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Project coordinator name **Cesare Bonacina**
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RISKSCRA

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Publishable Final Activity Report

Project coordinated by Dr. Cesare Bonacina

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Consorzio per la tutela del Pecorino Romano - Italy

Consejo Regulador de la D.O. "Queso Zamorano" - Spain

Chios Sheep Breeders Cooperative “Macedonia” – Greece

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INTRODUCTION

Considering the large amounts of sheep milk processed in the Mediterranean area, the dairy industry represents a strategic area within the agricultural and food economy. Milk sheep production is 1.3% of the total milk production. The Mediterranean basin, with 60% of total production, is the most important area. The dairy sheep industry is usually based on local breeds which are very well fit to their production areas, systems and environments.

One of the main problems across EU countries is to date represented by transmissible spongiform encephalopathies (TSEs). Scrapie is one of a number of TSEs and is a fatal brain disease of sheep and goats. Scrapie develops when the normal form of the Prion Protein (PrP) in a sheep's brain converts to an abnormal form. After a period of several months it causes nervous system dysfunction and, eventually, the death of the animal. Experimental studies have shown that bovine spongiform encephalopaty infection (BSE) is transmissible to sheep following oral and parental challenge. BSE affected sheep have a tissue distribution of infectivity and pathological changes similar to those of scrapie affected sheep. Now, abnormal prion proteins have been founded in the mammary glands of sheep affected by scrapie and mastitis. The suspect that TSE in sheep could be BSE rather than traditional scrapie lead European institutions to promote breeding plans for TSE resistance in the European countries. The PrP gene, which produces this PrP protein, also determines a sheep's resistance or susceptibility to TSE. The sheep PrP gene has two copies (alleles), one derived from each parent. Each codon on the gene translates into one of the 256 amino acids that form the PrP protein. Studies of the genetics of sheep have shown three particular codons on the alleles that indicate TSE resistance or susceptibility. The codons are 136, 154 and 171 and are based on variations of amino acids at the location. Five different scrapie related alleles in sheep have been identified: codon 136 codes for either the amino acid valine (V) or alanine (A), codon 154 codes for either histidine (H) or arginine (R), codon 171 codes for glutamine (Q), argininne (R) or Histidine (H). The three codons creates 15 genotypes classified into 5 categories of risk according to the British National Scrapie Plan (NSP). In all breeds the research suggest the ARR allele is the most resistant to scrapie. The VRQ allele is the most scrapie susceptible. The ARQ/ARQ genotype is susceptible to some forms of scrapie and very susceptible to experimental BSE. Genetic selection is being used as the primary mean of scrapie control in the UK. The British National Scrapie Plan will initially concentrate

on promoting the use of the ARR gene and excluding the VRQ gene and allow the continued use of sheep with the ARH, ARQ and AHQ genes.

Genotypes ranking in the British National Scrapie Plan

CLASS	GENOTYPE	CLASS OF RESISTANCE
NSP1	ARR/ARR	Highly resistant to Scrapie
NSP2	ARR/ARQ ARR/ARH ARR/AHQ	Resistant to Scrapie (genotype rare) (genotype rare)
NSP3	ARQ/ARQ ARQ/AHQ AHQ/AHQ ARH/ARH AHQ/AHQ ARQ/ARH	Little resistant to Scrapie (genotype rare) (genotype rare) (genotype rare) (genotype rare) (genotype rare)
NSP4	ARR/VRQ	Susceptibile to Scrapie
NSP5	ARQ/ VRQ VRQ/VRQ VRQ/ARH VRQ/AHQ VRQ/ARR	Highly Susceptible to Scrapie

The breeding program for genetic resistance is important to achieve a reduction of scrapie's incidence, but it is necessary to evaluate also other traits in breeding schemes in order to guarantee significant genetic improvement in dairy sheep industry. Italy and Greece are the EU countries with the largest amount of sheep milk annually produced (800,000 MT and 700,000 MT) (FAOSTAT, 2004), and respectively the second and the fourth largest sheep milk producers in the world. The sheep milk is mainly processed in dairy products (cheese, yoghurt, cream etc.). Dairy industry and consumers require constant supply of quality and safety milk from the farms. Therefore, successful production systems must combine high yields with quality and food safety features. Such systems will depend largely on genetic improvement programs, run by breeders' associations, which will guarantee animal health, in addition to high production and desirable

milk constitution. In this respect, it is important to control TSE, by assessing the current situation in the population, identifying genetically resistant animals, and designing and implementing genetic improvement program.

PROJECT OBJECTIVES

The possibility to increase consumers' security in relation to the assumption of scrapie-free dairy products will represent a competitive advantage on the market. This capability at enterprises' disposal can fulfill the current consumers' needs for food safety and therefore will directly turn into a quality factor capable to promote sheep dairy productions on a wider market at both national and international level, providing support to the whole sector. The implementation of a scrapie-free assurance for ovine milk should also lead in the future to a reconsideration of the milk's payment parameters according to its safety, thus providing dairies with an additional parameter for the selection of suppliers in order to achieve a better risk management. The main objective of this research was the development of analytical tools to assess and quantify scrapie risk in milk and the implementation of application by breeder organizations and dairy consortia throughout the production chain. The scientific work was performed by research Institution and Universities which prepared suitable analytical protocols for routine control purposes. The transfer of technology and knowledge from researchers to dairy technicians WAS possible by means of specific training courses.
The ultimate objective was to increase THE enterpriseS' competitiveness by developing and applying a safety control, in harmony with the advanced European strategies, thus stimulating the manufacturing of scrapie-free dairy products.

The main scientific and technological objectives of the research program were:

- Optimization of routinLY applicable methods for DNA extraction from milk
- Optimization of protocols for PrP genotyping and allele quantification
- Set up OF simple analytical procedures in order to monitor the dairy processing
- Introduction of a new analytical approach in the production chain to improve dairy product safety

PROJECT ORGANIZATION

The project was divided into 2 main phases which correspond to the main project objectives.

<table>
<tr><td rowspan="2">Phase 1: Optimization of a suitable protocol for dairy production chain</td><td>WP1 – Innovative analytical protocols to quantify scrapie risk in ovine milk from pooled samples</td></tr>
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In Phase 1 an exhaustive theoretical assessment of all possible approaches for the molecular analysis on PrP gene was provided by the RTDs and a selection of potential reference methods were defined on DNA from individual and bulk milk. Molecular methods were developed to study polymorphism at codons 136, 154 and 171 of the prion protein (PrP) gene. Moreover, new PrP alleles were searched in not previously studied sheep breeds. Furthermore the RTDs developed an integrated analytical protocol that can be used on a routine control by IAGs and SMEs technicians.

In Phase 2 specific theoretical and practical courses on main topics were organized by the RTDs to transfer the know-how to IAGs technicians and by IAGs technicians to sheep breeders and dairy producers. The aim of the scientific and technological transfer to the IAGs was the acquisition of the knowledge, the shift of the innovative technology and the adoption of the milk routine control. The innovative protocol was included in the IAG dairy production rules and regulations in order to certificate scrapie risk in dairy products.

ACTIVITIES PERFORMED

WP1- Innovative analytical protocols to quantify scrapie risk in ovine milk from pooled samples.
The innovative approach proposed by the project investigators was the identification and the quantification of the different alleles of PrP gene in bulk milk samples. New analytical protocols allowed to screen a large number of animals simultaneously, using a single analysis, in order to evaluate scrapie risk in milks used for cheese making.

WP2 – Set up of a routine procedure for a large scale application of a given analytical protocol.
The IAGs and SMEs provided guidelines for the sampling procedure at dairy farm and vat level in order to make an homogeneous and representative sampling. The protocols were adapted, by developing software tools, to obtain a routine procedure that will simplify the SME analytical goal to define the allelic profile of any given dairy product.

WP3 – Transfer of protocols to IAGs and SMEs and updating of self-control procedures
Theoretical and practical training courses were carried out by the RTDs researchers in order to transfer the new procedure to the IAGs and the IAGs furthermore prepared the SMEs technicians for the use of research results and equipment. The transfer of technical information and technology requirements were focused on the achievement of concrete results in terms of applicable new knowledge to improve safety of sheep dairy products. The overall goals of such a transfer activity were pursued by using: courses, stages, exchange of researchers, workshops and seminars.

WP4 – Drafting of a guarantee system for IAGs and SMEs involved in the ovine dairy product's chain and updating of product specifications.
The objectives of this WP were to obtain the guidelines to draft a guarantee system for the Italian, Spanish and Greek dairy industry based on a self-controllod traceability approach to assess the scrapie risk and to achieve a cheese certification for scrapie-risk. The introduction of a new parameter in the dairy production rules and regulations represent an added value that will allow to certify dairy products assuring production safety.

WP5 – Exploitation and dissemination of results
The identification and the organization of the activities were carried out in order to promote the use of the project's results and the dissemination of knowledge derived from the project. An exploitation plan containing an overview of the project outcomes was drafted a final term stages by the Riskscra consortium. The final piece of work was a critical review of the research work and technology transfer carried out in the project for the dissemination and dissemination strategy to be undertaken. The exploitation of the project results will start with the direct application of the new protocol

by the dairy consortia. A web site and more traditional tools like newsletters were used to reach a large target audience. The project's results were spread by means of technical publications directed to the sector operators. Furthermore the results should be published on the project web site dedicated to the diffusion of organizational information and intermediate and final results to all partners, also supporting a forum for the real time communication among partners.

WP0 – Coordination and project management.
The project was managed by the Project Management Committee that was in charge to coordinate and stimulate the various activities of the project. This WP included the scientific, technical and organizational project management together with the contractual and financial management.

METHODOLOGIES AND APPROACHES EMPLOYED

Quantification of PrP alleles to determine scrapie risk in ovine milk

Partners: ISILS, CTFPR, FPT, AGRS, IZCS/AGRIS

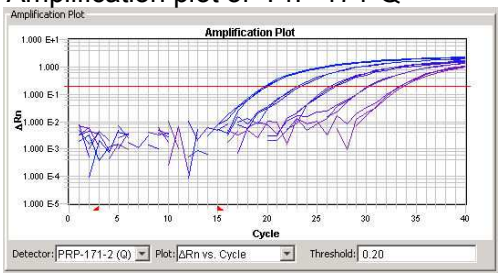
A new analytical protocol was developed to analyse genetic polymorphisms of *PrP* gene from DNA isolated to bulk milk. The protocol use specific calibration curves and permit to determine and quantify allelic variants of three codons associated to different levels of resistance for scrapie: 136 (*A/V*), 154 (*R/H*) and 171 (*Q/R/H*). The protocol was developed to analyse bulk milk samples, it supplies the information related to *PrP* allele frequencies in milk from several hundred animals.
DNA extraction was carried out by using a commercial kit (NucleoSpin® Food-Macherey-Nagel) with a modified procedure. The sample of DNA was amplified by Real Time PCR according to TaqMan MGB chemical. During the amplification cycle, the calibration curves were created by using known quantities (0.01%, 0.1%, 1%, 10% and 100%) of two variants of the investigated amplicon. After DNA amplification, the samples intensity fluorescence were plotted on the calibration curves that were constructed with the standard fluorescence values. In this way, it is possible to determine the percent value of the allelic variants. In particular, the analytical procedure was optimize to determine the allelic variants of codons 171 (*R/Q*) in order to evaluate the frequencies of the scrapie resistant alleles. The sperimental protocol was tested on mixes of DNA with known genotype. Repeatability and reproducibility were tested.

The results obtained are shown in the following table:

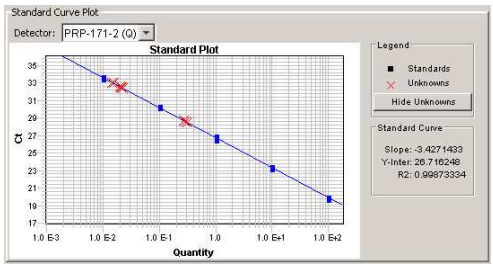
%Q expected	%R expected	Mean R%	Mean %Q	ssdr	ssdq
50	50	52.63	47.37	0.8353	0.835302
90	10	10.02	89.98	1.03345	1.033454
10	90	88.79	11.92	0.51901	0.519015

The protocol was validated on the field by measuring the percent of 171/R and 171/Q alleles in DNA samples from bulk milk sampled from 20 farms and tanks in dairies.

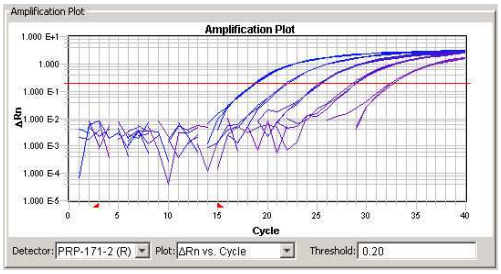
Amplification plot of "PrP-171-Q"



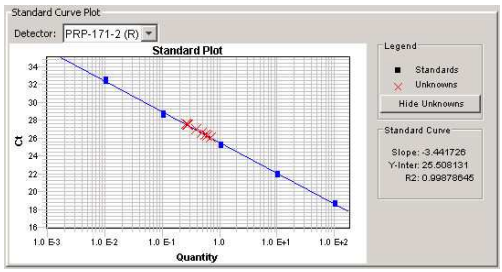
Standard Curve of "PrP-171-Q"



Amplification plot of "PrP-171-R"



Standard Curve of "PrP-171-R"



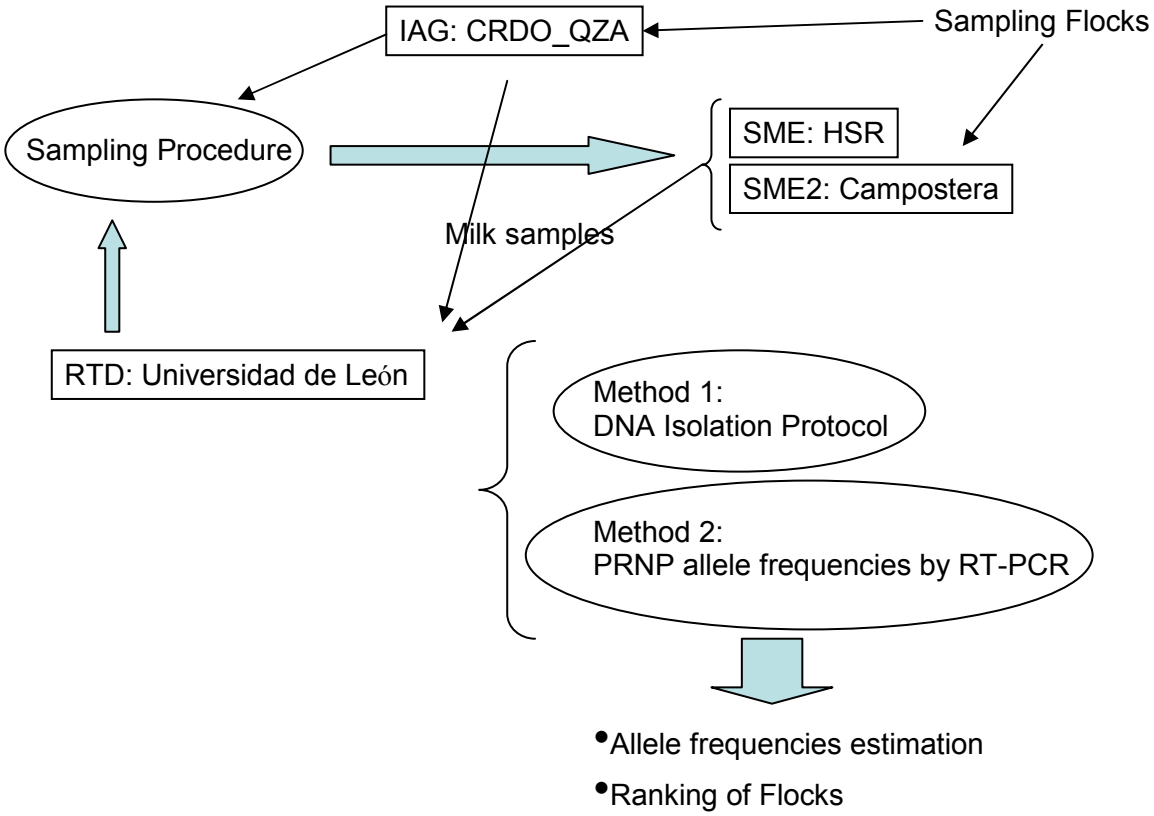
The Real Time PCR protocol was tested for the codon 171 using DNA mixtures at PrP171 in different percentages, milk mixtures from animals with known genotype and of milk from dairy farm.

Methods used for determining the scrapie risk in Zamorano Cheese Flocks

Partners: CRDO-QZA, ULE, HSR, Campostera

The Approach followed by Spanish Team is schematized in figure 1 and will be explained below:

Figure 1: Methodology developed by Spanish team



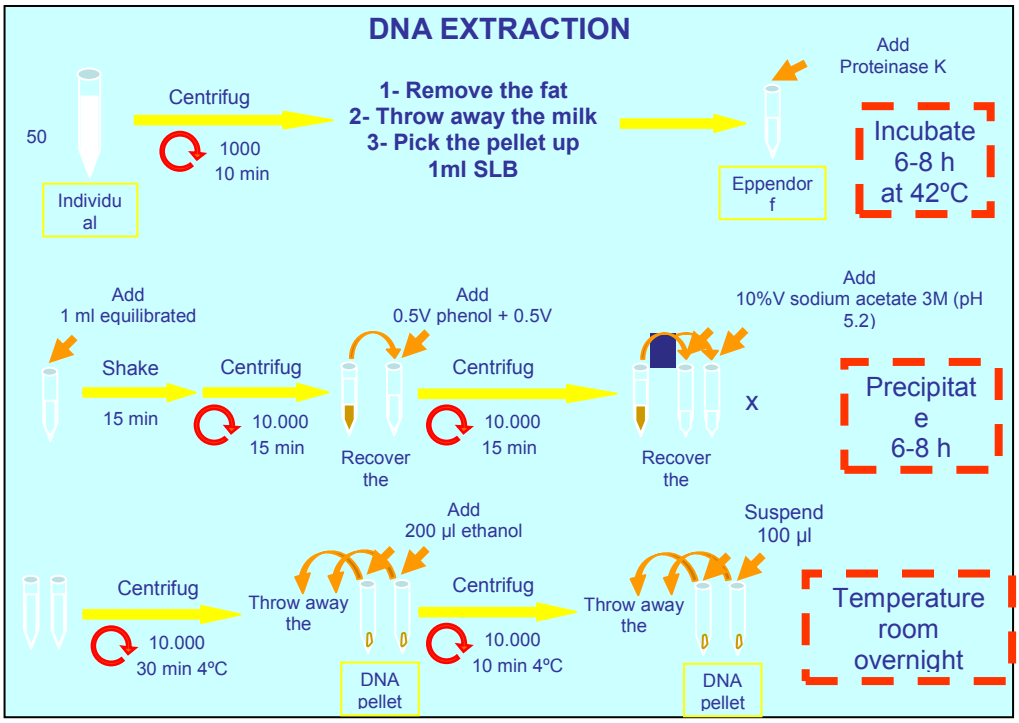
Firstly in a joint meeting between CRDO-QZA, Universidad de León and two SME participating in the project (HSR and Campostera) the general strategy for sampling was determined: all samples were taken at flock level. For this purpose, a general "Sampling Procedure" was determined concluding after different essays that the best procedure for sampling was:

Protocol "ES1": Sampling procedure at flock level

- Sampling procedure requires homogenization of bulk milk (manual)
- Samples are representative (tank) in a volume of 250 ml

Once milk was sampled the IAG and/or SMEs sent to the RTD laboratory where DNA was isolated using the Macherey-Nagel DNA isolation food kit protocol as indicated in figure 2.

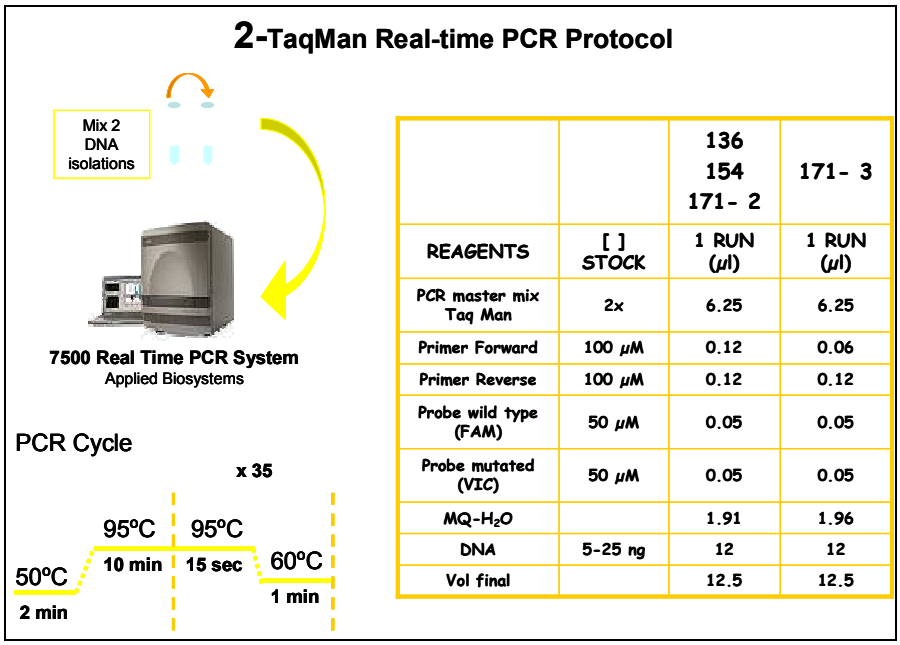
Protocol "ES2": DNA Isolation



Once DNA was isolated, estimation of allelic frequencies from codons 136, 154 and 171 at PRNP gene were estimated by a Real Time Procedure. For this purpose six TaqMan Probes were synthesized and a protocol established in order to estimate the relative frequencies of each allele at each codon. In this case, the quantifications were performed by the relative procedure or 2nd method. This procedure was tested on experimental bulk milk prepared using milk from genotyped animals and flocks where individual genotypes were known.

In this essays wee have detected a number of factors that could affect in PRNP genotyping: (i) Quality of DNA, (ii) Mutations in regions homologue to probe other than target SNPs, (iii) Milk yield and (iv) Somatic Cell Counts. These parameters are easily fixed if the milk sampling is near to official milk control. In this case both aspects could be used as co-factors in the analysis and corrected efficiently.

Protocol "ES3": RT-PCR assay



		136 154 171- 2	171- 3
REAGENTS	[ ] STOCK	1 RUN (µl)	1 RUN (µl)
PCR master mix Taq Man	2x	6.25	6.25
Primer Forward	100 µM	0.12	0.06
Primer Reverse	100 µM	0.12	0.12
Probe wild type (FAM)	50 µM	0.05	0.05
Probe mutated (VIC)	50 µM	0.05	0.05
MQ-H_2O		1.91	1.96
DNA	5-25 ng	12	12
Vol final		12.5	12.5

Once the robustness of the procedure was established in different field assays, our approach was used to estimate the scrapie resistance in most of theflocks contributing to Zamorano cheese. This approach (indicated in figure 1) was the main point where SMEs collaborated in the sampling procedure. Other flocks where sampled by IAG (CRDO-QZA). The general protocol established for sampling take into account that there is not a common protocol and that our protocol give good results in the conditions of Zamorano-cheese producers. We decide use this protocol for ranking the flocks 89 Flocks belonging to CRDO-QZA (120 in total) (~75%) were sampled by the protocol described before, briefly: Sampling of 250 ml of tank milk, DNA isolation: 3 replicas, determination of Allele frequencies (ARR, AHQ, ARQ, VRQ) by Real Time PCR and ranking using ARR frequencies.

School of Veterinary Medicine, Aristotle University of Thessaloniki – Greece

Milk samples from 1.013 purebred ewes of the Chios breed raised in 23 flocks were collected in collaboration with the Chios Sheep Breeders' Cooperative "Macedonia"). Genetic analyses considered the following steps:

1. **DNA extraction from individual ewe and bulk milk samples**

Six (6) different protocols were tested: (i) Nucleospin Blood commercial kit (Mackerey Nagel co), (ii) Nucleospin Tissue commercial kit (Mackerey Nagel co), (iii) modification of (i) with the addition of EDTA and chloroform for optimisation of milk casein and lipid dissolution, (iv) modification of (ii) with the addition of EDTA and chloroform for optimisation in milk casein and lipid dissolution, (v) phenol–chloroform protocol, based on these two substances, with increased centrifugation in order to obtain more DNA, and (vi) an in-house DNA extraction method, based on a silica guanidine protocol and home-made filters.

The six methods were evaluated for extraction efficiency, DNA quantity, DNA purity and DNA suitability for amplification. It was determined that a modified blood kit, enhanced with the addition of EDTA and chloroform, presented the most reliable, effective and economic method, and was identified as the protocol of choice for the analysis of individual and bulk milk samples.

2. PCR amplification methods and RFLP analyses

A PCR amplification – RFLP analysis protocol was developed and optimised. Three separate RFLP analyses were used in order to determine the polymorphisms at the three main codons {136 (A/V/T), 154 (R/H), 171(Q/R/H)} of the PrP gene locus. The first was based on the restriction enzyme *Bsp*H1, the second on *Bsp*D1 and the third on R*sa*I. Furthermore, a fourth restriction enzyme, MnlI, was used for the detection of polymorphisms at codon 141 (F/L). Polymorphisms at codons 141 are known to be associated with atypical forms of scrapie. In each case, two different PCR products were amplified: one with 259 bp (product A) and the other with 258 bp (product B). Both were amplified with the same up-stream primer (developed in-house). Product A was amplified with downstream primer H1, which creates an artificial recognition site for *Bsp*H1 when histidine occurs at codon 171. Product B was amplified with downstream primer D1, which creates an artificial recognition site for *Bsp*D1 when arginine occurs at codon 171. Recognition sites for *Bsp*H1 are formed when valine occurs at codon 136 and histidine occurs at codon 154. At the same time, in both PCR fragments, recognition site is formed for R*sa*I when threonine occurs at codon 136 and for MnLI when leucine occurs at codon 141. Half of product A was digested with *Bsp*H1and the other half was digested with MnlI. Half of PCR product B was double-digested with *Bsp*H1 and *Bsp*D1and the other half product B was digested with R*sa*I. Based on experiences from the first set of RFLP analyses using the above-mentioned procedure, improvements were sought regarding reproducibility of results. Therefore, a nested PCR concept was introduced. In this procedure, the entire PrP gene was first amplified in order to create a better template for the original RFLP PCR protocol. Including this additional step, the reproducibility of results was optimised, thereby decreasing genotyping costs. Therefore, nested PCR was

identified as our method of choice and was applied for the analysis of samples.

3. Development of real-time ligase chain reaction (LCR) for bulk milk analysis

A novel method for assessing the prevalence of a specific PrP polymorphism in the bulk milk was developed. This method was based on a three-step procedure (Figure 1). First, part of the PrP gene, containing the polymorphisms of interest, was amplified using a real-time PCR. The real-time PCR product was treated with exonuclease I and antarctic phosphatase in order to digest the single stranded DNA and dephosphorylate the remaining DNTPs, respectively. Dilutions of the treated PCR product were used as target in a novel real-time ligase chain reaction (real-time LCR) developed here for the detection of SNPs. This assay employs two adjacent oligonucleotides which hybridise to one strand of DNA template, and a complementary set of adjacent oligonucleotides which hybridise to the opposite strand. Thermostable DNA ligase links each set provided there is complementarity at the junction. In addition, because oligonucleotide products of each round serve as targets for the next one, the signal is amplified exponentially. The novelty in this LCR protocol was the utilisation of a fluorescent dye for quantifying the LCR product after each cycle. Ct values were obtained and used for constructing a standard curve for the quantification of the desired polymorphism in the samples (Figure 1).

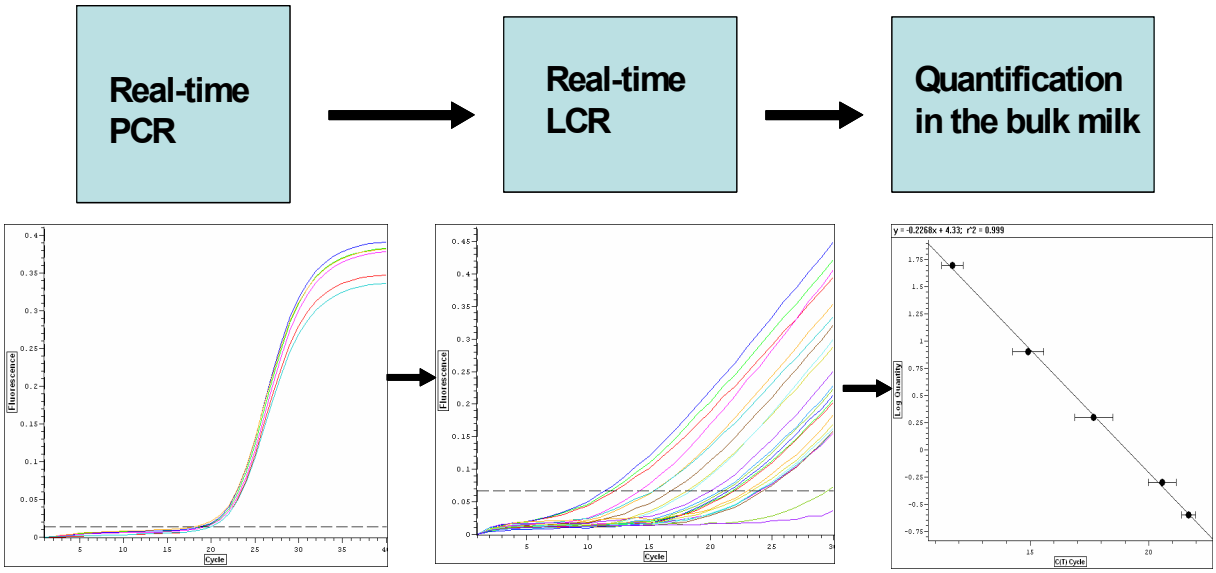


Figure 1. Schematic illustration of allele detection and quantification using the developed real-time ligase chain reaction.

Artificial sets of bulk milk containing different frequencies of VRQ polymorphism, created by mixing equimolar DNA extracts, were used to evaluate the method's sensitivity and accuracy of allele quantification. Although LCR was highly sensitive, non-specific ligation could still occur at

low levels. In an effort to further improve specificity, modifications of this method were tested. These involved the use of single nucleotide gap at the site of point mutation between the adjacent oligonucleotides. This gap was filled using a thermostable DNA polymerase in the presence of the appropriate mutant nucleotide in the reaction mixture adding substantial additional specificity to the reaction. On the basis of these principles we developed first a standard real-time LCR and then two modified Gap-LCR protocols.

The real-time LCR method and their modifications were tested for the detection and quantification of VRQ polymorphism in artificially formed equimolar bulk milk samples. The LCR assay was proved more efficient and sensitive than RFLP analysis for bulk milk samples. Results indicated that the minimum detectable allele frequency with LCR was not higher than 0.25%.
No significant difference were observed in the performance standard real-time LCR method and the two modified Gap-LCR protocols.

Faculty of Agriculture, University of Zagreb – Croatia

Individual milk or blood samples were taken from 2585 sheep (252 rams and 2333 ewes) randomly from 134 flocks of eight sheep breeds; Istrian sheep (IS), Cres Island sheep (CIS), Krk Island sheep (KIS), Rab Island sheep (RIS), Pag Island sheep (PIS), Licka Pramenka (LP), Dubrovacka Ruda (DR) and Dalmatian Pramenka (DP) as well as one farm named "Experimental farm Radosevic" (EFR). Dalmatian Pramenka is the highest numbered breed in Croatia so we took samples representing different areas/locations; a) Kornati Island (DPKI), Brac Island (DPBI), Vis Island (DPVI), hinterland area of Zadar and Sibenik towns (DPZSH), hinterland are of Split together with surroundings of town Sinj (DPSS) and surroundings of town Imotski (DPIS). For genotyping we used three different methods a) sequencing for 1003 individuals (according to Gmur et al. 2004), b) RFLP for 267 individuals (according to Yuzbasiyan-Gurkan et al., 1999 and Ekateriniadou et al. 2006) and c) RT-PCR for 1315 individuals (according to procedures used by RiskScra partners).

ACHIEVEMENT WITH REGARD TO THE STATE OF ART

The primary users of RISKSCRA Control System are dairy industries which will integrate the new procedures into milk quality analysis and possibly additional labellinginto commercial cheeses. In RISKSCRA, the sector is represented

by countries (Italy, Spain, Greece) that represent the most important sheep dairy production at European level and partners, Consorzio Tutela Pecorino Romano (CTFPR), Consejo Regulador de la D.O. "Queso Zamorano"-(CRDO-QZA), Chios Sheep Breeders Cooperative "Macedonia" (CHIOSGR) that were very vigorously involved in using the RISKSCRA protocols for the implementation of novel guidelines to dairy production rules and regulations.

Achivements in Italian Pecorino Romano cheese flocks (ISILS, CTFPR, FPT, AGRS, IZCS/AGRIS)

The results obtained from the project are an economic and effective tool for an active policy against scrapie, especially in relationship with recent conclusions and recommendations (Opinion of the Scientific Panel on Biological Hazard on the human and animal exposure risk related to Transmissible Spongiform Encephalopathies (TSEs) from milk and milk products derived from small Ruminants- Question No EFSA-Q-2008-310- Adopted on 22 October 2008).
The organisation by the consortium (IAG) of a service for the dairy enterprises (SMEs), performing the periodic self-control and routine analysis on the milk used in Pecorino Romano cheesemaking, could allow:

• to monitor the trend of the number of animals genetically susceptible to scrapie over time in large areas as well as in single breeding farms;

• to evaluate the program's efficiency based on the genotyping of single animals

• to increase, through prizes for milk production, the creation of resistant-genotype flocks

• to promote and reward the milk supply from scrapie resistant flocks;

• to characterize the milk used for cheese production in order to safeguard the consumers.

Achivements in Zamorano Cheese Flocks (CRDO-QZA, ULE, HSR, Campostera)

The main achievements were:

1. Establishment of a Sampling protocol for scrapie resistance determination at flock level
2. Laboratory Protocols for the determination of PRNP allele frequencies from bulk milk
3. Ranking of Zamorano Cheese flocks regarding scrapie resistance (rank is shown in figure 2)
4. Training courses for breeders belonging to PDO Zamorano Cheese
5. Training Courses for technicians of dairies contributing to Zamorano cheese
6. Dissemination of results and tools to make Zamorano cheese a safer food: book, website, booklets for food fairs, etc.

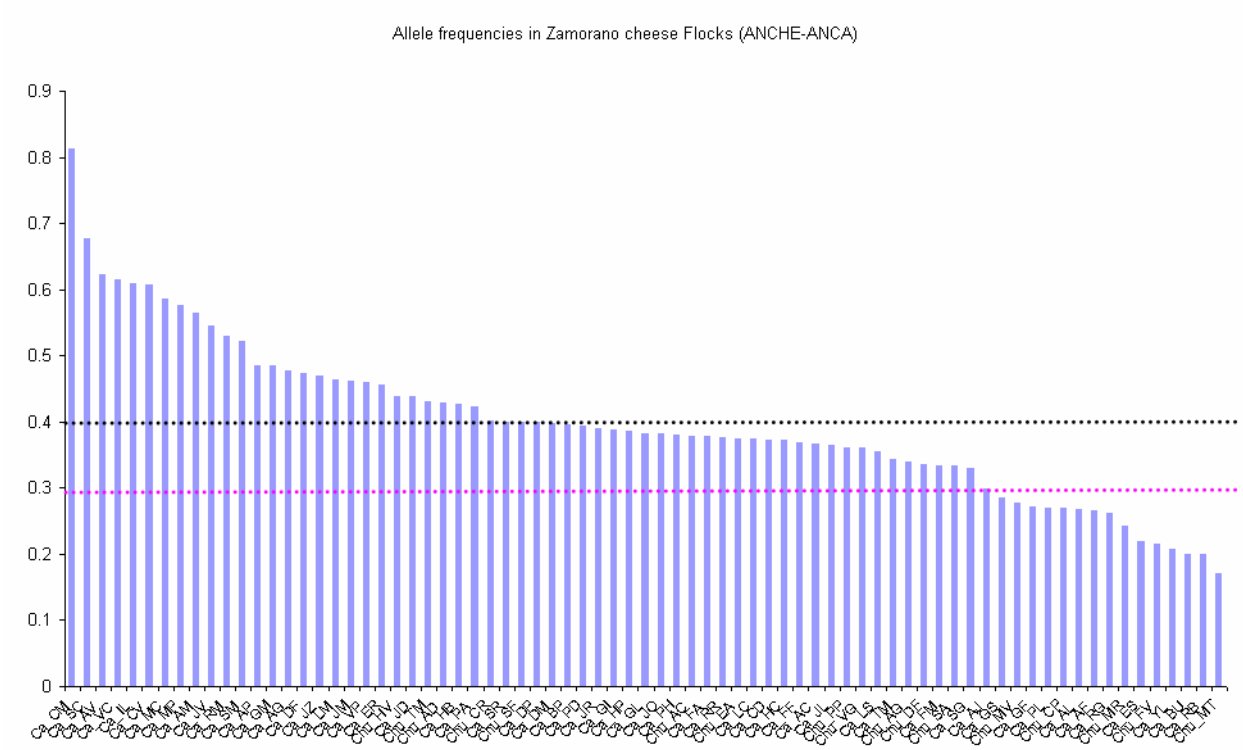


Figure 2: Zamorano Cheese rank based in ARR allele frequency.

Achievements in Greek flocks (AUTHVET, CHIOSGR)

The methodology developed can used at flock level as a pre-screening tool for undesirable scrapie alleles. If no such alleles are found in bulk milk there is no need to proceed with individual genotyping of each animal in the flock.

In the research sector, the developed methods can lend themselves for accurate genotypic identification studies of different organisms.

Achivements in Croatian flocks (UZA)

The results of Prnp screening (genotyping) obtained in this project for 252 rams and 2333 ewes that were sampled across eight breeds (one breed Dalmatian Pramenka included 6 subpopulations from of three were on islands) does provide detailed information about Prnp gene and genotypic frequencies in Croatia (results from the project are presented in more details in Appendices I, II, III, iV, V, VI, VII and VIII). The results coming from the project are the first results presenting Prnp status in Croatia and will act as a basis for a future “Croatian National Scrapie Resistance Plan”.

Achivements at a European level

The added value of carrying out the work at a European level is clear:

- There is a need to increase competitiveness of the sheep dairy industry at EU level, and not just in any given Member State, in order to place it in a leading position on a global scale competition. Providing SMEs dairy industries with access to innovative technology, such as RISKSCRA, will enable to rise their productivity.
- The problems inherent with scrapie detection need to be dealt with at European level. Needs and demands differ within Europe so there is a need for an integrated effort to ensure that the RISKSCRA system successfully serves the diverse requests of the Common Market.
- Given the free movement of goods within the EU, milk quality, as well as health and safety standards, need to be raised across all the Member States, as we are all common consumers.
- An integrated European effort and the pooling of resources and expertise will have a greater impact in helping the industry to fight the common problems they face in terms of quality productivity, as well as avoiding duplicity of research work, thus contributing to ERA objectives.
- In the light of the accession countries joining the EU, it is vital that a European-wide approach adopted to ensure the cohesion of the industry and the uniformity of standards.

The trans-national consortium of 4 SMEs, 5 RTDs and 4 IAGs is drawn from 3 Member States (Italy, Spain, Greece) and 1 Candidate State (Croatia). The composition reflects the international focus of both the skills and resources required for successfully achieving the goals of this project. The proposed development requires a combined level of human resources, equipment and facilities far beyond the capabilities of any of the SME participants alone, or even the combined resources of the proposers without EU support.
The teamwork of the partners involved will be emphasised by the fact that biomolecular experts, dairy farmers and dairy industries will work together with scientific and research centres, which will also contribute experience in technology transfer and technology dissemination. This teamwork improves both the economic and social cohesion of EU. We also envisage that such co-operation will extend well beyond the three year lifetime of the project as the members of the consortium work together to further develop and exploit the results and the technology, as well as to develop new applications.

Several RISKSCRA contributions are indicated in the Table below:

Contribution of Riskscra to:	**Dissemination goals of Riskscra**	**Tools of Riskscra**
Existing procedures for research on scrapie disease	Optimization of procedures to reduce TSE risk	High throughput quality control procedures of PrP allelic profiles
Current experimental approaches to studies scrapie diseases	Identification of a control process in dairy production chain	Critical analysis of existing control for scrapie diffusion
Existing terminology	Achieve a common language	Publication and dissemination of consensus reports and systematic reviews at International level Translation, adaptation and National dissemination of the documents released by the Consortium
Implementation of novel guidelines to dairy production rules and regulations	Spread awareness among IAG technicians of latest version of guidelines	Publication and dissemination of latest version of implemented guidelines to dairy industry specialists
Current healthcare approach	Identify the individual aspects that need improvement Favour an appropriate diagnostic approach in Europe	Systematic review of clinical and experimental data Proposal of analytical controls based on new methodologies and concepts

RISKSCRA project supports the Common Agricultural and Rural development policy whereby in the agricultural sector, and in rural areas, the EU is pursuing balanced economic growth and technological improvement.
The Lisbon Strategy focuses, among other things, on improving education and training, research and development and the promotion

of innovation and sustainability. These are exactly the results that the RISKSCRA project can deliver to the dairy industry. Investing in new technologies is one crucial element to exploiting opportunities for growth and employment in rural areas.

The project will thus contribute to implementing EU policies by promoting competitiveness, standardization, regulation and dissemination of best practices in the area of agriculture and more specifically sheep dairy industry. In terms of research and innovation, the objective of the new research and technology policy is to organise co-operation at different levels, co-ordinate national and European policies, encourage the networking of research teams and increase the mobility of individuals and ideas in order to reinforce European competitiveness.

This project, given its European and trans-national nature, will contribute to such policies by bringing European SME's from different sectors and countries in contact with Research Centres from across Europe. It will also contribute to create an European Research Area, the purpose of which is to establish a border-free zone for research, in which scientific resources will be better deployed to create more jobs and to improve Europe's competitiveness.

Contractors and principal investigators involved

Partner	Partner name	Partner acronym	Country	Principal investigators
1	Istituto Sperimentale Italiano "Lazzaro Spallanzani"	ISILS	Italy	Maria Feligini, Dr Caterina Cambuli, Dr Graziella Bongioni, Dr Natalia Buffoni, Dr
2	Consorzio Tutela Formaggio Pecorino Romano	CTFPR	Italy	Giovanni Galistu Santino Gattu
3	Chios Sheep Breeders' Cooperative "Macedonia"	CHIOSGR	Greece	Zoitsa Basdagianni, Dr Kostas Giannakopoulos Pasxalis Pouliopoulos
4	Consejo Regulador del Queso Zamorano	CRDO-QZA	Spain	Francisca Huertos Manuela Peláez Carlos Palacios Ignacio Almazán
5	Hijos de Salvador Rodriguez S.A.	HSR	Spain	Susana Alonso García Candelas López Vaquero Pedro Mielgo Rubio Dionisio Ferrero Núñez
6	Quesos Campostera S.L.	CAMPOSTERA	Spain	Marta Florez Santiago
7	F.lli Pinna Industria Casearia SpA	FPT	Italy	Gavino Murittu, Dr Giovanni Murru Pierluigi Pinna
8	Agriexport Sardegna Soc.Coop. srl	AGRS	Italy	Luca Cubeddu
9	Istituto Zootecnico Caseario per la Sardegna/Agris Sardegna	IZCS/ AGRIS	Italy	Antonello Carta, Dr Sabina Miari, Dr Caterina Maestrale, Dr
10	Dpto. Produccion Animal, Facultad de Veterinaria, Universidad de Leon	ULE	Spain	Juan José Arranz, Dr Susana Pedrosa Lorena Álvarez Alejandro Morán
11	Animal Science Department, Faculty of Agriculture, University of Zagreb	UZA	Croatia	Ino Curik, Dr Anton Kostelic, Dr Vlatka Cubric Curik, Dr Alen Dzidic, Dr Krešimir Salajpal, Dr
12	School of Veterinary Medicine, Aristotle University of Thessaloniki	AUTHVET	Greece	Georgios Banos, Prof Chrysostomos Dovas, Prof Androniki Psifidi, Ms

2. Dissemination and Use

Results of the Final Dissemination and Use Plan

The exploitation of the results was carried out by the RISKSCRA Consortium using two main approaches. The first approach concerns the internal exploitation while the second approach consists of a direct external exploitation of the project results. Application of the traceability guarantee system was extended from national to international market. In particular, regarding the exploitation of the results, each partner implemented their own exploitation actions to take advantage of both the knowledge acquired throughout the project and its tangible results. The major result consists of "*Guidelines to set up traceability in cheese making factories with associated scrapie risk level*". These guidelines can integrate IAG dairy production rules and regulations on the PrP allelic profile control.
Potential buyers/users of RISKSCRA products are any sheep dairy enterprises interested in implementing safety and commercial strategies or improving the existing ones by complementing them with RISKSCRA control system. These include: (i) producer consortia interested in improving traceability guarantee system along dairy production chain to associate the scrapie-risk to the origin of milk, (ii) breeder organizations interested in selection of genotype resistant to scrapie and (iii) institutions interested in controlling sheep milk related to scrapie-risk. Improving the quality of dairy products on the market increases the confidence of consumers and constitutes a basis for fair competition among dairy industries across Europe, again with added benefits for consumers. In addition, it enhances the European competitiveness in the global market. A further indirect application for breeder associations is the possibility to increase milk value by assuring the reduction of scrapie risk.

The exploitation activities of RISKSCRA consortium consisted of promoting and marketing the tools developed within RISKSCRA. The IAGs and SMEs exploited the Protocols carried out by RTDs to improve and manage their products' safety, thus increasing the consumers' interest and confidence towards ovine dairy products by offering assurance about scrapie risk's control

at *flock* level (breeders) by:
- Bulk milk samples collection for genetic analysis
- Breeding strategy including selection for PrP resistant alleles

at *dairy* level (SMEs) by:
- Differentiation of the milk collection from resistant-flocks
- Certification of cheese

at *Consortia* (IAGs) level by:
- Reference laboratory

- Guidelines for milk differentiation and differentiated payment of milk
- Supporting cheese certification

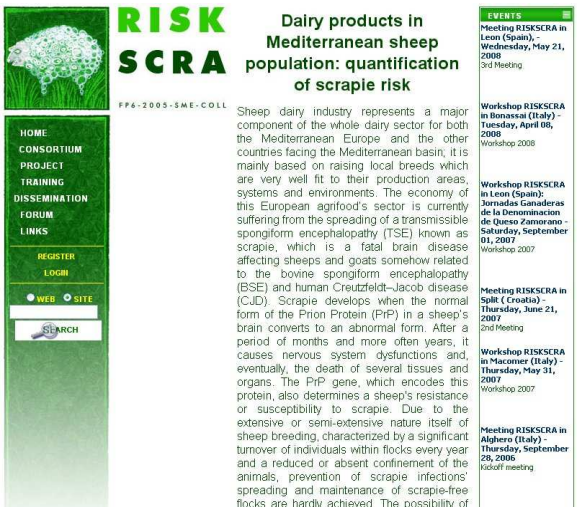
Recently, an article of Konold et al. (2008) and another study from Lacroux et al. (2008) independently demonstrated that Classical scrapie can be transmitted from susceptible ewe to transgenic mice via colostrum and milk. Subsequently the Panel on Biological Hazards (BIOHAZ) was asked from the European Commission to deliver a scientific opinion on the Human and animal exposure risk related to Transmissible Spongiform Encephalopathies (TSEs) from milk and milk products derived from small ruminants (The EFSA Journal, 2008, 849, 2-38). The Panel, according to the previous European Feed Safety Authority opinion (*The EFSA Journal (2006), 382, 1-46*) considered the conclusion of the article of Konold et al. (2008) as valid and recommended to perform research in order to characterise the exposure risk via milk especially for Atypical scrapie and BSE in small ruminants. In this context the RISKSCRA system strengthens the producer consortia position in existing markets improving the dairy product safety by introducing a new analytical approach in the SME production chain. RISKSCRA control system can benefit sheep dairy enterprises interested in implementing safety and commercial strategies or improving the existing ones by complementing them with the capability to quantify and manage the scrapie risk in the whole sector across the Mediterranean area.
We divided the actual outcome into two lines. During the first phase of the project RTDs defined a range of new protocols for RISKSCRA system use, in the second phase we transferred these protocols to IAG and SME technicians by appropriate training courses. The exploitation of project's outcomes started with the direct application of the methodologies at the IAG reference laboratories, which should be able to include the methods and the related parameters in the production control procedures and in the product label. The project results may also bring forth the possibility to reconsider the milk payment criteria, currently based on other criteria (e.g. milk quantity, protein, fat, somatic cells and bacterial content) by adding the absence of scrapie-susceptible genotypes. In the long term, the project scientific and technical outcomes should be made available to many SMEs and applicable to different dairy sectors across the EU.

Major dissemination events

The main RISKSCRA dissemination actions were:

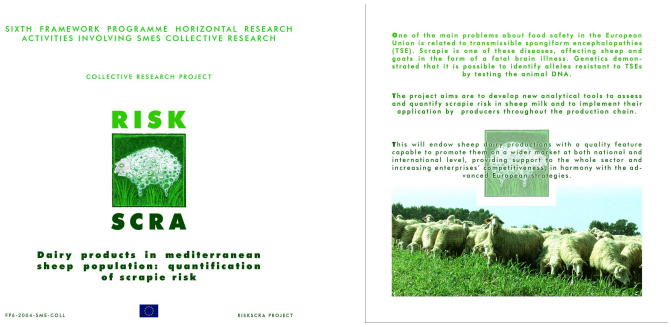
- *RISKSCRA Webpage*: A website was created in the first four months of the project (deliverable 20). Basic information about the project and its consortium was presented. An e-mail address allows users and interested people to ask questions about RISKSCRA and to describe their needs.

www.riskscra.eu.

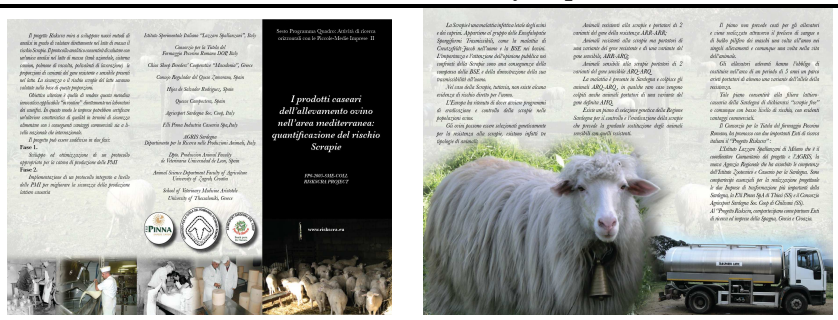


- *Newsletters*: The newsletter is one of the most important project deliverables; newsletters were published according to the delivery dates presented in the project's "Technical Annex" (deliverable 22). This will serve as reporting on scientific and technical activities of the project; it is public and will be used for dissemination.

- *Brochures*: The objectives of the brochures were to disseminate non-confidential information to the countries of Mediterranean area and the INCO countries where dairy sheep is economically significant. These brochures were produced in English and translated to the other languages if necessary. During the first phase of the project, additional brochures were produced in Italian to address mostly local sheep breeders and dairy producers.



. Brochure in English



Brochure in Italian

Brochures in Greek

Brochure in Spanish

- *Workshops and Seminars*: The IAG partners organized workshops that were one-day-events aiming to present the main RISKSCRA results to a broad interested audience including researchers, national authorities and user organizations. The RISKSCRA partners organized seminars where technical approaches and methodology were presented to user organizations with main focus on scope, impact and evaluation of results rather than methodological and technical details.
    - Workshop RISKSCRA in Macomer (Italy) - 31 May 2007
    - Workshop RISKSCRA in Leon (Spain): Jornadas Ganaderas de la Denominacion de Queso Zamorano – 1 September 2007

- In March 2008 a seminar was organized in Thessaloniki, Greece, where AUTHVET (RTD) researches met CHIOSGR (IAG) technicians in order to explain details about scrapie, what problems it causes and what solutions are needed. Also technical approaches and methodology were presented to CHIOSGR technicians. Finally, it was explained how future analytical protocols could be applied for the benefit of the breed. The seminar was held in Greek.
- Workshop RISKSCRA in Thessaloniki (Greece) - 3 October 2008
- In October 2008 a workshop was organized by CHIOSGR for farmers. During the workshop, scientists from AUTHVET presented seminars to technicians, staff and members of CHIOSGR. Moreover, the progress of RTD activities was presented and explained, and the future practical use of results was discussed.

The dissemination activities respected the intellectual property rights agreed in the Consortium Agreement.