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Mini Review

Implementation of contemporary DNA based techniques on traceability process of small ruminant species and products

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ABSTRACT

Traceability methods in livestock sector through the tracking of animal species, breed or even individuals, has become of utmost importance as a "vehicle" for ensuring consumers' food safety. The advent of new technology at DNA level has facilitated the convenience and the accuracy of the implementation of traceability methods. The scope of this review is to highlight the most up to date progress on DNA based approaches concerning the traceability procedures for small ruminant species and/or their products, giving emphasis on short tandem repeats (microsatellites) and single nucleotide polymorphisms. The conclusions of this review may be used either from the farmer or the State and other Organisations in order not only to certify traceability throughout the whole food process chain but to ensure also consumers' food safety.

KEYWORDS

DNA; Small ruminants; SNPs; STRs; Traceability

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INTRODUCTION

It is common sense that the term "traceability" may receive many definitions in accordance to the approach and the limits that each Organization, scheme, national or international legislation attempts to ensure or set. The most easily understandable approach defines traceability in livestock production as "*the ability to follow an animal or group of animals during all stages of its life*" (OIE, 2017). However, the best approach of the word is found in the fundamental law on food safety in Europe (European Commission, 2002), which is defined as "*the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution*". Since its application (January 1, 2005), the definition of a food sector traceability scheme has become obligatory in all EU countries. On the other part, under the implementation of the ISO 2205 standard norms, a traceability system aims to help an association to adjust with its specific targets and is applied when it is important to specify the history, or area of a product or its significant components (ISO, 2007). Internet sources (Wikipedia) defines the word "traceability" as the ability to substantiate historical events, position(s), place(s) or implementation of a procedure by terms of recorded documentation, while other approaches may include the ability to track any information up to a certain level, or to be able to chronologically relate identifiable entities in a verifiable way. (Murugappan and Prabha, 2017). In addition, traceability is also known as the 'one-step-back-one-step-forward' principle (ITC, 2015). However, most of the definitions, does not specify which points should be assesed or how to recall the origin, rendering the definition of the European Regulation, the most suitable in the agro-nutritional sector.

The aim and the importance of traceability process

Tracing the origin of animals' products is an important task of growing concern during the last decades, because of the increase consumers' attention on food quality and on products' authenticity (Rychlik et al., 2018). The stroke of many nutritional scandals (*i.e.*, dioxins, BSE, horse meat scandal etc), have provoked the increase of such concern. In line to the aforementioned, a simple consensus of the term "traceability" could be set as the ability to identify an animal and/or its product throughout the whole production chain But, is the traceability control throughout the whole food chain so important? Is it only considered as a matter of consumer's safety? The answer is "No", if the multipurpose approach regarding the importance of traceability control is considered. The latter is reflected by the following basic axes (Murphy et al., 2008; Rychlik et al., 2018):

a. *Farm management:*by means of offering a proof of ownership, a tool of selection or subsidy payment of a desired animal or protection against illegal activities (*e.g.*, thefts).

b. *Genetic management:*by means of pedigree or artificial insemination implementation.

c. *Biodiversity management:*as an assist to detect and notify outbreaks or to control animal grazing or assortment.

d. *Prevention/control of animal diseases:*by means of ensuring inspection, certification of animal's health and–tracking down the infected animals.

e. *Trade opportunities, control in products:*by means that traceability process may be a "vehicle" of protecting or prevent public health (food safety), deceptive practices and fraud in the market place (*e.g.*, geographic indication, food quality), adulteration and food contamination.

Methods of traceability

Several traceability methods have been described, based on the approach that traceability is achieved and on what data/information furnishes (Dalvit et al., 2007). On live animals, phenotypic characteristics such as coat colour, horns, tattoos, ear tags etc, have been widely used throughout decades as tracing method and reflect the simplest and the oldest markers used for this purpose. Reviewing all the methods used for tracing live animals, the method of the electronic RFID ear tag and the rumen bolus are the more sufficient by terms of implementation easiness and cost approach (Bowling et al., 2008; Awad 2016).

At biochemical level, enzyme-based methods have been widely used as a traceability tool (Dogu and Sireli, 2016). The developed methods focused either on the "isoelectric point" of the protein, or on agar gel immunodiffusion or counter immunoelectrophoresis or ELISA (Schwägele, 2005). Disadvantages of the above methods have been mainly described regarding the heat treatment, which might change the conformation of the proteins. In addition, the immunological methods already used, present serious drawbacks as they fail to analyze complex food matrices (Rodríguez et al., 2004).

As DNA technology is improved, new approaches based on the analysis at molecular gene level invented as a "vehicle" of ensuring traceability (**Figure 1**) in a more accurate way (Scarano and Rao, 2014; Guan et al., 2018). The ideal method is supposed to be reproducible, and thus to generate easily reliable data. DNA based

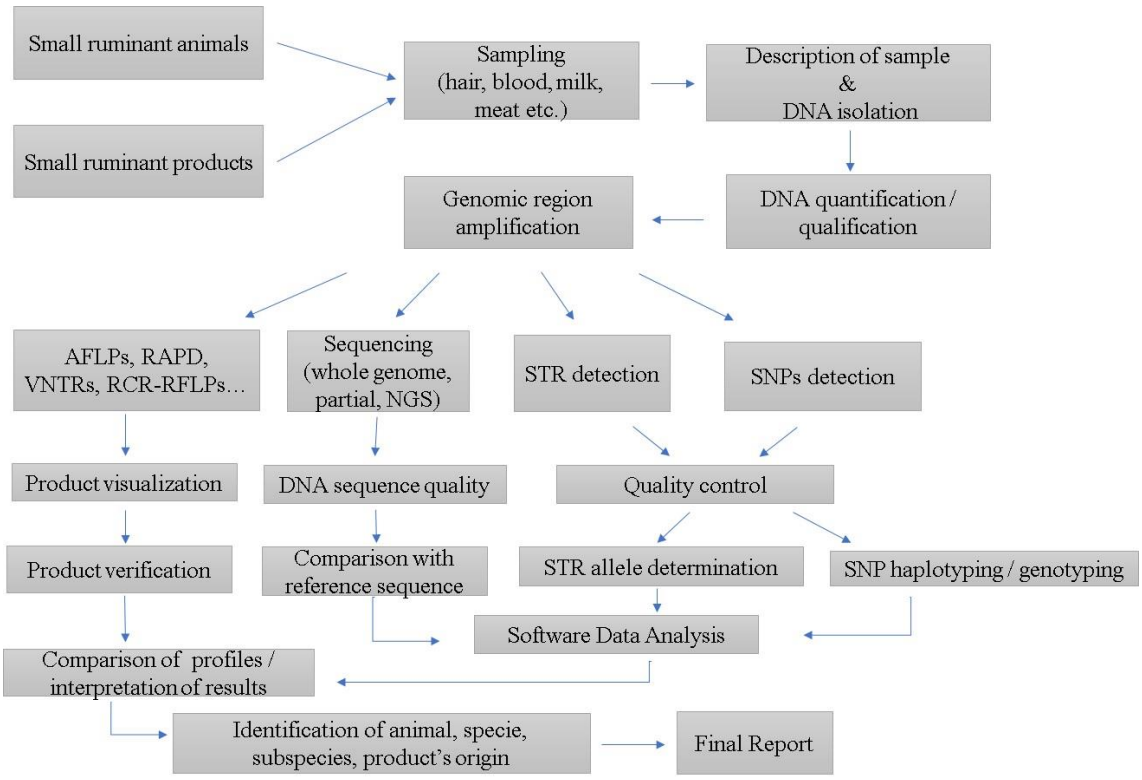

Figure 1. Traceability process on small ruminant species and their products using DNA based methods.

methods are usually focused on "polymorphic" DNA markers and they are grouped in two major categories: (a) methods that require the clone and/or the sequence of the marker, and (b) methods that the sequence of polymorphic location or the isolation and the cloning of a DNA fragment is not required. The first category includes markers such as Restriction Fragment Length Polymorphisms (RFLPs), microsatellites/short tandem repeats (STRs), Single Nucleotide Polymorphisms (SNPs) and specified DNA sequences, while the second one includes markers such as Amplified Fragment Length Polymorphisms (AFLPs), Variable Number of Tandem Repeats (VNTRs) and Random Amplified Polymorphic DNA (RAPDs).

Although the sequencing of the animal's DNA offers the most accurate way of an individual identification, it is not always feasible and cost affordable (Pillai et al., 2017). However, some recent research have been conducted on the matter of species discrimination (Bertolini et al., 2015; Giusti et al., 2017; Carvalho et al., 2017). On the other hand, PCR-RFLPs offer a more convenient, ease, cheap and time affordable way of traceability (Guan et al., 2018), but it cannot be implemented on all the desired cases, as cleavage sites of the restriction enzymes are case sensitive. However, many studies have been reported using this method for food traceability purposes (Guan et al., 2018). In addition, the results from AFLP and VNTR methods, although their lower cost, are quite difficult interpreted as the detection requires technical ability in casting and staining the gel. They are, also, more laborious to produce, renders high quality of DNA and the procedures request several steps (Marmiroli et al., 2003), rendering these methods not so desirable for implementation. De Marchi et al. (2003), managed to trace using AFLPs three local Italian chicken breeds, but improvement by means of cost diminish per sample, work input, reproducibility and precision of results should be obtained. Similarly, the distinguish of six different chicken breeds has been accomplished (Soattin et al., 2009), while recently, Zhao et al. (2018) reported the successful implementation of a combined use of eight primer pairs in order to distinguish the individuals of six cattle breeds.

RAPD has been implemented for evaluating commercial adulteration in an accurate and fast way (Ramella et al., 2005), but this method in many studies was used as a tool for investigating genetic diversity and for genetic population purposes rather than species identification

(Jawasreh et al., 2018). However, recently, some efforts using this technique have been conducted by means of fraud control (Cunha et al., 2016; Cunha and Domingues, 2017). On the other hand, mitochondrial DNA (mtDNA) genes offer several advantages in species discrimination tests, as it is maternally inherited. There are many references (Panwar et al., 2015; Floren et al., 2015; Jahura et al., 2016; Munira et al., 2016; Prusakova et al., 2018) that illustrate the effectiveness of mtDNA to discriminate adulteration *i.e.*, of meat, with different technique approaches (mitochondrial 12S ribosomal RNA gene or mito-chondrial cytochrome b gene). Implementing several PCR protocols detection of ovine (*Ovis aries*) and caprine (*Capra hircus*) sequences, in unprocessed and treated (with heat) meat blends containing several other species have been achieved, allowing, thus, the clear species identification with a detection limit of the 1% (wt/wt) for each species analyzed. Multi-specie discrimination has been also achieved based on mitochondrial DNA (Park et al., 2013; Prusakova et al., 2018).

In the dairy sector the higher market value of goat or sheep milk in many countries is related to an extended adulteration practice with bovine milk, which is cheaper. Many efforts have been conducted to detect such practices, especially using simple methods like PCR-RFLP analysis or using mtDNA (Golinelli et al., 2014; Tortorici et al., 2016; Barron et al., 2018). The sensitivity level can reach up to the 0.5%, depending on the raw material and the implemented methodology. Recently the use of more sensitive methods like End-Point PCR, RT-PCR and next generation sequencing (Di Pinto et al., 2017; Seçkin et al., 2017) revealed a great extent (over the 65% of the tested samples) of fraud in ovine or goat milk.

The above findings indicate the importance of the traceability procedure so as to prevent illegal practices, to preserve the quality of animal products and to protect consumers' health. Although mtDNA or PCR-RFLPs methods are not suitable for the discrimination of meat or milk at the breed level, an up to date research (Cunha et al., 2016), reported the precise identification of cheese adulteration made by milk from Serra da Estrela ewes, using the implementation of a RAPD method combined with sequenced characterized markers.

Although sheep and goats contribute considerably to the livestock sector, identification systems in order to traceback and traceforward the live animals and their products are poorly developed, and in some cases is not implemented any approach. However, it is recognised the need to link products with a specific breed not only for satisfying the consumer's desire, but also for ensuring the quality of several PDO or PGI products of dairy or meat origin (Negrini et al., 2008; Mateus and Russo-Almeida, 2015), which consecutively gain the increase preference of consumers. In this review the focus will shift on two DNA methods which rely on microsatellites (STRs) and Single Nucleotide Polymorphisms (SNPs) technology to cover genetic traceability demands in small ruminant species and their products. Both methods are considered reliable, worth of cost and time input and their results are repeatable reproduced. Multiplex PCR kits for STR implementation or SNPs panels have been developed or synthesised for species with invasive commercial interest. Although many studies have been conducted focusing on the traceability of products mainly on beef or porcine products using STRs or SNPs panels (Mateus and Russo-Almeida, 2015; Rogberg-Muñoz et al., 2016; Rębała et al., 2016; Kannur et al., 2017), the area of small ruminant species remains still not so over explored.

Implementation of microsatellites on traceability of small ruminant species and their products.

Microsatellite markers (STRs), defined as small tandem repeats of 2-6 bp widely spread across DNA (Gettings et al., 2015), have been widely used as a mean to determine the genetic variation in livestock populations. The latter reflects in the majority of the conducted studies to the genetic description of a breed (Ciani et al., 2013; Silva et al., 2017), or the evaluation of the genetic diversity (Ceccobelli et al., 2015; Cao et al., 2017; Nguluma et al., 2018), or genetic identification in animals or parentage assessment (Rosa et al., 2013; Clarke et al., 2014).

Although such approaches led to a different STRs pattern outputs, none of them have been highlighted as a tool for discriminating small ruminant breeds or their respective products. However, in some cases, the use of STRs in evaluating the genetic structure or diversity of various small ruminant populations gave the motivation to apply also this methodology as a discrimination tool of tracing the breed and/or the origin of their products and thus, protecting food authenticity. For instance, Koutsouli et al. (2007) managed to discriminate three indigenous Greek breeds and two foreign breeds, using a 12 set of microsatellites and breeding assignment approaches. In addition, a successful implementation of STRs have been reported to discriminate cheese starters and possible contaminating during cheese manufacturing (Giraud, et al., 2010). Similarly, the correct discrimination of seven purebred Italian ovine breeds have been conducted using

19 STR markers (Bramante et al., 2011). Moreover, Sardina et al. (2015) analyzed 20 microsatellites on three Italian goat breeds resulting firstly to the creation of a breed tracking system of dairy products made by Gergentana goat milk, and secondly to the fact that only three of the used STRs can be implemented to this system. Recently, Di Stasio et al. (2017) successfully developed a traceability method to verify the authenticity of Sambucano sheep a registered traditional Italian product, using 14 STRs. Obviously, a great number of studies using microsatellite markers have been conducted in small ruminant species, mainly on genetic structure and variability of the breeds or populations and with a minor effort to traceability purposes. However, in both cases unique discriminating or comparative DNA patterns for each breed exist as first outputs; but there is a need to summarize, organize, extend and diffuse all this amount of information, specialized in each breed, in a public database. All these patterns so far provided could serve then as a first approach in the development of a breed distinguished protocols based on DNA level (DNA bar coding system) for small ruminant species or products.

Implementation of single nucleotide polymorphism (SNPs) on traceability of small ruminant species and their products

DNA technology is evolving rapidly and STRs markers used for genetic diversity studies, and breed or individual assignment tests, are gradually being replaced by SNPs technology. The discrimination power of SNPs is exceptional as SNPs are abundant, more informative, less expensive and can be run automatically compared to STRs in massive tests. (Kawęcka et al., 2016). In several studies, the objective is to verify the parentage (Tortereau et al., 2017), to assign breeds and assess the genetic and population variability (Grasso et al., 2014; Edea et al., 2017) and more generally for phylogenetic and biodiversity purposes (Kawęcka et al., 2016; Leaché and Oaks, 2017). Subsequently this information is applied to the genetic identification of breed and furthermore to find possible adulteration practices on superior quality products from specific breed (*i.e.*, PDO cheese products and meat). The distinction and choice of DNA that will be able to discriminate different breeds should depend on the unique genetic "fingerprint" of each breed (Fontanesi, 2009). However, the creation of the contemporary breeds not only reflects the complicated connection among populations, but also the exchange of genetic material leading to a different levels of variability. In addition, as no biological reproductive barriers exist among breeds, distinction of their genetic mixture is not easily, leading to difficulties in specifying unique breed markers for traceability purposes. Although the implementation of novel genotyping methods using chip-technology that include thousands of SNPs, is a promising possibility for breed identification, the main drawback of this approach is relied on the fact that it is difficult to certify the origin for dairy products made by milk mixtures of different breeds (Fontanesi, 2009). The discovery of breed-exclusive markers, is expected to overcome the challenge regarding the intra-species identification products'.

Although many applications of SNP technology in animals have been reported (Gurgul et al., 2014), most of the research on traceability aspects using SNPs is focused on beef breeds discrimination for their meat (Orrù et al., 2009; Lasagna et al., 2015; Xu et al., 2018) or on dairy cattle breeds (Karniol et al. 2009; McClure et al., 2018) or swine breeds/products (Choi et al., 2015; Kwon et al., 2017). The discoveries on this issue concerning traceability studies in small ruminants are rather few. Initial research from Pariset et al. (2006) identified 37 SNPs, located within 27 genes in 16 animals from eight different European sheep breeds, which were associated with key metabolic pathways or potentially significant productive traits. The achieved information contributed sufficiently to reconstruct the history of each studied breed. In goat the research of Crepaldi and Nicoloso (2007) was focused on SNPs involved in pigmentation, as a tool to identify 4 goat breeds with different coat colour phenotypes (Camosciata delle Alpi, Blonde of Adamello, Saanen and Orbica), but resulted that a relative greater number of SNPs may be needed for traceability methods. Subsequently, Heaton et al. (2014) using a parentage SNPs approach managed to identify a SNPs panel for the North American sheep's identification, which according to the authors can be also used for global traceability efforts.

More recently, a panel of 249 SNPs, derived from a commercial 50K SNP chip, has been successfully used in an on-farm test on the Blanche du Massif Central breed resulting in more than 95% accuracy concerning the assignment of the tested sheeps to a distinct sire (Tortereau et al., 2017). In the same line seven SNPs have been successfully identified as potential markers to identify and trace the meat derived from sheep reared in north-western and eastern China (Wu et al., 2017).

Although SNPs provides a powerful tool both for genetic diversity and traceability purposes, it remains unexplored the possibilities of origin's discrimination in the situation of product adulteration or mixed final products.

Which method would be the perfect choice in the area of traceability control? SNPs or STRs?

The answer to this certain question is not so easy as many factors should be considered apart from the experienced personnel, labor timing and cost aspects. Even though STRS are considered highly informative, with a great level of polymorphism and wide-spread in the genome (Gettings et al., 2015), the STRs outputs obtained by different researchers or labs are not in all occasions compared to each other due to inconsistency of the genotype calling and/or mistakes in the output interpretation. Moreover, microsatellites are labor intensive, regarding the time needed for trained personnel to analyse the running output (Vignal et al., 2002).

The implementation of next generation sequencing and novel in-silico approaches, forced the use of SNPs to become more prominent (Heaton et al., 2002). Although by means of DNA information a two-allele marker may be interpreted as a movement to outdated methodologies, SNPs are considered as a powerful tool for genetic analysis purposes, mainly due to their abundance, stability, low cost and their ability to participate in high throughput analyses (Koopaee and Koshkoiyeh, 2014; Leaché and Oaks, 2017). In addition, SNPs not only have been successfully used in the approach and characterizations of quantitative trait loci (QTL) and the link of commercially important traits with specific genes (Chen and Abecasis 2007; Wollstein et al., 2007), but also in tracing specific individuals or breeds (Negrini et al., 2008) or other applications on animals (Koopaee and Koshkoiyeh, 2014). The first step for an efficient traceability method based on SNPs, is the set of a minimal number of marker that will be able to uniquely certify animals among pure breeds or cross-bred herds (Heaton et al., 2002). Generally, 2-5 SNPs for each STR are a prerequisite in order to have the same genetic information (Schopen et al., 2008; Koopaee and Koshkoiyeh, 2014). However, this is a matter either depending on the implemented purpose or the studied organism (Schopen et al., 2008; Lee et al., 2017) or on how informative is the set of SNPs used in each study (Glover et al., 2010). According to Fernández et al. (2013) for the genetic discrimination and parentage control in an Angus population, almost a double number of SNPs were needed in order to receive the same genetic information compared to STRs. Moreover, the reported SNPs sample matching probability showed that the 24 SNPs had an equivalent informative power with that of the minimal set of 10-11 STRs (recommended by ISAG (International Society for Animal Genetics).

Although SNPs reveal a great genetic power for diversity purposes, according to Karniol et al. (2009) the most important drawbacks compared to STRs are faced during the examination of samples containing DNA from different bovine breeds or animals. Such blends would not be easily identificated using SNPs. On the other hand, STRs would reveal rapidly the mixed samples by determining the existence of more than two alleles per STR.

To the best of our knowledge, no further analysis to this field have been conducted in the area of small ruminants. However, it would be useful if such information can be retrieved in the future, in order not only to be more precise by terms of cost implication of SNPs or STRS in a routine control of food safety or adulteration, but also to identify the higher informative SNPs loci that may lead researchers to a powerful approach of traceability control of animals and their products.

CONCLUSION

Considering the positive impact of traceability throughout the whole food chain, we can conclude that this impact not only can lead to an increase of its acceptance but also to the distribution of an added value of the final product among all stakeholders. All participants at national and international level should insist on its importance. As the cost of SNPs examination decreases, more studies will be designed, and more results are expected to provide the available information to trace animal products to their breed of origin. However, genetic patterns revealed by STRs or SNPs should be interpreted in a simpler way (*i.e.*, handling data with friendly to use software outputs) in order the final implementation of the method to be the easiest by means of final results and by means of a massive routine protocol use.

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CONFLICT OF INTEREST

There is no conflict of interest to declare.

AUTHORS' CONTRIBUTION

Author contributed equally to the preparation of the certain review.

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