activity *in vitro* are present in Limousine and Piedmontese cattle breeds.
- *Rs*384467435 is the only missense single nucleotide variant for which the mutant allele is recorded in homozygosis in approximately 4% of Limousine and Piedmontese cattle.
- The polymorphisms here detected might affect the kinetics and the toxicity of xenobiotics metabolised by CYP3A in cattle.

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Introduction

In adult humans, the cytochrome P450 3A (CYP3A) is the most important subfamily of drug metabolising enzymes owing to its broad substrate specificity (70% of clinically used drugs, natural, anthropogenic and endogenous compounds). Several genetic, environmental and physiological factors may affect CYP3A expression and activity. In particular, human *CYP3A4/5* genetic variants significantly contribute to ethnic, intra- and inter-individual variability in xenobiotic metabolism, leading to alterations in pharmacokinetics, clinical response and/or toxicity (Klein and Zanger 2013; Werk and Cascorbi 2014; Hohmann et al. 2016).

Cattle is one of the most important farm animal species, kept worldwide for milk and meat production. The bovine CYP3A (bCYP3A) subfamily consists of three genes, i.e. *CYP3A28*, *CYP3A38*, and *CYP3A48* (Zancanella et al. 2010). They contribute to the oxidative metabolism of drugs commonly used in bovine practice, such as the macrocyclic lactone moxidectin, the pleuromutilin and macrolide antibiotics (e.g. tiamulin and tilmicosin, respectively) as well as the ionophore antibiotic monensin (Zweers-Zeilmaker et al. 1999; Dupuy et al. 2001; Nebbia et al. 2001). Furthermore, they are responsible for the bioactivation of natural toxins of current growing interest,

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i.e. aflatoxins and ergot alkaloids (Kuilman et al. 2000; Sales et al. 2012; Rosenkrans and Ezell 2015).

Breed-differences in bCYP3As expression and catalytic activity have been previously highlighted (Dacasto et al. 2005; Giantin et al. 2008; Ashwell et al. 2011; Zancanella et al. 2014). Conversely, little information is available on b*CYP3A* genetic polymorphisms and their possible consequences on the kinetics of xenobiotics, except for some effects on productivity traits (Sales et al. 2012, 2013). As an example, Brahman cattle show a greater tolerance to fescue toxicosis than Angus cattle (Brown et al. 1993), and this different sensitivity to ergot alkaloids has been associated to the presence of a polymorphism in *CYP3A48* coding sequence (Sales et al. 2012). Specifically, cows with the mutant allele in homozygosis and grazing toxic tall fescue tended to reduce milk production and butterfat percentage, probably due to an altered CYP protein structure and function (Sales et al. 2012). These same authors identified further polymorphisms within the *CYP3A48* regulatory region, and speculated that the reduction in calf growth was attributable to the consumption of ergot alkaloids (metabolised by CYP3A), either acquired directly by grazing than indirectly (as metabolites) through the milk (Sales et al. 2013).

Recently, thirteen b*CYP3A* missense single nucleotide variants (SNVs) were identified in Piedmontese beef cattle, thanks to the *CYP3A* gene cluster deep sequencing of 300 bulls. Subsequently, their functional impact was assessed by heterologous expression and measurement of CYP3A-dependent catalytic activity (Giantin et al. 2019). Five SNVs out of thirteen showed differences in the metabolism of CYP3A probe substrates: three *CYP3A28* SNVs halved testosterone (TST) 6β-hydroxylation; one *CYP3A38* variant triplicated TST 16β-hydroxylation, while a *CYP3A48* SNV showed enhanced nifedipine oxidation. Interestingly, the reduction of TST 6β-hydroxylation obtained *in vitro* for *rs*384467435 was confirmed *ex vivo* in liver microsomes isolated from bulls homozygous for this same SNV (Giantin et al. 2019).

In food-producing species, inter-individual variability in xenobiotics metabolism might result in the accumulation of harmful residues in foodstuffs. Therefore, pharmacogenetic approaches are fundamental to either understand the fate of xenobiotics in the living animal than estimate the consumers' risk of exposure to noxious chemicals (Hu et al. 2016; Jiang et al. 2018; Giantin et al. 2019). To improve our knowledge on this aspect, this study aims to measure the frequency of five harmful missense *CYP3A* SNVs in a population of Limousine cattle, a French meat cattle breed widely bred in Northern Italy (Mastrangelo et al. 2018). The five selected SNVs have been previously identified in Piedmontese breed and they were shown to affect CYP3A catalytic activity *in vitro* (Giantin et al. 2019).

Materials and methods

The genotyping of five *CYP3A* missense SNVs (Table 1) was carried out on 215 Limousine cattle (female, 1–17 years old), bred in three different districts of Veneto Region (Italy): Rovigo ($n = 129$), Adria ($n = 69$), and Feltre ($n = 17$).

Genomic DNA was isolated from archived whole blood samples collected within the framework of statutory surveillance activities for infectious diseases by district Official Veterinarians. The DNeasy Blood and Tissue Kit (Qiagen, Milan, Italy) was used and the nucleic acid extracts were then quantified by NanoDrop ND1000 Spectrophotometer (Thermoscientific, Waltham, Massachusetts, USA).

Genotyping was performed using FRET Hybprobe probes (Wittwer et al. 2001), as previously reported (Giantin et al. 2019). After PCR amplification, the melting profile of the heteroduplexes amplicon-probe, analysed using LightCycler 480 software release 1.5 (Roche, Basel, Switzerland), allowed the discrimination into wild-type (WT/WT), heterozygote (WT/MUT) and mutant (MUT/MUT) genotypes. For the correct interpretation of melting profiles, reference DNA samples corresponding to the different genotypes were used. They were selected after PCR end-point amplification, cloning, plasmid purification and confirmatory Sanger sequencing of at least ten colonies.

The genotype frequency of each variant was examined for deviations from Hardy-Weinberg Equilibrium (HWE) within the population using the Chi-square test implemented in the online calculator (https://www.coursehero.com/file/8442059/Court-lab-HW-calculator/). A p value $< .05$ indicated a deviation from HWE.

Results and discussion

The estimation of *CYP3A* pattern of allelic distribution in different ethnic groups is an approach widely used in human pharmacogenetics for its clinical implications and the actual need for personalised therapies (Hu et al. 2005; Szalai et al. 2015; Hu et al. 2017). Conversely, this study represents one of the few examples of the estimation of *CYP3A* genetic variants prevalence in bovine breeds (Sales et al. 2012, 2013;

Table 1. Cattle cytochrome P450 3A missense single-nucleotide variants subject of investigation in the present study: IDs, functional impact on CYP3A activity and frequency in Limousine cattle breed.

Ref. ID	DNA variant*	Amino acid variant	Impact on CYP3A activity	Genotype frequency (%, n)	Minor allele's frequency	Chi-square test *p* value**
*rs*384467435	NM_001099367.1:c.589G > A	NP_001092837.1:p.Gly197Ser	−50% TST 6β-hydroxylation	WT/WT: 72.1% (155/215) WT/MUT: 24.2% (52/215) MUT/MUT: 3.7% (8/215)	0.16	0.179
*rs*433125080	NM_001099367.1:c.866C > T	NP_001092837.1: p.Ala289Val	−50% TST 6β-hydroxylation	WT/WT: 100% (215/215)	–	Not applicable
*rs*454167819	NM_001099367.1:c.1162A > G	NP_001092837.1:p.Ile388Val	−50% TST 6β-hydroxylation	WT/WT: 94.4% (204/215) WT/MUT: 5.1% (11/215) MUT/MUT: 0% (0/215)	0.03	Not applicable
NA	NM_001075888.2:c.1122G > T	NP_001069356.1:p.Glu374Asp	+258% TST 16β-hydroxylation	WT/WT: 100% (215/215)	–	Not applicable
*rs*137124349	NM_174513.3:c.1051G > A	NP_776956.2:p.Val351Ile	+317% NIF oxydation	ND	–	Not applicable

Ala: alanine; Asp: aspartic acid; CYP3A: cytochrome P450 3A; Glu: glutamic acid; Gly: glycine; Ile: isoleucine; MUT/MUT: mutant genotype; NA: not available; ND: not detectable; NIF: nifedipine; Ref. ID: reference identification tag; Ser: serine; TST: testosterone; Val: valine; WT/MUT: heterozygous genotype; WT/WT: wild type genotype.
*Variant nomenclature is consistent with the previous paper of Giantin et al. (2019), in which UMD3.1 assembly was used as reference genome. **Hardy-Weinberg Equilibrium (HWE) was assessed by means of Chi-square test. A value of $p > .05$ was obtained for *rs*384467435, denoting a consistency with HWE.

Giantin et al. 2019). The genotyping results are summed up in Table 1 and described below.

Among the five target SNVs, only two of them (namely, NM_001099367.1:c.589G > A, and NM_001099367.1:c.1162A > G) were successfully identified and quantified in the tested population.

The NM_001099367.1:c.589G > A assay, distinguishing a SNV in exon 7 of *CYP3A28,* and causing the amino acid change Gly to Ser at residue 197, showed three different melting profiles (Figure 1A). The MUT/MUT genotype showed a single peak, with a melting temperature (Tm) of 60 °C; the WT/WT genotype, a peak at 66 °C; finally, the WT/MUT genotype showed both peaks, as expected. The mutant allele was detected both in homozygosis and heterozygosis in the 4% and 24.2% of animals, respectively. The frequency of MUT/MUT and WT/MUT genotypes was quite similar to that previously observed in Piedmontese cattle (3.4% and 17.7%, respectively: Giantin et al. 2019). However in Limousine population, in opposition to what observed in Piedmontese population, this SNV was consistent with HWE. This SNV is present in the official public repositories with the reference ID *rs*384467435, thus meaning that this variant is also present in Hereford cattle, whose DNA was used as template for the UMD3.1 bovine genome sequencing. Looking at the results as a whole, we might infer that this SNV is present in at least three different cattle breeds (Limousine, Piedmontese and Hereford). Therefore, we might suggest that rs384467435 is not a breed-specific SNV, and it could be subjected to selection only in Piedmontese population. Unfortunately the lack of data about the presence (and frequency) of such a SNV in other cattle breeds prevents us from inferring conclusive remarks. Anyway, this SNV is worth of mention, because in a recent study it was demonstrated to be the only missense variant (out of thirteen) enable to significantly reduce TST hydroxylation either *in vitro,* i.e. in V79 cells transiently transfected with *CYP3A28* mutated sequence, than *ex vivo,* in liver microsomes isolated from 300 Piedmontese bulls (Giantin et al. 2019).

In the cohort of Limousine cattle, the genotyping of NM_001099367.1:c.1162A > G (identified in public databases as *rs*454167819) showed the alternative nucleobase only in heterozygosis (Table 1 and Figure 1B). Indeed, only two different melting profiles were obtained. The WT/WT genotype was characterised by a single peak at 66 °C, while WT/MUT by 2 peaks at 60 °C and 66 °C (Figure 1B). All the animals showing the WT/MUT genotype (5.1%) were from the Rovigo district, presumably from the same progeny.

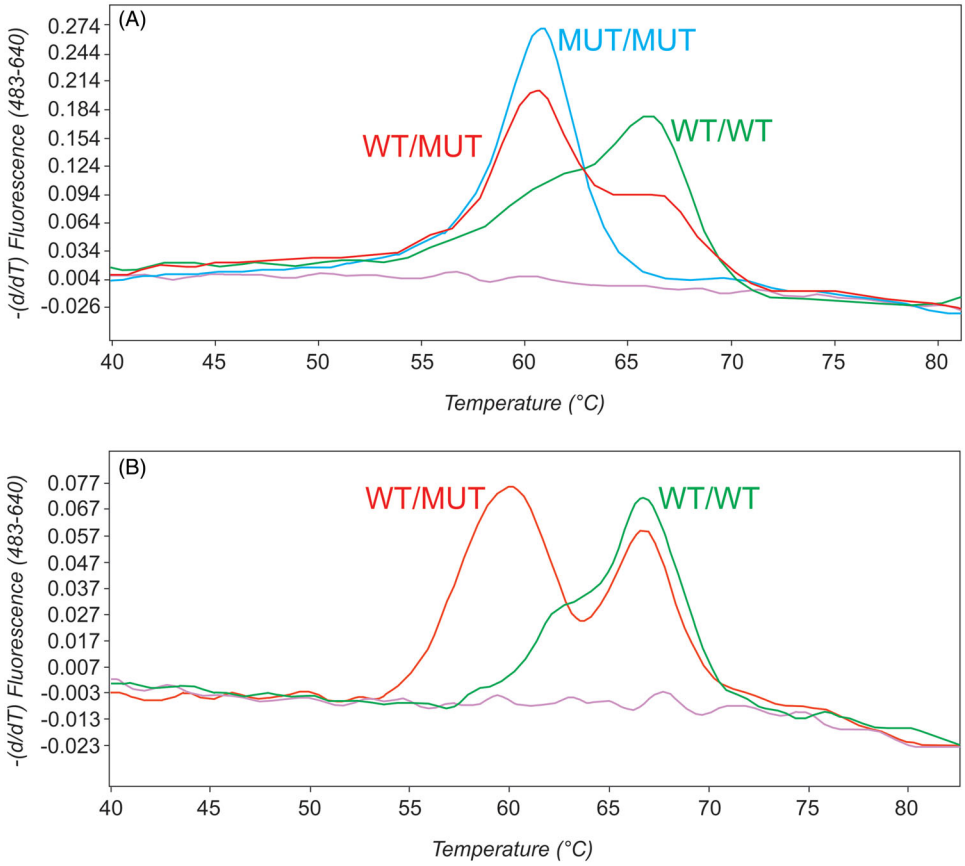

Figure 1. Output of NM_001099367.1:c.589G > A (A) and NM_001099367.1:c.1162A > G (B) melting curve genotyping assays (LightCycler 480 software release 1.5, Roche). WT/WT (wild type), WT/MUT (heterozygous), and MUT/MUT (mutant) genotypes are indicated with green, red and blue curves, respectively. The BLANK (no template control) is shown in purple.

The percentage of WT/MUT Limousine cows was lower when compared to that observed in Piedmontese cattle (i.e. 13%; Giantin et al. 2019), while the MUT/MUT genotype was completely absent in both breeds. This SNV has been proved to halve CYP3A28 catalytic activity (Giantin et al. 2019). Therefore, both WT/MUT and, even more dramatically, MUT/MUT genotypes might significantly affect veterinary drug kinetics, with consequences on their efficacy and residue formation. The low allele frequency might result from a man-driven selection (Bosse et al. 2019), yet the phenotype associated to this allele is still unknown. Nevertheless, further population studies encompassing a wider number of animals are required to confirm this hypothesis.

Concerning the remaining three target variants, two of them, i.e. NM_001099367.1:c.866C > T (*rs*433125080) and NM_001075888.2:c.1122G > T (a novel SNV recently identified in Piedmontese breed: Giantin et al. 2019), were completely absent in the Limousine population. The third one, i.e. NM_174513.3:c.1051G > A (*rs*137124349), was not detectable for technical limitations. Also in Piedmontese breed, NM_001099367.1:c.866C > T and NM_001075888.2:c.1122G > T were not detected or identified in only 1 bull out of 300, respectively (Giantin et al. 2019). This would confirm the extremely low frequency of these variants in both breeds. We would rule out a low efficiency of the assays because the probes allowed the identification of further missense SNVs in heterozygosis: *rs*481349500 and NM_001075888.2:c.1126T > C. Conversely, the FRET assay for NM_174513.3:c.1051G > A, previously set up and used for Piedmontese cattle genotyping (minor allele frequency 0.21: Giantin et al. 2019), was not efficient enough in Limousine samples because of the presence of an additional variant at residue 350. This synonymous variant (NM_174513.3:c.1050T > C or *rs*379152445), confirmed by Sanger sequencing, probably interfered with probes binding and therefore altered the melting curve profile. In this challenging situation, the amplicon cloning followed by Sanger sequencing is potentially the only viable strategy to assign the proper genotype.

Limousine and Piedmontese meat cattle breeds have been here considered because in a previous study they already showed differences in CYP3A catalytic activity (Dacasto et al. 2005). These two breeds share *rs*384467435 and *rs*454167819 missense

variants with some slight differences in frequency, potentially attributable to the main features of the breed itself. Specifically, Limousine is a cosmopolitan breed imported in Italy, used worldwide for beef production in pure or cross-breeding systems, and showing a moderate level of genetic diversity compared to foremost Italian breeds (Mastrangelo et al. 2018). Conversely, Piedmontese is a local breed that has benefitted from proper breed management and has a sufficiently large effective population size and a low degree of recent consanguinity (Mastrangelo et al. 2018). In perspective, it could be of interest to extend the evaluation of these same SNVs in further local breeds (e.g. Rendena, Burlina), confined in small geographical areas and generally characterised by a low genetic diversity and a small population size.

The minor allele frequency (MAF) of the variants here identified resembles the one found in Nellore cattle in the xenobiotic sensor gene *NR1I3* (Nuclear receptor 1 family I member 3, also known as Constitutive Androstane Receptor: Alexandre et al. 2014), and is lower than the one described in Braham and Angus and reciprocal crosses in *CYP3A48* by Sales et al. (2012, 2013). Even if low, the MAF here calculated should not be viewed as bad and might be regarded as a great opportunity to use these markers for rapidly increase the net effect of a chosen phenotype (Alexandre et al. 2014).

In the above mentioned studies (Sales et al. 2012, 2013; Alexandre et al. 2014), genotypes were associated with feed efficiency traits. However, feed efficiency in beef cattle is a phenotypic trait where several cellular processes and gene networks take part, such as cellular growth and proliferation, cell signalling, drug metabolism, protein synthesis, lipid metabolism and carbohydrate metabolism (Chen et al. 2011). All this makes feed efficiency phenotype a polygenic character, and it is difficult for a single gene to hardly affect it (Moore et al. 2009), unless this gene plays a fundamental role nor it is involved in many pathways (Alexandre et al. 2014).

Conversely, CYPs are a superfamily of genes basically involved in the biotransformation of xenobiotics and endogenous compounds (e.g. steroids, vitamin D); hence, a single genetic variant may potentially result in an altered phenotype. In human medicine, differences in CYP catalytic activity due to genetic polymorphisms may be of clinical significance with respect to drug efficacy, drug–drug interactions as well as overall xenobiotic toxicity (Werk and Cascorbi 2014; Szalai et al. 2015). In veterinary medicine, and particularly in food-producing species, these genetic alterations might impact also on the levels of harmful or unpleasant residues in foodstuffs. Notably, recent studies have described the effect of swine *CYP3A29* and *CYP2E1* SNVs on aflatoxin B1 and T-2 toxin bioactivation and skatole metabolism, respectively (Mörlein et al. 2012; Cheng et al. 2014; Wu et al. 2016; Zadinová et al. 2017; Jiang et al. 2018). Present results, and particularly those obtained for bCYP3A missense variant *rs*384467435 (MAF = 0.16), are therefore potentially relevant for the veterinary community, and should be considered in future pharmacogenetics studies. Anyway, the validation of genotype results with perspective phenotypic investigations, based on pharmacokinetics studies and residue analyses in genotyped cattle treated with drugs known to be metabolised by CYP3A (e.g. macrolides, macrocyclic lactones, …), is strongly recommended.

Finally, a further challenging issue in perspective could be to estimate the differential allelic-specific expression in cattle heterozygous for *CYP3A* SNVs, potentially resulting in changes in gene expression levels and, consequently, in further phenotypic variability (Hirota et al. 2004; Ghotbi et al. 2009).

Conclusions

In conclusion, this study confirm the presence of two (*rs*384467435 and *rs*454167819) out of five CYP3A missense variants affecting CYP3A activity *in vitro* in female Limousine cattle. Since the presence of these SNVs might result in differential clinical (e.g. a different drug mean residency time) and toxicological (e.g. increasing amounts of harmful residues) responses, further phenotypic insights are required in perspective.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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