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COLLANA DI MONOGRAFIE

VETERINARIA ITALIANA
RIVISTA DI SANITÀ PUBBLICA VETERINARIA

Networking: tool for an excellent research

Atti del 4° Convegno Nazionale sulla Ricerca in Sanità Pubblica Veterinaria

Roma, 6 aprile 2017

26

Ministero della Salute
Direzione generale della sanità animale
e dei farmaci veterinari - Ufficio II

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A public veterinary health without borders to face new emergencies

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Edited by
Marina Bagni, Antonio Petrini, Antonio Lavazza

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Prefazione

Coordino Veterinaria Italiana da cinque anni, un lustro fortunato per partecipare ai cambiamenti radicali dell'editoria scientifica. Gli ingressi dei modelli ad accesso aperto e di business reader-pay rappresentano senza alcun dubbio due delle tappe più importanti di questa profonda metamorfosi. L'accesso aperto, pur se ha reso il mercato più competitivo, ha infatti favorito la diffusione dell'informazione velocizzando il processo di fruizione e ampliando il pubblico dei lettori mentre il modello di business reader-pay, in affiancamento o sostituzione di quello author-pay, ha snaturato la logica della diffusione dei contenuti e ha imposto agli editori una rigorosa autodisciplina nella selezione dei lavori poiché l'aspirante autore è in realtà un potenziale cliente.

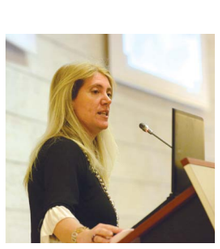
Ho vissuto con orgoglio, timore ed esaltazione gli esordi alla guida della rivista. L'orgoglio per la fiducia accordata; il timore di non essere all'altezza; l'esaltazione nella molteplicità di idee da declinare nel nuovo progetto, nel rispetto del rigore scientifico. In questi anni abbiamo sempre riservato particolare attenzione agli articoli presentati dai colleghi ricercatori degli Istituti, nella personale convinzione che siano testimoni del valore della ricerca italiana.

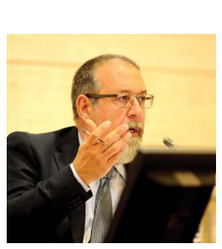
L'offerta di ospitare i Proceedings del IV Convegno nazionale sulla ricerca in sanità pubblica veterinaria era pertanto preziosa e irrinunciabile perché sanciva il compimento di una specifica policy che ha fondato anche sulla collaborazione con il Ministero e con tutti gli Istituti Zooprofilattici la solidità della proposta editoriale della rivista. Tutti i contributi presentati hanno ribadito l'enorme valore della rete a servizio della ricerca d'eccellenza che ritengo indispensabile per affrontare le odierne sfide emergenti. Proprio questa sinergia ha permesso infatti la pubblicazione di contenuti qualificati in tempi brevi nel rispetto del processo di revisione, garanzia scientifica nella presentazione dei risultati alla comunità internazionale. Verrebbe da dire un esercizio di networking editoriale che ha sottolineato il valore di un terreno comune sul sapere condiviso per superare una sfida altrettanto emergente: documentare la ricerca riducendo sensibilmente il divario tra l'accettazione e la visibilità sulle banche dati dei lavori presentati; solo così si può accrescere in competitività e guadagnare la fiducia dei potenziali partners internazionali

per tessere collaborazioni e scommettere sul futuro. Sarà questo il percorso sul quale la rivista continuerà a camminare, con ancora più impegno, insieme a quanti vorranno mettersi in gioco con noi.

Ringrazio pertanto il Ministero della Salute, l'Istituto Superiore di Sanità e gli Istituti Zooprofilattici Sperimentali, che hanno proposto e sostenuto la rivista. Un ringraziamento particolare va a Romano Marabelli, che da sempre ci accompagna e confida nel mio operato, e al gruppo di lavoro di Veterinaria Italiana che da anni supporta e dà concretezza al mio progetto.

Giovanni Savini
Editor-in-Chief





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Marina Bagni e Marco Ianniello
Ministero della salute, Direzione generale della sanità animale e dei farmaci veterinari, Ufficio II

COLLANA DI MONOGRAFIE 26

Introduction

This Conference is a moment of constructive dialogue between the researchers of the veterinary public health sector from the EU countries, and represent an important opportunity to strengthening the link between Science and its practical application for the protection of public health. This is also an important moment for exchange, which will allow us to assess the national research programmes and to identify the weaknesses still existing.

This event is organized by the Directorate General for Animal Health and Veterinary Medicinal Products that I am privileged to lead, in cooperation with the Istituti Zooprofilattici Sperimentali and the Istituto Superiore di Sanità. The 2017 edition is devoted to report the Italian experiences made throughout the national territory, with the support of the Ministry of Health's research funds, which turn into Science and expertise to be used even at international level. The context in which the EU researchers of the veterinary public health are working is actually a virtual meeting and discussion place that we must promote. In fact, this is an international network, through which flows of ideas "without frontiers" meet, intertwine and develop new models, methods and tools for veterinary public health activities.

In this framework, the Italian Istituti Zooprofilattici Sperimentali represent an unique and valuable institution in the international scene. Their contribution in animal health and food safety sectors is undeniable and essential. Moreover, the contribution to scientific research is an added value allowing our country to share knowledge and good practices by the means of cross-border cooperation projects with developing and in transition countries. The political and technical support of this scientific network based on a mutual trust and collaboration means creating a safety and protection network against health and environmental risks and starting relationships and opportunities for Italian agri-food enterprises. The research activity performed by the IZSs and their NRCs (National Reference Centres) plays a fundamental role in studying and developing sanitary strategies for the continuous improvement and implementation of those already established and for the standardization and validation of diagnostic techniques and operational protocols.

All this makes our country an example of excellence in such sector and gives us the chance to establish relevant international collaborations. For about a decade, through the active participation in initiatives financed by the

European Commission like the ERAnet actions and several other EU tools of research coordination, we are making an important effort to build an international coordination of veterinary research. Each Member State has its own characteristics and specialization. If turned into strengths, such diversity represents the way to a solid and shared scientific basis and a correct approach to the protection of public and animal health.

In this perspective, both information and communication are also essential. Conferences like the one we are about to begin constitute a fundamental resource to the Italian Ministry of Health and a necessary initiative through which rebuild communication with its scientists, with those managing public health in the field and with citizens. On the other hand, research institutions operating in the veterinary public health, the Istituti Zooprofilattici Sperimentali and the ISS constantly create and share knowledge that we use in order to reach the objectives of the National Health Service.

Silvio Borrello

Head of General Directorate of
Animal Health and Veterinary Medicinal Products
Ministry of Health

The fundamental role of Research coordination: from EU to a global level

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Keywords
Animal health,
Research coordination,
Gap analysis,
Prioritisation,
Research roadmaps.

Summary
Animal disease risks have increased over recent decades, especially as a result of the increased globalisation of trade and animal product movements, and the consequent transfer of associated fast evolving pathogens. To address these challenges research activities need to be more focused and better coordinated through greater international collaboration, to ensure that the national policies and strategies regarding trans-boundary and other animal diseases (including zoonoses), that protect the sustainability of the livestock sector and animal health industries, are underpinned by efficient and effective research. Coordination of research has evolved from the formation of the European Collaborative Working Group on Animal Health and Welfare research to the establishment of the global International Research Consortium on Animal Health with the aim to coordinate research at international level to contribute to new and improved animal health strategies for at least 30 priority diseases/infections/issues.

Riassunto
Negli anni recenti è aumentato il rischio dell'insorgenza di malattie infettive soprattutto a causa degli effetti della globalizzazione e della maggiore mobilizzazione di animali e prodotti, a cui si associa una veloce evoluzione dei meccanismi patogenetici. Per poter affrontare queste sfide è necessario che la ricerca, anche a livello internazionale, sia ben indirizzata e coordinata affinchè l'efficienza e l'efficacia di essa siano da supporto alle strategie rivolte al contenimento delle maggiori malattie infettive degli animali, incluse le zoonosi, a tutela della sostenibilità della zootecnia. Il coordinamento internazionale della ricerca in sanità animale si è sviluppato molto a partire dalle attività del Collaborative Working Group (CWG AHW) in ambito SCAR fino alla realizzazione di un Consorzio Internazionale della Ricerca in sanità animale (IRC Staridaz) che opera a livello globale per coordinare la ricerca e contribuire alla definizione di nuove e migliori strategie per fronteggiare le priorità sanitarie veterinarie da affrontare nei prossimi 30 anni.

Introduction

Animal diseases can cause serious social, economic and environmental damage, impact on animal welfare and in some cases directly threaten human health. Disease risks have increased over recent decades, especially as a result of the increased globalisation of trade and animal product movements, and the consequent transfer of associated fast evolving pathogens. Research on infectious diseases of animals is poorly funded compared to the human equivalent, despite 60% of all human infectious diseases and around 75% of emerging infectious diseases being zoonotic. However, more could be achieved, even with the current level of investment, through the coordination of this research effort and the sharing of results.

Collaborative Working Group and ERA-NETs

In Europe, despite Animal Health Policy being developed at the EU level and complementary EU initiatives to support animal health research capacity and collaborative research activities, there was little coordination of the national animal health research programmes across Member States to underpin and support these activities and the needs of the European livestock industries.

A Collaborative Working Group on Animal Health and Welfare Research under the EU Standing Committee on Agriculture Research (SCAR) was formed in 2005 with the goal of providing a forum leading to improved collaboration on research prioritisation and procurement, creating the necessary critical mass and focus to deliver the animal health research needs of our policy makers and the European livestock industry. The specific objective of the Collaborative Working Group (CWG) is to develop a durable focused network of national research funders in Member and Associated States of the EU for the purpose of sharing information, coordinating activities and working towards a common research agenda and mutual research funding activities in the field of animal health and welfare, including fish health and welfare, and including those conditions which also pose a threat to human health. With the CWG established to coordinate public funding it was important that similar structures were organised to coordinate industry supported research and provide a platform with which the CWG could interact. The EU Pig Innovation Group (EU PIG) was initiated by the CWG and the European Cattle Innovation Platform (ECIP) was established with CWG support. Both groups have now become Thematic Networks under the European Innovation Partnership on Agricultural Productivity and Sustainability (EIP-AGRI) as EU PIG and EU Dairy respectively. Around the same time as the CWG was formed the European Technology Platform for Global Animal Health was developed, producing a Vision Statement, Strategic Research Agenda and Action Plan. The disease information sheets developed by the associated DISCONTOOLS project are currently maintained and updated with support from CWG partners.

Aware of the success of the CWG in initiating coordination of research activities, the European Commission made funding available for the development of an ERA-Net (European Research Area Network) on infectious diseases of animals and this was taken forward as the EMIDA (Coordination of European Research on Emerging and Major Infectious Diseases of Livestock) ERA-Net. The ERA-Net scheme was developed by the EC to improve cooperation and coordination of research activities carried out at national or regional level in the Member States and Associated States through a) the networking of research activities conducted at national or regional level, and b) the mutual opening of national and regional research programmes.

The overall aim of EMIDA, which ran from 2008-2011, was to build on and accelerate the work of the SCAR CWG in delivering its objectives. It developed systems for sharing information, ran two common calls funding 26 transnational projects with a total value of approximately €43 million and developed a long-term Strategic Research Agenda. Wishing to build on the success of EMIDA, the European Commission decided to provide further support to the area funding a second initiative, the Animal Health and Welfare (ANIHWA) ERA-Net, which ran from 2012-2015 and included animal welfare as well as animal health, funding a further 32 transnational projects, with a total value of approximately €32 million, from three common calls.

Going global

An increasing number of the major disease problems or threats faced by the livestock industry are of a global nature. Global challenges need global solutions and these can only be achieved in the required timeframe through a common and coordinated global research effort. To achieve this, an international forum of R&D programme owners/managers and international organisations (STAR-IDAZ) was established in 2011, with EU funding, to share information, improve collaboration on research activities and work towards common research agendas and coordinated research funding on the major animal diseases affecting livestock production and/or human health. STAR-IDAZ (Global Strategic Alliances for the Coordination of Research on the Major Infectious Diseases of Animals and Zoonoses) was successful in establishing, through its global and regional activities, a network of organisations managing research budgets or programmes in approximately 50 countries that are committed to working together.

Building on the achievements of the EU-funded STAR-IDAZ Global Network, an International Research Consortium (IRC) has been established with a higher level of commitment to collaborate. It aims to strengthen the linkages between and reduce the duplication of global research effort on high priority infectious diseases of animals (including zoonoses), maximise the efficient use of expertise and resources and accelerate coordinated development of control methods. The partners that have, to date, signed the Letter of Intent to participate include national funding bodies/programme owners, research institutes which function as programme owners, charities, pharmaceutical industry and representatives of the diagnostic industry, together having a combined five-year research budget of $2.5 billion.

The agreed aim of the STAR-IDAZ IRC is to coordinate research at international level to contribute to new and improved animal health strategies for at least 30 priority diseases/infections/issues. The deliverables will include candidate vaccines, diagnostics, therapeutic, procedures and key scientific information/tools to support risk analysis and disease control. To achieve these goals Working Groups consisting of researchers will be established for each of the priority topics. Guided by a Scientific Committee, these groups will perform research gap analyses to identify where along the research pipeline, from fundamental to applied science, knowledge gaps exist. The identified gaps will then be used to develop research roadmaps so that research can be focused in a logical manner to address these needs. The Scientific Committee, consisting of independent experts, will present the gap analyses to the IRC partners (Executive Committee) and advise them on how their programmes might be aligned. A Scientific Secretariat, led by Defra and involving CABI, OIE, IFAH and BBSRC has been established to provide the Working Groups with literature reviews and support them in their gap analyses, support the Scientific Committee and Executive Committee logistically and facilitate information exchange within and between all three levels.

The initial identified priorities include Influenza, Foot and Mouth Disease, Coronaviruses, African Swine Fever, Pox Virus Infections, Bovine Tuberculosis, Brucellosis, Mastitis, PRRSV, Porcine Respiratory Disease Complex, Helminths including anthelmintic resistance, Vector-borne diseases and Innovative anti-infective approaches, including alternatives to anti-microbial agents. However, many of the challenges are common to a number of diseases so working groups will also be set up on cross-cutting issues such as Vaccinology (tools and technologies), Diagnostics and Animal genomics/genetics for animal health. The development of an ERA-Net Co-fund on veterinary vaccinology is currently being promoted.

Animal disease research can be expensive, especially where containment facilities are required. We can't all do everything. Coordination of animal health research on priority topics at a global level will help avoid duplication, fragmentation, redundancy and gaps in coverage and therefore ensure that the results from research investment can be rapidly translated to improved disease control strategies, including diagnostics, vaccines and treatments. As well as maximising the impact of existing investment, global coordination of efforts will also help raise substantial additional investment for research in this area.

Figure 1. *Official launch of IRC Staridaz at the EU Conference "Designing the path: A strategic approach to EU agricultural research & innovation", Brussels 27 Jan 2016 presented by Phil Hogan, European Commissioner for Agriculture and Rural Development, Monique Eloit, Director General, World Organisation for Animal Health (OIE) and Professor Ian Boyd, Chief Scientific Adviser, DEFRA, United Kingdom, and Countries delegates.*

Figure 2. *Nairobi partners meeting of International Research Consortium - IRC Staridaz 30th Jan-2nd Feb 2017.*

Understanding the evolutionary dynamics of avian influenza: the impact of cross-border and multidisciplinary collaboration

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Summary

Avian influenza virus represents a serious threat to the poultry industry. In the last decade the increasing number of outbreaks in domestic birds has been in part attributed to the emergence and spread of a highly pathogenic influenza virus of the H5 subtype of Asiatic origin. Since 2005, this virus has been responsible for multiple outbreaks which have greatly impacted on global economy and provoked sporadic cases of human infections. One of the most outstanding characteristics of this virus is its ability to mutate and produce new viral variants. This property results in an unusual capacity to adapt to different environments and escape existing control measures. It is therefore essential to further study the evolutionary dynamics of this virus if we want to understand the factors that contribute to its persistence, co-circulation and spread as this would allow us to develop more efficient monitoring and control programs. Nowadays new sequencing technologies can be used to understand viral diversity and evolution with extraordinary accuracy. In this context, F7 European Union (EU) calls have offered researchers the opportunity to share their knowledge by engaging in multidisciplinary research, which has led to a better understanding of the micro and macro-evolution of avian influenza viruses.

Keywords

Avian influenza,
Evolution,
Network.

Riassunto

Il virus dell'influenza aviaria rappresenta una grave minaccia per il settore avicolo. Nell'ultimo decennio si è assistito ad un'allarmante crescita nel numero di focolai di influenza nei volatili domestici in gran parte legata all'emergere e diffondersi di un ceppo influenzale di sottotipo H5 ad alta patogenicità di origine Asiatica. Questo virus è stato responsabile dal 2005 di molteplici ondate epidemiche che hanno sconvolto l'economia globale e provocato sporadici casi di infezione nell'uomo. Uno degli aspetti più caratteristici di questo virus è la sua capacità di mutare che si riflette in un'abilità inusuale di dare origine a nuove varianti virali, di adattarsi ad ambienti molto diversi e di sfuggire ai presidi disponibili per il suo controllo. Comprendere le dinamiche evolutive di questo virus è quindi essenziale per predirne la diffusione e sviluppare strumenti di monitoraggio e controllo più efficaci. Le tecnologie di sequenziamento di nuova generazione consentono oggi di indagare l'evoluzione del virus ad un livello di risoluzione straordinario. In questo ambito, le attività supportate dal settimo programma quadro della comunità europea hanno offerto agli studiosi l'opportunità di condividere le loro conoscenze in una ricerca multidisciplinare che ha portato ad una migliore comprensione dei meccanismi di macro e micro-evoluzione dell'influenza aviaria.

Avian influenza represents one of the major concerns to animal health. Outbreaks of highly pathogenic avian influenza (HPAI) cause huge economic losses to the poultry industries and directly affect food security and the livelihood of rural areas in developing countries. The incidence of the disease has greatly increased in the last decade compared to the previous 40 years, in particular as a consequence of the spread of the HPAI viruses of the H5 subtype descendent of the H5N1 virus A/goose/Guangdong/1/1996 (Gs/GD), which was first detected in China in 1996. The sporadic cases of human infections caused by viruses belonging to the Gs/GD lineage have also focused the attention on the potential threat to human health. All these factors have increased the awareness of international organizations on the need to create bridges among scientists and develop systems to integrate their knowledge for a better understanding of influenza dynamics. The evolution of the Gs/GD H5 HA since 1996 has been characterized by a wide genetic diversity among circulating strains. Multiple avian influenza A hemagglutinin (HA) clades continue to emerge, evolve and co-circulate in different regions and species, acquiring distinct antigenic properties and zoonotic potentials in their evolutionary process. The fast growing innovations in the field of sequencing technology and associated bioinformatics offer new opportunities to improve our knowledge on virus evolution and to promptly detect strains with pandemic potential. Therefore, bringing together the expertise of bioinformaticians and virologists has rapidly become imperative to expand our understanding of the genetic variability of the Asian H5 strains.

Seventh Framework Programme and Horizon 2020 funded research projects have offered a unique opportunity to foster the collaboration of European and International teams with complementary expertise, paving the way for the development of new research approaches on AI evolution.

A striking example of what can be achieved through an interdisciplinary and global approach to research is well represented by the results of a recent study aimed to investigate the global spread of clade 2.3.4.4 during 2014-2015 (Lycett *et al*. 2016). Between 2014 and 2015, HPAI viruses of the subtype H5N8 (clade 2.3.4.4) and its derivatives spread not only from South-East Asia to Europe, but also to North America, causing several outbreaks in poultry holdings and a loss of over 50 million heads of poultry (Adlhoch *et al*. 2014, Lee *et al*. 2015, Pasick *et al*. 2015). A global analysis of the available genetic, epidemiological, ornithological and trade data from affected countries involving over 30 institutes worldwide and 39 scientists with virological, epidemiological and bioinformatics background was performed under the lead of European researchers supported, among the others, by EU funded projects. The evolutionary and spatial analyses implemented by the newly created "Global consortium for H5N8 and related influenza viruses" on the sequences obtained from the viruses identified in infected wild and domestic birds indicated that the main routes of spread of this virus were most probably via long-distance flights of infected migratory wild birds. In particular, the movement of infected birds seemed to have occurred in two distinct phases, namely i) in spring 2014, from South Korea or from other unsampled locations in the region to northern breeding grounds, and ii) in autumn 2014 from these breeding grounds along the migration routes to the wintering sites in North America and Europe. Not only did the global and interdisciplinary team compare the genetic code of viruses collected from 16 different countries but also performed a qualitative analysis of epidemiological data from outbreak investigations, as well as from reviewed data on import/export movements to evaluate the risk of spread of the virus through international trade. This turned out not to have been one of the main causes of the diffusion of the virus. On the contrary, in most of the outbreaks direct or indirect contacts with wild birds was found to be the most likely route of virus introduction in the poultry sector, which confirmed the results of the genetic investigations. The analyses also highlighted that greater surveillance of wild birds at known breeding areas (circumpolar sub-arctic breeding areas) could help to provide early warning of threats of influenza viruses. The relevance of the findings of the "Global consortium for H5N8 and related influenza viruses" found further confirmation in the recent events involving the 2.3.4.4 clade; in June 2016, a HPAIV H5Nx of the same clade (2.3.4.4) was detected in several waterbird species in Russia and a few months later the virus started to spread westward through migratory birds causing outbreaks in several European, Middle Eastern and African countries.

With the spread of HPAI Gs/GD H5 in large parts of the world, several countries decided to use vaccination as part of their control strategy. Influenza A viruses evolve rapidly in response to selection pressures generated through vaccine protection (Cattoli *et al*. 2011), and the emergence of virus strains, for which existing vaccines are not well matched and offer little protection, continuously challenges the effectiveness of vaccines in the field (Connie *et al*. 2013, Kim *et al*. 2010). A more extensive knowledge of the mechanisms underlying intra-host evolution of avian influenza viruses circulating in vaccinated poultry populations could be of help to formulate and adopt more adequate vaccine strategies. The application of high throughput sequencing platforms in this field has increased the resolution with which these processes can now be studied. In this respect, through the activities of a project funded by the European Commission's Seventh Framework Programme, bioinformaticians and virologists have joined their expertise to explore the impact of vaccine immune pressure on viral population evolution during infection with the HPAI Gs/GD H5 (Milani *et al.* 2017). More specifically, a deep sequencing analysis of clinical samples collected in the framework of an experimental study conducted to assess the protection efficacy of two distinct vaccines against a HPAI H5N1 virus of the 2.2.1 clade was performed. In particular, two experimental challenge groups mimicking different levels of immunity were created to explore the way in which viruses evolve within hosts under different immune pressures. The results obtained by

applying a deep sequencing approach to samples collected from the two experimental groups and to the challenge virus suggested that a suboptimal level of antibody protection may favour the increase of viral population heterogeneity from the early stages of infection and may promote the selection of minority variants, some of which may be involved in antigenic drift.

The results described herein are representative of the powerful achievements that an informed and integrated use of today's technologies may have on our ability to understand, predict and control the evolution of influenza viruses. The greatest challenge we will have to face in the next future is to find a way to motivate scientists to promptly unite and share information to combat the avian influenza virus, which knows no boundaries.

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GOAT-TSE-FREE: European network to investigate genetic scrapie resistance in goats and its application in breeding plans

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Keywords
Scrapie,
Genetic resistance,
Selection,
Goats.

Summary
Breeding programs to control classical scrapie in goats, as it happens in sheep, have not yet been applied, but they would be desirable in this species too. Genetic analysis of the goat PRNP gene revealed many polymorphisms and various European studies have suggested that some of them can modulate scrapie susceptibility in goats as well. The most promising results have been obtained for codon 222, carrying lysine (K), that appeared a promising candidate for selective culling strategies in scrapie-affected herds and for possible breeding programs for TSE resistance. The GOAT-TSE-FREE project, a big European network of labs, carried out all the studies necessary to assess the resistance given by K222. Great attention was put on designing studies able to produce complementary and robust data through collaboration, coordination, exchange of samples, and sharing of the results. Joining the efforts in a network of excellence allowed to produce a big amount of data, in a relatively short time and in an efficient and cost effective way, avoiding also duplications of researches. These data were useful to confirm the association with resistance of K222 allele and its usefulness to control classical scrapie in goats through genetic selection. Besides the publication of scientific articles, the remarkable applicability of the research done by the network gave as outcome the request, by the European Commission, of an EFSA Opinion on genetic resistance to TSEs in goats. The data of the GOAT-TSE-FREE project will be certainly considered in the Opinion and it is likely that they will play a key role in the decision on the future implementation of the breeding programs.

Riassunto
Così come già avviene negli ovini, per controllare la scrapie nelle capre è auspicabile l'applicazione di piani di selezione genetica. L'analisi del gene PRNP caprino ha svelato che esso è interessato da numerosi polimorfismi, alcuni di questi con ruolo di modulazione della suscettibilità alla scrapie. I risultati più promettenti sono stati ottenuti per il codone 222 in cui la presenza di lisina (K) sembra essere associata a resistenza elevata, rendendo questa mutazione un target candidato per l'applicazione di strategie di abbattimento pianificato in mandrie colpite da scrapie e per eventuali programmi di allevamento per la resistenza TSE. Il progetto europeo GOAT-TSE-FREE, che ha coinvolto numerosi laboratori europei, ha svolto tutti gli studi necessari a confermare la resistenza di K222. Grande attenzione è stata riservata alla progettazione dei diversi studi, puntando su collaborazione, coordinamento, scambio di campioni e condivisione dei risultati. Unire gli sforzi in un grande network ha permesso di produrre, in modo efficiente ed evitando duplicazioni, una mole di dati utile a confermare la resistenza di K222. La notevole applicabilità del progetto ha determinato la produzione di pubblicazioni scientifiche e la richiesta della Commissione Europea all'EFSA di una Opinion sulla resistenza genetica alle TSE nelle capre. I dati del progetto GOAT-TSE-FREE saranno certamente presi in considerazione nella Opinion e probabilmente avranno un ruolo chiave sulla decisione di intraprendere i piani di selezione.

Introduction

Scrapie is a naturally occurring transmissible spongiform encephalopathy (TSE) of sheep and goats, characterized by the accumulation of an abnormal isoform (PrPSc) of a host-encoded cellular prion protein in the central nervous system. Natural scrapie in goats was first reported in France, followed by other cases worldwide: a state of the art review on goat scrapie in the European Union, including its epidemiology, was published by Vaccari *et al*. in 2009. Goats are susceptible not only to classical scrapie, but also to atypical/Nor98 scrapie. Notably, the only two confirmed cases of natural bovine spongiform encephalopathy (BSE) in small ruminants were reported in goats in France (Eloit *et al*. 2005) and in a retrospective study in the UK (Jeffrey *et al*. 2006).

Although scrapie is an infectious disease, the susceptibility of sheep is strongly influenced by polymorphisms of the prion protein gene (PRNP). PRNP haplotypes valine/arginine/glutamine (VRQ) and alanine/arginine/glutamine (ARQ) at codons 136, 154, 171, respectively, are associated with high susceptibility to classical scrapie, whereas the ARR haplotype has been linked to resistance. Accordingly, the EU has implemented breeding programs to increase scrapie resistance in sheep populations. In compliance with regulation (EC) 999/2001, as amended, several Member States are now increasing the frequency of the ARR haplotype. A similar approach has not yet been applied in goats, but it would be desirable in this species too, given that scrapie poses a problem for the economy and animal welfare, and that goats, often bred in mixed flocks with sheep, can play a role in maintaining the circulation of scrapie and affect consequently sheep exposure.

The research on genetic resistance to scrapie in goats: from separate studies to a collaborative network

Genetic analysis of the goat PRNP gene revealed many polymorphisms, and various European studies have suggested that some of them can modulate scrapie susceptibility in goats as well. The most promising results have been obtained for codon 222, carrying lysine (K), that appeared a promising candidate for selective culling strategies in scrapie-affected herds and for possible breeding programs for TSE resistance. The association of this allele with resistance to scrapie was first reported in Italy, in two separate case-control studies (Acutis *et al*. 2006, Vaccari *et al*. 2006). Then the same association was reported in field studies in France and Greece (Barillet *et al*. 2009, Bouzalas *et al*. 2010). These relevant results encouraged laboratories, working on TSE genetic resistance in different European countries, to join their efforts in coordinate and collaborative studies to produce all the necessary scientific evidences to confirm the resistance association of K222 and to build a solid background for the future application of breeding programs in goats. As a consequence, in the last decade, both national and European projects were funded thanks to a network of laboratories that grew more and more. In Italy, IZSPLV and ISS joined other European labs in a first European project (Neuroprion). From this, a second European project originated (GoatBSE), involving also IZSSA. The last evolution has been the extensive project GOAT-TSE-FREE, that involved 15 European labs, including IZSPLV, ISS, IZSSA, IZSSI, from 12 Member States. Especially in the GOAT-TSE-FREE project, all the studies necessary to assess the resistance given by K222 were carried out. In doing so, great attention was put on designing studies able to produce complementary and robust data through collaboration, coordination, exchange of samples and sharing of the results. Field studies, experimental studies, bioassays and in vitro conversion studies were carried out in the different labs or in subgroups of labs, considering also the host variability (different breeds) and the pathogen variability (different TSE agents, different scrapie strains). Significant results were obtained, showing a high resistance of K222 versus classical scrapie and bovine BSE, confirming that the K222 allele con be considered as target in breeding programs for resistance to classical scrapie in goats. Moreover the network put in place actions 1) to sensitize and inform breeders about the possibility of selecting resistant goats and 2) to know the frequency of the K222 allele in the different European breeds, a necessary starting point for the implementation and organization of the breeding programs. For these actions, a great support was given in Italy by the Ministry of Health, who coordinated samples collection on the national territory and promoted the connection with breeders. To inform breeders, in Italy a brochure, with all the information regarding K222, was produced and distributed to breeders. Meeting with breeders in different Italian regions were organized as well. All the European partners carried out surveys on their main national goat breeds to estimate the K222 allele frequency. Overall, a low frequency of the K222 allele was found in European breeds, except in Italian Southern breeds, that showed high frequencies of K222, in some of them reaching 30%. These data indicate that breeding programs can be feasible but that they need to be carefully planned in order to increase the resistant population of goats, without losing genetic variability.

In conclusion, joining the efforts in a big network of excellence allowed to produce, in a relatively short time, in an efficient and cost effective way, avoiding duplications of researches, a big amount of data useful to confirm the association with resistance of K222 allele and its usefulness to control classical scrapie In goats through genetic selection. Besides the publication of scientific articles, the remarkable applicability of the research done by the network gave as outcome the request to EFSA, by the European Commission, of an Opinion on genetic resistance to TSEs in goats. The data of the GOAT-TSE-FREE project will be certainly considered in the Opinion and it is likely that they will play a key role in the decision on the future implementation of the breeding programs.

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Microevolution of Anthrax from a Young Ancestor (M.A.Y.A.) suggests a soil-borne life cycle of Bacillus anthracis

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Summary
During an anthrax outbreak at the Pollino National Park (Basilicata, Italy) in 2004, diseased cattle were buried and from these anthrax-foci *Bacillus anthracis* endospores still diffuse to the surface. Frequent isolation of *B. anthracis* from soil lacking one or both virulence plasmids, could suggest a limited soil-borne life cycle of *B. anthracis*. To test this hypothesis we investigated possible microevolution at two natural anthrax foci from the 2004 outbreak conducting biomolecular analysis for different isolates sampled in the near-surface and in the deep layer soil (near the carcass). Thanks to Single Nucleotide Repeats assay it has been revealed the presence of 5 sub-genotypes above all among in the near-surface isolates. Taken together, our results could suggest a limited soil-borne life cycle of *B. anthracis*.

Keywords
Anthrax,
Bacillus anthracis,
Microevolution,
Soilborne.

Riassunto
Durante l'epidemia di carbonchio ematico verificatasi nel 2004 nel Parco Nazionale del Pollino (Basilicata, Italia), diversi bovini morti furono sepolti in una vasta area. È noto che ceppi di *Bacillus anthracis* isolati da vecchi siti di sepoltura possono risultare privi di uno o entrambi i plasmidi (pXO1 e pXO2) e questo potrebbe suggerire un ciclo vitale del batterio in ambiente esterno. Per verificare tale ipotesi e per verificare l'esistenza di una potenziale micro-evoluzione, sono state condotte analisi biomolecolari su ceppi di antrace isolati da siti in cui erano stati infossati animali morti di antrace. Sono stati esaminati numerosi ceppi isolati da campioni di terra prelevati a diverse profondità. Attraverso la SNR analysis è stata evidenziata la presenza di 5 sub-genotipi differenti. La biodiversità dei sub-genotipi era più varia in superficie rispetto a quella registrata vicino la carcassa infossata. Questi risultati potrebbero suggerire l'effettiva esistenza di un *soil-borne life cycle* di *B. anthracis*.

Introduction

Anthrax is a non-contagious infectious disease that mainly afflicts domestic and wild ruminants but other animals including horses, donkeys, pigs, as well as humans are also susceptible to infection. The disease is characterised by a rapid lympho-haematogenous spread and production of toxins leading to internal hemorrhage and bleedings from orifices and death (Turnbull, 2008). Endospores are able to survive in extreme and unfavorable environmental conditions and remain viable in the soil over a long period of time. Besides low interspecies diversity of the *Bacillus cereus sensu lato* group on the genomic level, *Bacillus anthracis* itself can be considered almost as a clonal organism with little, if at all, horizontal gene-transfer. Analysis of genomic data revealed variations mainly in Variable Number of Tandem Repeats (VNTR), Single Nucleotide Repeats (SNR) and Single Nucleotide Polymorphisms (SNP). In Italy, anthrax tends to occur where infected animals have been buried in the past. In the region of Basilicata in southern Italy, anthrax outbreaks are typically isolated, self-containing, and involve unvaccinated herbivores. During the summer of 2004, because of favorable weather conditions at "Pollino National Park", a larger anthrax epizootic occurred. The affected area comprised 13 settlements and involved animals from 41 farms over an area of about 900 km^2, with a livestock population of about 7,000 cattle and 33,000 sheep or goats. Several animal carcasses were buried on high-altitude pastures. From these infective bodies massive amounts of *B. anthracis* endospores were released into the surrounding soil, contaminating the surface above the burial site (Fasanella, *unpublished communication*) probably due to diffusion into puddles of standing waters after heavy rainfall (Hugh-Jones and Blackburn 2009). Despite the prevailing theory that in nature *B. anthracis* is an obligate pathogen restricted to a metabolically inactive endospore state outside the host, an early hypothesis (Van Ness 1971) postulated multiplication of *B. anthracis* in "incubator areas" (soils rich in organic matter and calcium with a pH above 6.0 and an ambient temperature above 15.5°C). To test the hypothesis of a soil-borne lifecycle of *B. anthracis*, analysis of the genetic diversity among strains isolated from soil samples taken near the carcass (200 cm depth) and of near-surface derived isolates (from 5 cm to 100 cm depth) were conducted at three documented (Fasanella, *unpublished data*) anthrax carcass burial sites within Pollino National Park.

Material and methods

Collection of soil samples and isolation of *Bacillus anthracis* from soil samples

Soil samples for this study were collected in May 2014 at Pollino National Park situated in the Basilicata region in the South of Italy. Three different sites A, B and C were chosen. Three individual spots were selected for sampling from each of the three burial sites. Soil samples were taken with a soil auger from 5, 40, 50, 60, 70, 80, 90, 100 and 200 cm below the surface. The Ground Anthrax Bacillus Refined Isolation (GABRI) was performed as described by Fasanella and colleagues (Fasanella *et al.* 2013).

Extraction of DNA, PCR, canSNPs, MLVA 15-loci, SNRs

For the isolation of DNA from inactivated bacteria suspensions, DNeasy Blood and Tissue Kit (Qiagen) was used. Real-time PCR for detection of dhp61 (chromosomal markers), capC (pXO2) and pagA (pXO1) were performed. For canSNPs typing of the *B. anthracis* isolates Mismatch Amplification Mutation Assays (Melt-MAMA) were performed (Birdsell *et al.* 2012), then were performed Multi locus VNTR (variable number of tandem repeats) analysis (MLVA) 15-loci. To determine the sub-genotypes 4 loci (HM1, 2, 6 e 13) were analyzed through Single Nucleotide Repeat (SNR) analysis (Kenefic *et al.* 2008).

Whole genome sequencing and (HRM-SNP)

Whole genome sequencing of DNA of *B. anthracis*-isolates from Pollino National Park was carried out with the Ion PGM (Life Technologies). For this, library preparation was performed according to the Ion Xpress Plus Fragment Library Preparation Guide (Life Technologies) using a sonication method involving the Bioruptor system UCD-200 (Life Technologies) and subsequent end-repair (Life Technologies). Sequencing output files were mapped to the genome *B. anthracis* Ames ancestor [GenBank entries: chromosome: AE017334.2; pXO1 AE017336.2; pXO2: AE017335.3) using bowtie2 (Langmead and Salzberg 2012)]. In order to validate characteristic SNPs identified by whole genome sequencing in Pollino isolates and to determine the distribution of these SNPs in the other isolates, high-resolution melt (HRM) PCR assays were designed surrounding the SNP regions. To verify results from SNR- and SNP-typing Sanger sequencing was conducted.

Results

All isolates retrieved by the GABRI method were identified as *B. anthracis* by diagnostic real time PCR on chromosomal markers dhp61, pagA (pXO1)

Table I. *Distribution of sub-genotypes of* Bacillus anthracis *in relation to the depth of the soil.* It can be observed that the greater variability is present in the upper layers.

SITE A (99 isolates)								
5 cm	**40 cm**	**50 cm**	**60 cm**	**70 cm**	**80 cm**	**90 cm**	**100 cm**	**200 cm**
33 isolates	4 isolates	11 isolates	15 isolates	7 isolates	5 isolates	2 isolates	16 isolates	6 isolates
30 subGT1	3 subGT1	11 subGT1	14 subGT1	7 subGT1	5 subGT1	2 subGT1	15 subGT1	6 subGT1
3 subGT3	1 subGT3		1 subGT4				1 subGT4	
SITE C (103 isolates)								
5 cm	**40 cm**		**60 cm**			**100 cm**	**150 cm**	**200 cm**
53 isolates	24 isolates		5 isolates			15 isolates	3 isolates	3 isolates
46 subGT1	20 subGT1		5 subGT1			14 subGT1	3 subGT1	2 subGT1
5 subGT2	2 subGT2					1 subGT3		1 subGT2
1 subGT3	2 subGT4							
1 subGT5								

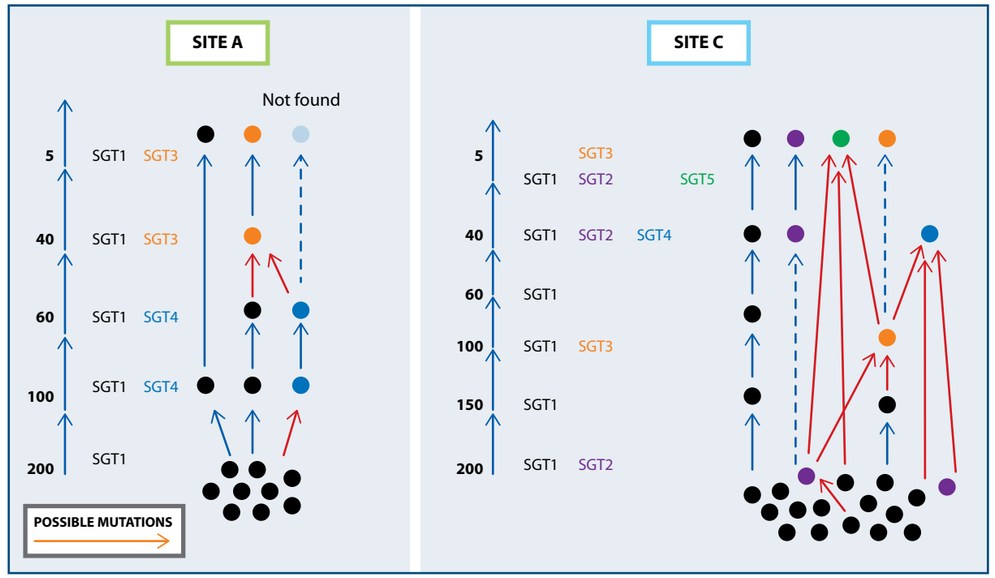

Figure 1. *Graphic representation of the microevolution of* Bacillus anthracis *in burial sites examined in this study.*

and capC (pXO2). Notably, one isolate was found to lack virulence plasmid pXO1. No *B. anthracis* could be isolated from soil samples of site B. All isolates belonged to the A. Br. 011/009 canSNP group. 114 randomly chosen soil isolates were screened for variations in their 15 MLVA markers and all isolates were assigned to the MLVA cluster A1.a. According to their SNR profiles, each isolate was assigned to a SNR-specific sub-genotype (SGT1-5). The majority (182 isolates) belonged to SGT1 (Table 1). Among 99 colonies in SITE A, thanks SNR analysis, were identified SGT1 as dominant within all soil samples, 4 out of 99 belonged to SGT3 present in 5 cm and 40 cm soil layer, 2 out of 99 belonged to SGT4 identified in 60 cm and 100 cm soil samples. In SITE C the SGT1 was the dominant sub-genotype in all soil samples, while 8 out of 103 isolates belonged to SGT2 (5 belonged to 5 cm soil samples, 2 to 40 cm, 1 to 200 cm), 2 out of 103 isolates belonged to SGT4 and they were identified in 40 cm depth and 1 isolate with SGT5 belonged to 5 cm sample. The great abundance of isolates is within 5 cm soil samples. Sub-genotypes variations too are more in the near surface soil rather than in the near carcass soil. This is a resume to follow how the possible mutations occur. In site A, within 100 cm, a new SGT4 appears and could be the result of a mutation of the previous SGT1. The same situation is present in 60 cm depth while in the soil sample of 40 cm a new SGT is revealed: SGT3. Within site C the soil sample probed at 200 cm depth revealed the SGT1 as dominant. At 150 cm just SGT1 was recovered. SGT3 has resulted with SGT1 in a soil sampled at 100 cm with the dominant SGT1 and probably SGT3 was the result of a possible mutations of the underlying SGT1. In 40 cm depth 2 possible mutations occurred, resulting in SGT2 and SGT4. Noteworthy is the situation in 5 cm where the possible mutations showed SGT2, SGT3, SGT5. Whole Genome Sequencing revealed six SNPs in 18 Pollino isolates.

Conclusion

In conclusion near the carcass soil harbors a very high endospores burden. Considering that mutation events of bacteria can occur during host infection, we should find near the carcass different sub-genotypes, contrariwise we found near the carcass the dominant genotype SGT1 while we found a higher diversity going up towards the surface (Figure 1). It is important to consider that endospores reach the surface and accumulate via physical diffusion and during this "ascent" the bacterium could find favorable conditions that promote germination and thus mutation events. The distribution of sub-genotypes in different layers of soil, together with the recognition of loss of pXO1 in a near-surface isolate, clearly suggest a soil-borne life cycle of *B. anthracis*. We hypothesized that a soil-borne life cycle of *B. anthracis* exists at anthrax burial sites leading to microevolution increasing the genetic diversity from the depth of the carcass to the near-surface. However, finding a SNP containing pXO1-negative strain and a strain harboring two SNPs in near-surface soil of burial site C indicates that at least some or limited soil replication takes place in at least one of the two burial sites. Although an unambiguous explanation cannot be provided for the obscure processes of *B. anthracis* in soil environments, this study was the first to address this issue in situ. To obtain reliable information on the genetic diversity at such sites and thus, detect potential changes, it would be crucial to test a sufficiently large subpopulation of the monomorphic pathogen by massive whole genome sequencing which might be an easy feat in the near future. Apart from a genomic approach, the unprecedented detection of the elusive vegetative forms of *B. anthracis* in soil environments would clearly bring the existence of soil-borne life cycle in *B. anthracis* to the light of the day.

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Quality and quantity IZS research assessment: methodological approaches and future directions

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Summary
The costs for Research are increasing in a context of general reduction of funds available to finance it: this situation requires the development of a new procedure in the management of research financed with public funds. An efficient evaluation system is essential and in this article we analyze the financing system of the "Current" Research of IIZZSS by the Directorate General for Animal Health and veterinary medicine (DGSAF) of the Italian Ministry of Health. The management procedure and the methodology for assessment and monitoring of research activities of II.ZZ.SS are described. The history of this process and the annual review of funds allocation is the confirmation that a system based on scientific production is synonymous of the Institute's high performance level. The aim of this paper is to highlight the evaluation activities of IIZZSS, mainly based on bibliometric value of the normalized Impact Factor (NIF). The NIF, together with the evaluation of the Impact Factor linked to the publications of the National Reference Centers, contributes to the correct evaluation of the Institutes.

Keywords
Public research, Animal health, Evaluation.

Riassunto
I costi per la Ricerca aumentano a fronte di una riduzione dei fondi disponibili per il suo finanziamento: ciò impone l'elaborazione di una nuova procedura nella gestione della Ricerca finanziata con fondi pubblici. In questo contesto un Sistema di valutazione efficace è non sono necessario ma indispensabile. In questo articolo analizziamo il sistema di finanziamento della Ricerca Corrente degli IIZZSS da parte del Ministero della Salute, Direzione generale della sanità animale e del farmaco veterinario (DGSAF). Quella illustrata è la procedura di gestione e la metodologia adottata sia per la valutazione che per il monitoraggio delle attività di ricerca che la DGSAF finanzia agli II.ZZ.SS.. La storia di questo processo e la revisione annuale della assegnazione dei fondi conferma come un sistema fondato sulla produzione scientifica sia sinonimo di elevati livelli di performance dell'Istituto. Obiettivo del lavoro è mettere in evidenza il punto di forza dell'attività di valutazione degli IIZZSS, basato principalmente sull'indice bibliometrico dell'Impact Factor normalizzato. Questo, insieme alla valutazione dell'Impact Factor tracciante ovvero legato alle pubblicazioni dei Centri di Referenza Nazionale nella propria area tematica, concorre alla buona valutazione dell'attività degli istituti.

The Directorate General for Animal Health and Veterinary Medicinal Products (DGSAF) of Italian Ministry of Health is involved in the progress, financing, and management of high quality biomedical and health research, with the purpose to plan activities with the aim to improve the health status of the overall population.

The Italian legislation (Legislative Decree n. 502/1992) states the concept of "sanitary research" and its dual meaning: operational and knowledge-based. Over the years, the sanitary research has become a fundamental activity of the National Sanitary Service (SSN) with the broad-ranging aim to contribute to the citizen health and quality of life, rather than just supporting scientific and technological progress, as in the first declaration. To date, in agreement with the Italian Regions, the Ministry of Health provides a National Program of Sanitary Research (PNRS) that identifies the strategies, the funds allocation, and the areas of interest. The performance-based funds allocation system is divided into "current research" and "finalized research": the "current research" funds are devoted to the IRCCS and the IIZZSS in their relevant operating areas; to the "finalized research" can access also other institutions such as ISS, Regions, INAIL and ANSSR.

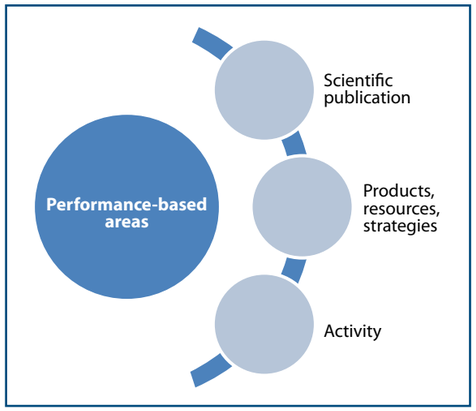

Figure 1. *Key areas for the assessment IIZZSS research.*

It is important to highlight that the Ministry of Education, University and Research (MIUR) is competent for the fundamental research – also called pure research –, while the Ministry of Health is responsible for the knowledge production with an experimental and practical application on sanitary aspects. At the international level, the recent document describing Europe 2020 plan - EU's growth strategy (EC 2014) focuses on a common action to face the challenges of globalization: the creation of national infrastructures for research concurring at pan-Europe level, the clustering of projects, the investment on the human resources are technical and cultural reactions to face the crisis (Messori and Bagni 2015).

In Italy, in the field of sanitary research, a national coordination has been created identifying priority, development, and innovation. At the same time, a technical sanitary committee (CTS) provides the rules and verifies the research results.

Thus, since 2003 the IIZZSS RC funding allocation is decided on the basis of a set of indicators (IZS evaluation grid) and the relevant score. Every three years, the IZS evaluation grid is reexamined, in order to maintain a dynamic approach to IIZZSS evaluation based on a weighted matrix and considering three topics:

- scientific production (article published by researcher and by National reference Centre, dissemination of outputs) 55%;
- products, resources, and strategy (networking, national and international collaboration, participation to European Projects) 30%; and
- activities (National References Laboratories, Project activities and results, Learning activities, support to the academic learning courses – thesis, master) 15%.

Over the years, the performance-based funding system has deeply changed. The "value" of scientific publications has increased from 20% of 2003 to 55% of 2016 [using the international assessment system and the recent development of bibliometric techniques as objective quality indicators

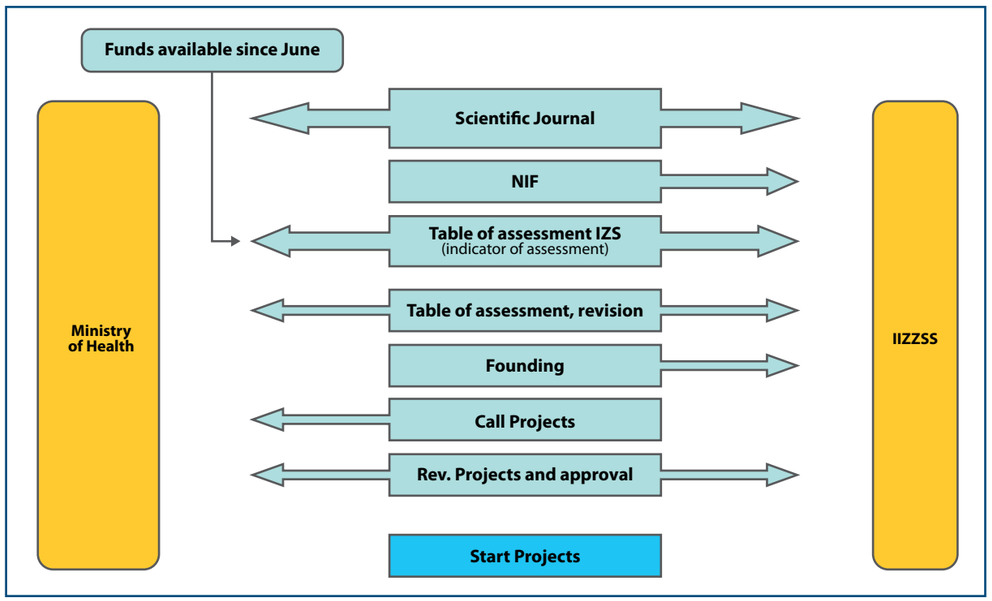

Figure 2. *Workflow of RC assessment 2016.*

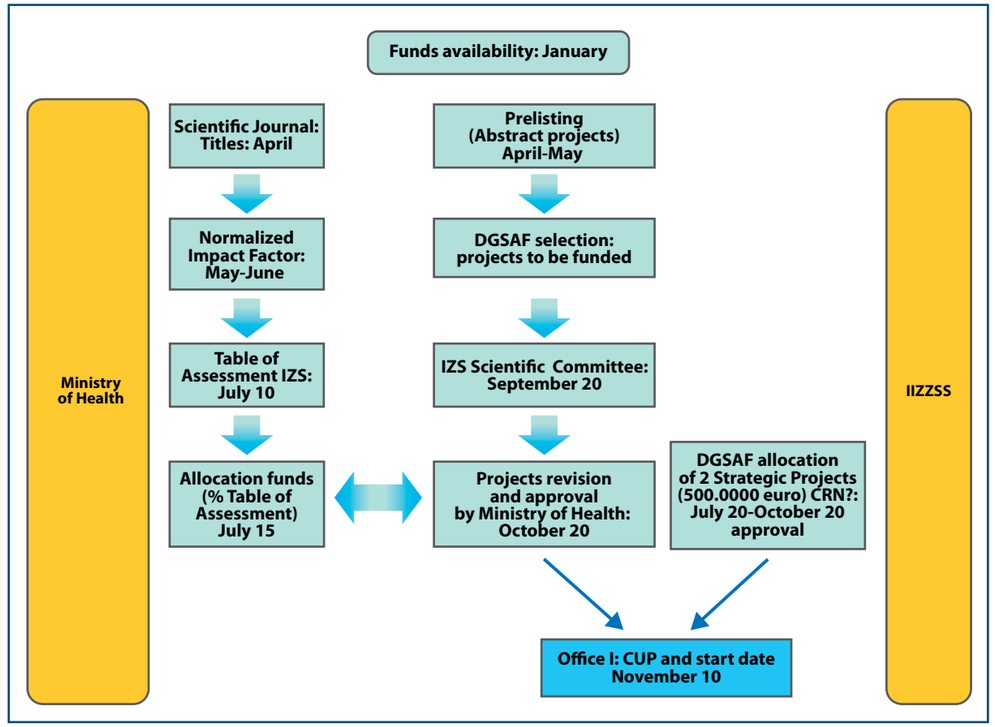

Figure 3. *Workflow Current Research (RC) 2017 assessment.* The goal is start the projects in the referring year of funding.

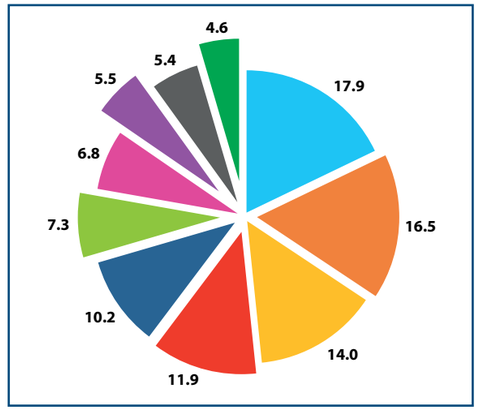

Figure 4. *IIZZSS/RC Funds 2016.*

(Todeschini and Baccini 2016)]. In contrast to the controversial events that occurred in the VQR Italian Research Assessment Exercise (Franceschini and Maisano 2017, Piazzini 2017), the CTS has adopted a Normalized Impact Factor (NIF), that represents an adjusted method for calculating the citation rate of biomedical journal. Also if it is well-known that NIF scores vary between disciplines and these differences cause unfairness in the evaluation of research institutes, the main advantages of relying on NIF include the extension of the index to inter-discipline journal comparison and the stability of normalization constants during years (Pudovkin and Garfield 2004, Owlia *et al.* 2011). In order to calculate the scientific production, also no-IF journals with an editorial board and a peer review system are considered; to these journals a value of 0.1 NIF are given. In Figure 2 we show the workflow of RC assessment / General Secretariat, Directorate General for Animal Health and Veterinary Medicinal Products until 2016. From the year 2017, on the basis of a new annual management procedure of research of DGSAF, the funds will be available on January of each years (the previous years it was on June); consequently, the start of the projects is foreseen for the end of the same year. The differences in founding of IIZZSS is than resulting from a meritocratic and transparent methodology, and constitute a drive for each Institute to improve investments and programming the research in order to gain a better position in the rank list. In this regard, we would highlight the importance of endorsing a robust methodological approach that emphasizes the study of complex systems and the use of foresight methodologies applied to veterinary public health management (Messori *et al.* 2017). Every three years the veterinary DG re-adapt the list of research topics on animal health, welfare and food safety on the basis of national needs and suggestions orientation coming from the international strategic research agendas (OIE, FAO, WHO and EFSA). Finally, the setting out key concepts are to stress the importance of networking and the "One Health, One Medicine, One Knowledge" paradigm (Craddock and Hinchliffe 2014).

A special thanks to Antonella Lonigro and Manuele Pecci for their contribute on the regular updating of IIZZSS' database.

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The innate immune response to non-infectious stressors: human and animal models

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Summary
The achievement of high production levels leads to a greater difficulty of farm animals to adapt to the environment. This translates into occurrence of multifactorial diseases and use of drugs. A new conceptual framework must underlie the disease control strategies in this area. This implies the recognition of the innate immune system as a foundation of environmental adaptation after exposure to both infectious and non-infectious stressors. In addition to microbial stressors, the non-microbial ones are recognized because of common signalling pathways based on Damage-Associated Molecular Patterns (DAMPs) and *de novo* expression of diverse stress proteins on the cell surface. Therefore, sensing, signalling and effector mechanisms of the innate immune system are remarkably similar for both infectious and non–infectious stimuli, albeit differently modulated. Disease may be the final outcome in humans and animals in established models of innate immune response to non-infectious, physical, chemical, metabolic and psychotic stressors. The timely investigation of these coping responses by means of proper clinical immunology and chemistry parameters can be the basis of successful predictive and prognostic approaches. The outlined conceptual framework can be the foundation of a successful "One Health" approach and conducive to fruitful links between pre-clinical and clinical research centres.

Keywords
Environment,
Adaptation,
Innate immunity,
Disease,
Animals,
Humans.

Riassunto
Il raggiungimento di elevati livelli produttivi implica una maggiore difficoltà di adattamento degli animali all'ambiente d'allevamento, che determina l'insorgenza di malattie condizionate e l'uso di farmaci. Una nuova cornice concettuale deve essere alla base delle strategie di controllo delle malattie in questo settore. Ciò implica il riconoscimento del sistema immunitario innato come componente fondamentale di adattamento ambientale a stressori infettivi e non infettivi. Oltre agli stressori microbici, quelli non-microbici sono riconosciuti a causa di vie di segnalazione comuni basate su Damage-Associated Molecular Patterns (DAMPs) ed espressione *de novo* di diverse proteine dello stress sulla superficie cellulare. Pertanto, rilevamento, segnalazione e meccanismi effettoriali del sistema immunitario innato, pure diversamente modulati, sono molto simili per gli stimoli infettivi e non infettivi. La malattia può essere il risultato finale nell'uomo e negli animali di risposte immunitarie innate non controllate a stressori fisici, chimici, metabolici e psicotici. La valutazione tempestiva di queste risposte di adattamento tramite adeguati parametri di immunologia e chimica clinica può essere alla base di efficaci procedure diagnostiche predittive e prognostiche. Il quadro concettuale delineato può essere il fondamento di un approccio "One Health", atto a stimolare collegamenti fruttuosi tra centri di ricerca pre-clinica e clinica di medicina umana e veterinaria.

Introduction

The concepts outlined in this manuscript are detailed and explained in a recent "One Health" book, which describes the correlations between the innate immune response to non-infectious stressors and the onset of different diseases in humans and animals (Amadori 2016).

The achievement of high production levels implies a greater difficulty of farm animals to adapt to the environment. This translates into occurrence of multifactorial diseases and use of drugs, which is of major concern in terms of food safety. These disease events arise from failure of environmental adaptation of farm animals following an overload of the homeostatic regulation systems and serious breaks of the physiological balance. In a global view, healthy subjects experience a condition of "sterile inflammation", i.e. an inflammatory response to the complex of microbial, physical, metabolic and psychotic stressors associated with their environment. These stressors are able to elicit an inflammatory response of the innate immune system on the basis of two distinct markers of exposure: 1) abnormal accumulation in the extracellular space of cell damage products (Damage-Associated Molecular Patterns, DAMPs); 2) neo-antigens on the cell surface (stress antigens). The inflammatory response is particularly supported by myeloid leukocyte cells (granulocytes and monocytes), but other tissues and cell types may also be involved. Such a response aims to restore homeostasis of the subject after exposure to diverse environmental *noxae*. At the same time, the immune system exerts a fundamental control over the inflammatory process, to prevent tissue damage and immuno-pathological phenomena, as well as an excessive expenditure of metabolic energy due to inflammatory processes. Such a metabolic cost is partly due to the energy expenditure of leucocytes and partly to the reduced efficiency in the use of energy (Krebs cycle with oxidative phosphorylation) in the presence of an ongoing inflammatory response. These can be defined as quantitative changes of a baseline profile of "homeostatic inflammation". This means that the body produces under steady-state conditions low amounts of inflammatory mediators such as free nucleic acids and fatty acids, to determine a state of ready responsiveness and efficiency of the innate immune system. In particular, this underlies the well-known phenomenon of constitutive expression of different interferon molecules in healthy subjects, in the complete absence of microbial infections. However, if the secretion of inflammatory mediators runs out of control and reaches high levels, the homeostatic control systems may be inadequate and the host may enter the stage of "irreversible inflammation." This is a very dangerous condition, since it predisposes to tissue damage and the onset of diverse disease conditions, within which one can highlight or not a microbial component.

Non-infectious stressors

The innate immune system can recognize a large array of metabolic changes in tissues and organs like acidosis, osmolarity changes, hypoxia, accumulation of Reactive Oxygen Species (ROS), alterations of the energy status (ATP/AMP ratio), lack of amino acids, increase of non-esterified fatty acids (NEFA) (Amadori 2016), as shown in Figure 1. This outlines the major role of the signaling pathway consisting of phosphoinositide 3(Pi3) -kinase, Akt and mechanistic target of rapamycin (mTor), which is a key regulator of innate immune responses to environmental stress. Among Mitogen-Activated Protein Kinases (MAPK), p38 plays a crucial role in the regulation of mTor activity. p38 can be activated by Toll-like Receptors (TLR) ligands, cytokines and, most important, by diverse physicochemical, non-infectious stress signals. p38

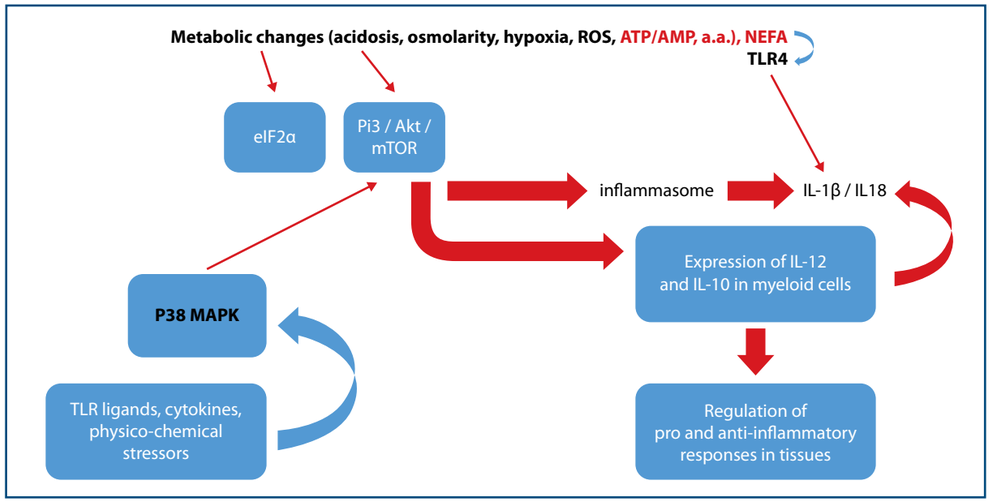

Figure 1. *The innate immune system can sense metabolic stress as a result of fundamental microenvironment changes and tissue damages.* The Pi3/Akt/mTOR and eIF2α pathways play a major role in conveying information to the innate immune system. Among products of metabolic stress, non-esterified fatty acids (NEFA) can directly signal through Toll-like receptor 4 (TLR4).

and Pi3-driven signals coordinately act on mTor to regulate the expression of IL-12 and IL-10 in myeloid immune cells (Amadori 2016). Accordingly, abnormal inflammatory responses and activation of the innate immune system (cytokines, acute phase responses) can be detected in high-yielding dairy cows submitted to the metabolic stress of lactation onset. Truck transportation and early weaning induce a Type I interferon response in cattle and pigs, and heat stress can induce an increase of serum IL-4, IL-6 and TNF-α, as well as a significant decrease of serum IFN-γ levels over the heat stress period (Amadori 2016). These data are fully in agreement with previous findings in humans after traumatic and burn injuries, which confirm a major down-regulation of the T helper (Th)1 and an up-regulation of the Th2 response. The innate immune response to endocrine disruptors is a fascinating issue, largely investigated in fish models, because the fish immune system is a potential target for environmental endocrine disruptors. Endocrine disruptors give rise to oxidative stress with accumulation of ROS, as shown e.g. in a Zebrafish model of exposure to atrazine (Amadori 2016). ROS cause the release of DAMPs and relevant innate immune responses. Mycotoxins can also cause oxidative stress (Amadori 2016) and release of pro-inflammatory cytokines (IL-1 beta, IL-6 and TNF-alpha), and acute phase proteins like haptoglobin and serum amyloid A.

Disease models

The aforementioned mechanisms of response to non-infectious stressors underlie the onset of several production diseases in farm animals, as well diverse disease cases in humans like Systemic Inflammatory Response Syndrome (SIRS), metastatization of tumors, obesity-related diseases, schizophrenia and other psychoses, autoimmune diseases (Lupus Erythematosus, Sjögren's Syndrome), pulmonary hypertension, rheumatoid arthritis, Crohn's Disease, Atherosclerosis (Amadori 2016). Innate immune responses to non-infectious stressors are involved in the pathogenesis of all these disease conditions (Amadori 2016).

Predictive diagnostics and prognosis

In humans, cytokines, Acute Phase Proteins (APP) and leukocyte responses have been characterized as predictive and/or prognostic parameters in a wide range of diseases, such as cold or flu syndromes, heat stress, spondyloarthritis. In dairy cattle, both positive and negative APP and cytokine responses are correlated with disease occurrence and subsequent culling from the herd. Hemolytic complement, serum bactericidal activity, lysozyme, haptoglobin were shown to vary prior to, as well as following disease outbreaks sustained by opportunistic microbial agents in pigs (Amadori 2016).

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Antimicrobial Resistant Pathogens at the animal-human interface: the EMIDA-ERA-net applied research on Livestock-Associated MRSA

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Keywords
Livestock-Associated MRSA, Epidemiology, Molecular characterisation, Colonisation, Transmission, Zoonoses

Summary
The General Diagnostic Department at Istituto Zooprofilattico Sperimentale del Lazio e Toscana (IZSLT), National Reference Laboratory for Antimicrobial Resistance, has been involved in the last decade in several international research projects, dealing with animal and zoonotic pathogens, and related antimicrobial resistance issues. The most recent (and ongoing) experiences include partnership in a Research Project within the European Seventh Framework Programme (Ecology from Farm to Fork Of microbial drug Resistance and Transmission, EFFORT, http://www.effort-against-amr.eu/), and the one in the research granted by the European Food Safety Authority, EFSA (call GP/EFSA/AFSCO/2015/01), entitled "Establishing Next Generation sequencing Ability for Genomic analysis in Europe", ENGAGE, dealing with different aspects of classical and molecular epidemiology and the use of more recent molecular and bioinformatic tools such as those related with Next Generation Sequencing technology. Here we report the main results of the (concluded) Research Project within the EMIDA-ERANET (first call for transnational Research Projects) network "Methicillin-resistant *Staphylococcus aureus* lineages in primary productions: multi-host pathogen, spill-over and spill-back between animals and humans?", LA-MRSA.

** Details on results are available in the scientific papers published, funded by the Project grants.*

Riassunto
La Struttura complessa Diagnostica Generale (NRL-AR) presso l'Istituto Zooprofilattico Sperimentale del Lazio e Toscana (IZSLT), ha operato negli ultimi dieci anni in diversi progetti di ricerca internazionale, relativi a vari aspetti di malattie degli animali, zoonosi e antibioticoresistenza correlata. Le esperienze più recenti (tuttora in corso) includono un progetto di ricerca nell'ambito del settimo programma quadro europeo (Ecology from Farm to Fork Of microbial drug Resistance and Transmission, EFFORT, http://www.effort-against-amr.eu/), e quello nella ricerca finanziata dalla European Food Safety Authority, EFSA (GP/EFSA/AFSCO/2015/01), dal titolo "Establishing Next Generation sequencing Ability for Genomic analysis in Europe", ENGAGE; hanno per oggetto diversi aspetti di epidemiologia classica e molecolare e l'uso dei più recenti strumenti molecolari e bioinformatici come quelli correlati alla tecnologia "Next Generation Sequencing". Qui riportiamo i principali risultati del Progetto di ricerca (concluso) nel network EMIDA-ERANET (first call for transnational Research Projects): "Methicillin-resistant *Staphylococcus aureus* lineages in primary productions: multi-host pathogen, spill-over and spill-back between animals and humans?", LA-MRSA.

** Il dettaglio dei risultati è accessibile nelle pubblicazioni segnalate in bibliografia con un asterisco.*

Aims of the Project "LA-MRSA" and partnership

Staphylococcus aureus is a major pathogen causing considerable human morbidity and mortality worldwide, it is a leading cause of infections of some economically important livestock species, and, as a prominent bacterial cause of contagious bovine mastitis, is a major economic burden for the dairy cattle industry. Methicillin-resistant *S. aureus* (MRSA) lineages are cause of healthcare- and community-associated infections, which are

a major burden of disease on a global scale. In the last decade, MRSA Sequence Type (ST)398 has found an ecological niche in pig, cattle and poultry industry, although other MRSA lineages (e.g. ST1, ST5, ST9, ST97, ST130, ST433), have been identified in farmed animals worldwide (Argudín *et al.* 2016). All these lineages are currently termed "livestock-associated MRSA" (LA-MRSA). The Project's aims are to contribute to the better understanding of the epidemiology of MRSA in farm animals by developing improved detection, typing and monitoring methods and to contribute to the assessment of possible control options.

IZSLT, Diagnostic Dept., NRL-AR, coordinated the Project "Methicillin-resistant *Staphylococcus aureus* lineages in primary productions: multi-host pathogen, spill-over and spill-back between animals and humans?", LA-MRSA, within the EMIDA-ERANET (first call for transnational Research Projects) network (Project proposal ranked 1st place). Partnership included Veterinary Public Health Institutions of some EU Member States (DTU-Food, BfR (D), VISAVET, University Complutense (E), CODA-CERVA (B), two Italian Istituti Zooprofilattici (IZS), IZS Lombardia ed Emilia-Romagna, IZS Piemonte Liguria e Valle d'Aosta, and one private company (Congen, D) with previous experience in the development of diagnostics.

Workpackages

The Project was composed of five Workpackages (WP), with 1-4 WPs dealing with applied research/scientific aspects, and WP5 dedicated to management activities and coordinated by the Project leader at IZSLT.

WP1: Descriptive and Quantitative Epidemiology

The WP was intended to assess the diffusion and the relationships of LA-MRSA clones, including ST(CC)398, ST9, ST(CC)1, ST(CC)97, others) in primary productions in the European Union, with particular attention to lineages of human origin circulating in farm animals.

WP2: Genotyping and molecular epidemiology

The WP was intended to:

1. provide genotypic characterisation of MRSA clones obtained by means of population studies or passive surveillance in farm animals (i.e. spa-types, Sequence Types, *SCCmec* cassettes, and the presence of genetic determinants for additional virulence factors, including leukotoxins, e.g. Panton-Valentine toxin, and other virulence factors and toxins);
2. assess the presence of different multi-resistance patterns and its distribution among different MRSA clones and lineages.

WP3: Colonisation and its dynamics

The WP was intended to: 1. explore the possible patterns of diffusion of MRSA colonization within pig holdings (e.g. longitudinal studies within holdings at different phases/ages); 2. study the effects of Cleaning & Disinfection procedures on environmental contamination in different phases (e.g. gestation to fattening pens).

WP4: Development of diagnostics for detection and risk assessment

The WP was aimed at developing innovative real-time PCR based screening consequently to be used as sensitive tools to obtain comparable data on the prevalence and characteristics of LA-MRSA clones. This approach could have been used also in Official Controls and Own checks along the food chain (e.g. at animal/sample/holding level).

Main results

The main results of the Project were disseminated by means of reports, conferences, continuing education activities at both agricultural and veterinary public health level, and most importantly, through peer review journals. A summary of results of WPs 1-3 are reported in this paper.

WP1: MRSA are widespread in animal production in the partner states, although at different prevalences. In most member states and production lines the LA-MRSA CC398 clearly predominates. Unfortunately, the lack of Regulations, or Decisions at EU level for monitoring this zoonotic pathogen has contributed to delays in discussing the opportunity for systematically monitoring LA-MRSA in farm animals in the EU with the aim of providing possible risk management options. From cross-sectional studies and passive monitoring in farmed animals in Italy it was demonstrated that LA-MRSA of three major clones (CC398, CC1, and CC97) are widespread in the most important Italian primary productions, with highest prevalence rates in fattening pigs, although veal calves, dairy cattle, and broiler chickens harbour these multidrug-resistant clones. On the human side, no current common strategy at supranational level on screening policy options is available (e.g. at admission, or risk patients only, admission to ICU etc., and systematic genotyping of isolates). For instance, in Italy reliable information on frequencies of Community Acquired-MRSA clones is not currently available. As a consequence, estimates on prevalence/incidence of LA-MRSA clones in humans and their trends in these countries are currently out of reach.

WP2: Isolates detected in population studies of WP1 were stored for selecting a collection to be characterised by a panel of consensus molecular methods (MLST, *SCCmec* Typing, PFGE, macroarray on hundreds of genetic loci encoding for virulence, superantigens/toxins and antimicrobial resistance genes).

As a general rule, LA-MRSA of the major Clonal Complexes (CC398, CC1, CC97) in the EU countries under study display multi-drug resistance not only towards all beta-lactams (*mecA* or occasionally *mecC* gene), by also towards many other classes of antibiotics used in food animal primary productions, all of which are also Medically Important Antibiotics (including Critically Important or Highly Important ones, according to WHO definitions). Metal resistance, almost invariably present as co-resistance to antibiotics, is another feature of the LA-MRSA clones studied (Argudín *et al.* 2016*). The majority of the isolates carried the gene *czrC* (zinc resistance), usually associated with the carriage of *tet*(K) and a subtype of *SCCmecV*. Thus, as previously observed, zinc resistance co-selects also for beta-lactam resistance (all beta-lactams) and viceversa. The high prevalence of *copB* (copper resistance) in different LA-MRSA with different *SCCmec* cassettes may indicate a plasmid origin. These results suggests that the use of metal containing compounds in animal productions might be an important selection factor for LA-MRSA.

Additionally, beside resistance genes, they carry several virulence and pathogenicity genes and may represent a serious therapeutic challenge in case of invasive infections in humans (Alba *et al.* 2015*, Feltrin *et al.* 2016*, Alba *et al.* 2016*, Argudín *et al.* 2016*).

Most importantly there is genetic evidence of a spill-back of the major LA-MRSA clones to humans, as demonstrated by the very close genetic relationship between isolates detected in animals and humans, which in many cases makes isolates of both sources indistinguishable with the molecular methods employed. However, also a Whole Genome Sequence approach on CC338 isolates (Alba *et al.* 2016*, Larsen *et al.* 2016) demonstrated that human isolates are interspersed and highly closely related to animal isolates in the SNP-based phylogeny. Moreover, a high genetic relatedness of CC1 isolates from Italian cattle herds and humans (Alba *et al.* 2015*) is evident. In this latter case, there is also evidence that isolates of this clone, increasingly associated with mastitis in dairy cattle, have acquired a genetic background which is typical of human-adapted *S. aureus*/MRSA clones (i.e. immune evasion cluster genes, IEC), which facilitates colonisation and possible infection in the exposed human host. Additionally, CC97 is one of the major *S. aureus* in bovines, where it is associated with mastitis: recently the livestock origin of some human pandemic CC97 MRSA lineages has been demonstrated, resulting in two emergent human epidemic CC97 CA-MRSA clones, deriving from livestock-to-human host jumps (Spoor *et al.* 2013). In recent years CC97 has become one of the major MRSA lineages detected in Italian farmed animals. These are multidrug-resistant (MDR) isolates colonizing pigs and causing infection/mastitis in dairy cattle. In this latter clone, only a minority of isolates harbour genes overtly associated with human adaptation (i.e. the IEC genes), while other marker genes clearly point to a bovine origin. It has been hypothesized, based on genetic background and on geographical distribution of positive herds, that it may have originated as a MSSA in dairy cattle and then acquired its MDR traits in the pig host, because of the high selection pressure with antibiotics in the pig industry, and then spilled back to cattle in areas with high densities of both dairy and pig farms (Feltrin *et al.* 2016*).

WP3: Colonisation and its dynamics

a. Experimental colonisation and transmission. An experimental longitudinal study on colonization and transmission with a CC1, spa type t127 *SCCmecV* LA-MRSA, a clone commonly found in Italian pig holdings. (i e. a non-CC398 LA-MRSA) was conducted in piglets, in order to evaluate its capability of colonizing and to be transmitted among piglets. All experimentally contaminated piglets (Group A) resulted colonized and quickly transmitted the experiment strain via direct contact or via environmental contamination to LA-MRSA-negative piglets (Group B). Piglets of both experimental groups, once become MRSA- positive, proved to be persistently positive during all the experiment period (44 dpc, Group A; 42 dpe, Group B), thus demonstrating stable and long-lasting colonization. No selection pressure was exerted through medicated feed or water during the whole duration of the experiment. In conclusion, the results of this study add further knowledge on attitudes of CC1 LA-MRSA to colonize pigs, and on the dynamics of colonization and transmission processes, both suggesting good host adaptation, and should be considered for future risk management options aiming at control or even to eradication of LA-MRSA.
b. Environmental persistence: The aim of the study was to better understand MRSA

environmental contamination in pig holdings in relation to the productive phase and the application of cleaning and disinfection practices (C&D). Dust samples from farrowing crates, weaning boxes, growing and finishing pens were collected in six holdings, from each herd environment prior to and after C&D and cultured for MRSA. The proportion of positive samples prior to C&D was lower in fattening than in other phases. The proportion of positive samples prior to and after C&D was 121/240 (50%) and 46/240 (19%) respectively. In the mixed effects logistic regression analysis both productive phase and C&D were associated with the probability of having a positive sample, although the C&D effect was different in the different productive phases. In particular the effect of C&D was stronger in farrowing crates than in the other productive phases. Although current practices of cleaning and disinfection reduce MRSA environmental contamination, they are likely to be inadequate to its elimination. However, a strict application of hygienic protocols can lead to a marked reduction of MRSA environmental contamination (Merialdi *et al.* 2013*).

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Study of Babesia bigemina *surface antigen immunogenicity for new diagnostic method development and determination of possible vaccine targets*

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Keywords
Animal movements, Diseases modelling, Early detection, Epidemic emergency, Network analysis, Risk assessment, Risk maps.

Summary
Cattle babesiosis is a tick-borne disease transmitted by haemoprotozoa such as *Babesia bigemina* and *Babesia bovis*. The pathology affects cattle worldwide, strongly reducing meat and milk production. Among *B. bigemina* surface antigens, the Apical Membrane Antigen 1 (AMA-1) is one of the most investigated protein for vaccine and diagnostic purposes. This study aimed to the molecular characterization of Apical Membrane Antigen 1 (AMA-1) from several Apicomplexa and to the analysis of its ability to induce IFN-γ production by infected bovine lymphocyte. Bioinformatics analysis showed highly conserved regions among AMA-1 proteins from different Apicomplexa, as the Signal Peptide, the transmembrane domain or the cysteines involved in disulphuric bonds. Peripheral Blood Mononuclear Cells (PBMCs) collected from *B. bigemina* infected cattle *in vitro* re-exposed to *B. bigemina* AMA-1 produced an INF-γ amount almost twice that the uninfected animals. The study allowed obtaining new knowledge about AMA-1 in related organisms and on its immunogenicity in *B. bigemina*. IFN-γ has a key role in the immunity pathway elicited by the host against *Babesia* infection and *B. bigemina* AMA-1ability to induce the production of IFN-γ would suggest its ability to stimulate host immune reaction. These data could be useful to design diagnostic and vaccine strategies in *Babesia* infected animals.

Riassunto
La babesiosi bovina è una malattia trasmessa da zecche causata da emoprotozoi quali *Babesia bigemina* e *Babesia bovis*. L'infezione colpisce i bovini in tutto il mondo, riducendo la produzione di carne e latte. L'Apical Membrane Antigen-1 (AMA-1) è uno degli antigeni di superficie di *B. bigemina* maggiormente indagati a scopo diagnostico e vaccinale. Lo studio mira alla caratterizzazione molecolare di AMA-1 in diversi Apicomplexa e all'analisi della sua capacità di indurre la produzione di IFN-γ in linfociti di bovini naturalmente infetti. L'analisi bioinformatica ha mostrato la presenza di regioni altamente conservate nelle proteine AMA-1 degli Apicomplexa, quali il Peptide Segnale, il dominio transmembrana e le cisteine implicate nella formazione dei legami disolfuro. Le cellule mononucleate del sangue periferico (PBMCs), isolate da bovini infetti da *B. bigemina*, hanno prodotto, in seguito a riesposizione in vitro a AMA-1 di *B. bigemina*, una quantità di IFN-γ circa doppia rispetto a quelli isolati da animali non infetti. L'IFN-γ ha un ruolo chiave nel processo immunitario dell'ospite nei confronti di *Babesia* e la capacità di AMA-1 di *B. bigemina* di indurre la produzione di IFN-γ suggerisce la sua abilità di stimolare il sistema immunitario. Questi dati potrebbero essere utili per prospettare strategie diagnostiche e vaccinali in animali infettati con *Babesia*.

Introduction

Cattle Babesiosis is one of the most common diseases affecting livestock worldwide. It is caused by intraerythrocytic protozoa of *Babesia* genus (Piroplasmida order, Apicomplexa phylum) (Levine 1971). The disease is endemic in Mediterranean countries and in South America and represents a serious limit on livestock productivity. *Babesia bigemina* is a bovine pathogen whose transmission occurs by means of Ixodidae ticks of *Rhipicephalus*

genus. Affected cattle suffer from severe anaemia and haemoglobinuria, fever, ataxia, anorexia and, in severe cases, neurological symptoms and disseminated intravascular coagulation (Bock *et al.* 2004). Several efforts have been directed to vaccine development and more advanced researches are addressed to the study of surface molecules, in order to detect molecules that are conserved in different species of *Babesia* and immunogenic. This would ensure a vaccine effective on a wide scale. The study was aimed to the characterization of AMA-1 in different Apicomplexa and to the analysis of *B. bigemina* AMA-1 ability to stimulate production of IFN-γ, a key cytokine able to induce innate and adaptive immunity, in lymphocytes of cattle naturally infected.

Material and methods

Bioinformatics and sequence analysis

AMA-1 sequences from several Apicomplexa (access numbers reported in Table 1) were aligned using the software Clustal W2.0.10 (Larkin *et al.* 2007) and Bioedit (Tom Hall Ibis Biosciences, Carlsbad, CA, USA). The software MEGA (Tamura *et al.* 2007) was used to calculate the percentage of similarity among each of the analyzed sequences. All results are based on pairwise analysis of the sequences and on the Maximum Composite Likelihood method in MEGA4. To obtain the main predictive features of the protein, the sequences were submitted to EXPASY proteomic server (Gasteiger *et al.* 2003). Each aminoacidic sequence was analyzed for presence of possible signal peptides using Signal P software (Center for Biological Sequence Analysis, University of Denmark, Copenhagen, Denmark), trans-membrane helixes using TMHMM software (Center for Biological Sequence Analysis) and disulphide bonds (DiANNA 1.1 web server – Ferre and Clote 2005).

Table I. *Access numbers of AMA-1 amino acid sequences from different Apicomplexa analysed in the text.* Positions of the signal peptide (SP) and transmembrane helix (TM) are reported.

Organism	Accession number	SP	TM
Babesia bigemina	GQ257738 (ITA1)	30-31	513-531
Babesia bovis	XM_001610993.1	39-40	523-541
Babesia gibsoni	FJ804755.1	32-33	513-535
Babesia divergens	EU486539	30-31	524-541
Plasmodium falciparum	XM_001347979.1	24-25	547-566
Plasmodium knowlesi	XM_002259303.1	21-22	485-507
Theileria annulata	M_949044.1	-	693-715
Theileria parva	XM_761078.1	-	697-719
Toxoplasma gondii	XM_002364710.1	41-42	485-507

B. bigemina AMA-1 production in *E. coli*

AMA-1 codifying region cloned into the expression vector pET160/GW/D-TOPO (Life Technologies Corporation™) was used to transform *E. coli* BL21 Star™ (DE3) cells and the protein was expressed and purified as previously described (Torina *et al.* 2016). Protein extracts were analyzed by SDS-gel electrophoresis following 0.005% Blue Coomassie staining (Coomassie Brillant Blue R-250) (w/v) and quantified by Bradford protein assay.

IFN-γ production by infected bovine lymphocytes stimulated by *B.bigemina* AMA-1

IFN-γ production after *in vitro* exposure to *B. bigemina* AMA-1 was analysed on peripheral blood mononuclear cells (PBMC) from naturally *B. bigemina* infected and uninfected bovines. PBMC were separated by centrifugation of heparinized bovine blood on Lympholyte solution (Cedarlane labs, Canada) and quantified by Trypan blue dye exclusion test. Cells were resuspended (1 x 106 /ml) in RPMI 1640 medium plus FCS 10%, glutamine 1%, streptomycin, penicillin, gentamycin and Hepes (EuroClone, Italy). Cells were stimulated with recombinant *B. bigemina* AMA-1 protein, at different concentrations (140 µg/ml, 14 µg/ml and 2.8 µg/ml). Suitable negative (RPMI without stimuli) and positive (*Staphylococcus* enterotoxin B-SEB 4 µg/ml) controls were used. Culture supernatants were collected and analysed by ELISA for Bovine IFN-γ reagent set (Mabtech, Sweden), following manufacturer's specifications. At the end of the reaction, the Optical Density (OD) was measured at 450 nm. IFN-γ titers were considered positive when they yielded an $OD_{450\ nm}$ value at least twice as high as the negative control supernatant. Titers were expressed as the $OD_{450\ nm}$ value for the lowest antigen concentration and compared between immune and control cattle using an ANOVA test (P = 0.05).

Results

Bioinformatics analysis of *B. bigemina* AMA-1 orthologous proteins in Apicomplexa

Amino acid sequences of AMA-1 orthologous proteins were analyzed from different organisms belonging to several Apicomplexa genera. Sequences analysis showed the presence of several highly conserved regions between the examined organisms. The average evolutionary distance was of 0.869 and 0.921 for amino acids and nucleotides sequences, respectively. Analysis of the predictive

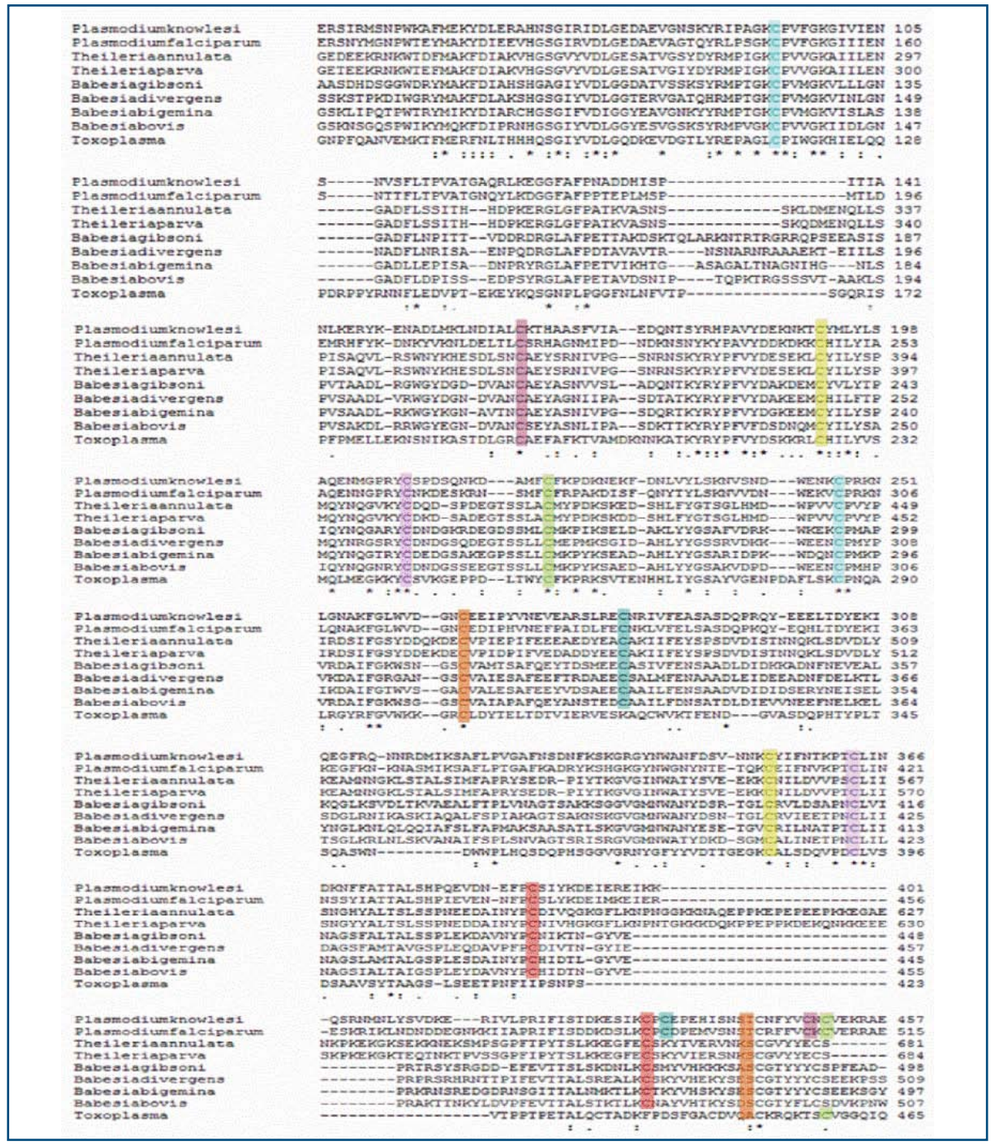

Figure 1. *Multiple alignment among AMA-1 aminoacidic sequences of the analysed organisms.* Cysteines that bioinformatic analysis identified as involved in disulfide bond formation in *Babesia bigemina* are highlighted.

protein showed that all the organisms, with the exception of *Theileria* species, contained a signal peptide addressing the protein to the membrane and all of the species had a trans-membrane helix in the C-terminal region of the protein (Table 1). *B. bigemina* AMA-1 protein contains 16 cysteines potentially involved in disulfide bond formation. The multi-sequence alignment among the AMA-1 proteins of the analysed species showed that almost all these cysteines are conserved (Figure 1), suggesting that they could have a significant importance for the protein structure.

Study of *in vitro* production of IFN-γ in response to stimulation by *B. bigemina* AMA-1

Analysis of *B. bigemina* AMA-1 ability to induce the production of IFN-γ by lymphocytes obtained from infected cattle, showed that supernatants collected

Table II. *Produced IFN-γ (pg/ml) in* Babesia bigemina *positive and negative bovine.*

	SEB (Pos.)	AMA-1 (140 µg/ml)	AMA-1 (14 µg/ml)	AMA-1 (2.8 µg/ml)	RPMI (Neg)
B. bigemina positive bovines	> 500 pg/ml	220 ± 19	105 ± 12	115 ± 10	< 5 pg/ml
B. bigemina negative bovines	> 500 pg/ml	35 ± 4	35 ± 2	35 ± 3	< 5 pg/ml

from lymphocytes obtained by PBMCs from infected animals stimulated with all concentrations of AMA-1 antigens contained an amount of IFN-γ at least double with respect to negative animals (Table 2).

Discussion

AMA-1 proteins have been characterized only in few Apicomplexa and for some of them a role in erythrocyte invasion process has been described (Latitha *et al.* 2008, Mital *et al.* 2005, Montero *et al.* 2009). *B. bigemina* AMA-1 protein showed many common features with other proteins of AMA-1 family (Torina *et al.* 2010). Comparative analysis between the protein sequences of AMA-1 of different Apicomplexa have shown a high degree of conservation among the functional domains of the proteins.

It has been previously reported, IFN-γ has a key role in the immune pathway elicited by the host against *Babesia* infection (Brown *et al.* 2006). The analysis of IFN-γ production following *B. bigemina* AMA-1 *in vitro* stimulation of bovine lymphocytes showed that the production of IFN- γ is significantly higher in samples from infected bovine subjected to *in vitro* stimulation, rather than in cattle that have never been in contact with the parasite, thus suggesting that this cytokine release could be specifically induced by the contact with the antigen. The recovery of a *B. bovis* infection strongly depends by the activation of parasites killing obtained by IFN-γ activated macrophages. In persistently *B. bovis* infected animals, antigen-specificCD4$^+$ T lymphocytes play a central role in the adaptive immune response. Being *B. bovis* and *B. bigemina* phylogenetically related, it can be assumed that a similar mechanism of stimulating IFN-γ production may act, playing an important role in the complex immune system response against *Babesia*. The ability to induce the production of this crucial cytokine would thus suggest its ability to stimulate host immune reaction, and then its suitability for an inclusion in a subunit vaccine.

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The Mediterranean Italian buffalo: research for supply chain sustainability

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Summary
The report describes the activities developed by the CReNBuF to support the buffalo industry through three research projects. These activities focus on biosecurity, welfare and farm products, the results of each projects have been integrated in order to provide practical tools for use by farmers company veterinarians and the NHS. The results obtained can also be applied to other sectors and constitute a working model for the integration of different public bodies, various skills and livestock rearing.

Keywords
Buffalo,
Animal welfare,
Risk assessment,
Traceability,
Information system,
Biosecurity.

Riassunto
Il lavoro descrive le attività del CReNBuF a sostegno della filiera bufalina attraverso tre progetti di ricerca. Il percorso è stato sviluppato ponendo l'attenzione su biosicurezza, benessere e produzioni aziendali e integrando i risultati ottenuti dalle singole progettualità per fornire degli strumenti pratici sia agli allevatori che ai medici veterinari aziendali e del SSN. Le conclusioni possono costituire un esempio esportabile anche su altre filiere e rappresentano un modello di lavoro integrato tra diversi enti pubblici e diverse professionalità anche del mondo allevatoriale.

Material and methods

The Directorate General of the European Commission for Health of Consumers, Food and Veterinary Office, in its report on the mission carried out in 2009 in the Province of Caserta to assess implementation of the program for the eradication of buffalo brucellosis, recommended that the Italian Authority should intensify biosecurity measures in order to pursue the objectives set out in paragraph 1 c) of the Annex to decision 2008/341/CE. Subsequently, a research program was devised with the objective of identifying and implementing appropriate measures to prevent the spread of infectious diseases through the application of management systems in biosecurity, livestock hygiene and animal welfare, environmental protection and preservation. Named, "Program for the biosafety of buffalo farms", this project is complementary to the prophylactic activities implemented by the Veterinary Services. The project covered five main areas:

1. research activities designed to expand knowledge on the spread of infectious diseases, in particular concerning brucellosis, in relation to the specific characteristics of buffalo breeding; and the drafting of guidelines for a biosafety manual;
2. assistance for buffalo breeders through the creation of a task force of pratictioners charged with gathering productive and reproductive data and identifying gaps to be filled regarding welfare and biosecurity conditions, in order to make the necessary managerial and structural adjustments. This action aims to improve knowledge and the applicability of corporate biosecurity plans for farms. Development of a specific checklist for the assessment of biosafety levels;
3. drafting and implementing a specific biosafety manual for application on the farm;
4. the application and testing of a protocol of biotechnology applied to reproduction, in order to limit sexually transmitted diseases;
5. specific training on biosafety, animal health, production and reproduction, through specialized courses for pratictioners and farmers. Calls for the participation of farmers and for the recruitment of freelance operators were issued.

The activities were supported through the nationwide application of the system for traceability of the buffalo industry developed within the ministerial project "Development, validation and verification of the applicability of a computer system to be used for the management of traceability in the buffalo industry" and the scheme for assessing welfare and biosecurity on buffalo farms developed in the project "Ruminant welfare®", the leading unit being the CReNBA.

In the project named "Ruminant welfare®", the CReNBA, as leading unit, enlisted the support of various operating units, particularly the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM), whose National Reference Centre on Water Buffalo Farming and Production, Hygiene and Technologies (CReNBuf) developed a scheme for assessing welfare and biosecurity on buffalo farms. The method chosen for the development of the project was the Risk Assessment Methodology in Animal Welfare suggested by the European Food Safety Authority (EFSA 2017). Building on the CReNBA's work on the welfare of dairy cows (Bertocchi L. et al. 2014), Guidance on Risk Assessment for Animal Welfare of European Food Safety Authority (EFSA 2012), European Welfare Quality® in buffalo (De Rosa *et al.* 2015) minimum regulatory provisions, bibliography, and the support of the expert group, the activity of the CReNBuf started in march 2015.

Specifically, risk assessment in animal welfare was carried out in the following phases:

1. identification of the target population;
2. hazard identification (non-ABMs);
3. identification of the hazard threshold level;
4. identification of adverse effects (ABMs);
5. measurement of adverse effects;
6. evaluation of hazard magnitude;
7. evaluation of adverse-effect magnitude;
8. application of the checklist in a significant number of pilot farms;
9. formulation of an explanatory regulation for the checklist;
10. assessment of data reproducibility by means of statistical analysis;
11. implementation of the checklist on digital media;
12. organization of training courses for evaluators.

The target population was identified on the basis of the experience yielded by the CreNBA's work on dairy cows, the scientific literature on buffalo breeding, the individual experiences of the expert board members, and the 87 farm visits undertaken in the period May-September 2015 by the staff of the CreNBuF. The population consisted of buffaloes raised in loose housing systems, and was divided into the following sub groups: lactation, dry period, heifers and calves. Furthermore, the consequences of hazards on animals and threshold levels regarding positive and negative changes in health conditions were identified and expressed as Animal Based Measures (ABM). The system utilized consists of 83 observations (Items) in a multiple-choice check-list. Each Item was scored according to three choice categories: "unacceptable", "acceptable" and "excellent". In borderline situations, the veterinarian performing the assessment must always bear in mind that the worst condition (unacceptable) and the best condition (excellent) should only be assigned if there is clear negative or clear positive evidence. Regarding hazards, evaluation was performed by using parameters divided into two macro-areas: Area A (23 items) "Farm management and personnel"; Area B (29 items) "Facilities and equipment", and identifying their respective thresholds. Animal-based measures were assessed in point 14 of Area C. Finally, the buffalo farming welfare assessment system was integrated with parameters for analysing conditions of biosecurity (Area E) and the inspection of alarm systems (Area D). The check-list was completed in an average time of 140 minutes. The herds evaluated consisted of an average of 582 head, with a minimum of 130 and a maximum of 1152 animals. The average overall welfare value (0-100% average recorded score) was 70.35%; the values of the specific areas were: Area A 69.13%; Area B 66.50%; Area C 79.94%, Biosafety 56.61% and major risks 57.58%. Legislative non-compliance was recorded in 9.09% of farms, in which visual and tactile contact between the calves was lacking.

In the project named "Development, validation and verification of the applicability of a computer system to be used for the management of traceability in the buffalo industry" an on-line system was developed for ascertaining traceability in buffalo rearing and the production of milk and milk derivatives. The system was submitted to the Ministry of Agriculture and the Ministry of Health, which jointly promoted the enactment of specific legislation for the mandatory application of traceability. This on-line system is now applied nationwide and is managed by the National Reference Centre on Water Buffalo Farming and Production, Hygiene and Technologies, at the IZSM, and by the national agricultural information system (SIAN) of the Ministry of Agriculture, in

applicative cooperation, with contributions by the Agribusiness Quality Department (DQA) with regard to DOP Cheese farms. As described in the decree of 14 September 2014. In the informatics portal (www.tracciabilitabufala.it / www.mipaaf.sian.it), buffalo breeders are obliged to upload the following data to the platform "Traceability of buffalo industry": daily quantities of milk produced by all the lactating buffaloes in the herd and the persons to whom it is supplied. Cheese factories that utilize buffalo milk are required to upload the following information to the platform:

a. the quantities of buffalo milk and other buffalo products , also in frozen form, purchased for the production of processed products, as well as the names of individual suppliers;
b. the quantities of products: Campana Buffalo-milk Mozzarella DOP;
c. the quantities of products: buffalo-milk Mozzarella (non-DOP);
d. the quantities of other processed products resulting from the use of buffalo milk, such as "ricotta", yogurt, "provola", butter, ice-cream, etc.;
e. the quantities of buffalo milk and semi-finished products stocked.

Intermediaries are obliged to upload the following data to the platform:

a. the daily quantities of buffalo milk and other buffalo products purchased, also in frozen form, with the indication of each supplier;
b. the daily quantities of buffalo milk and other buffalo products sold, also in frozen form, with an indication of each recipient.

Both series of the above data have to be uploaded to the platform on a daily basis no later than the second day of the week following their quantification. Furthermore, every month, breeders must submit data on individual animals acquired to the central platform. The software, has a section dedicated to the operators of the service / call centre.

Results

This check-list, which was developed through collaboration between the CReNBA and CReNBuf in the project named "Ruminant welfare®", constitutes an impartial, reproducible, functional instrument based on risk analysis. It enables a numerical animal welfare index to be assigned to each farm, furthermore through the data collected in each Area, provides veterinarians and breeders with tools for improving farm management and structures while respecting the farm's sustainability. The use of this tool will yield a uniform assessment of the level of welfare of buffalo farms, and is preparatory to the development of a Ministerial trademark for animal welfare, which will meet consumers' needs and grant recognition to those farmers who respect the parameters of well-being. The Traceability system developed in the project named "Development, validation and verification of the applicability of a computer system to be used for the management of traceability in the buffalo industry" provides operators with a real-time picture of the management of personal data and alerts, access logs and monitoring of production and sales. These data can be consulted by the inspectors of the Ministry of Agriculture and the Ministry of Health in real time, through the issue of passwords.

The production of 1827 farmers, 518 non-DOP cheese farms, 98 DOP-cheese farms, 43 non-DOP intermediaries, and 14 DOP intermediaries nationwide is currently traced, and 94% of farmers comply with the decree. In the two years since the project was first implemented, no winter milk surplus has arisen, as was the case in previous years. In addition, there farmers' milk revenues have increased and the gap between winter and summer milk prices has narrowed.

These tools have been applied to the "biosecurity program". Further results of this project are the development of a check-list for biosafety in buffalo herds and a on-line farm management program accessible from any device with an internet connection. This program includes the compilation of check-lists, the loading of images and documents, and reporting on all activities, achievements, and progress. The project is currently attended by 350 farms, supported by 56 veterinarians.

Conclusion

The research activities have been implemented throughout the territory country, providing real support for the buffalo industry. The results obtained can also be applied to other sectors and constitute a working model for the integration of different public bodies, various skills and livestock rearing.

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Foresight methods for a SRA on animal health: the experience within the Collaborative Working Group on Animal Health and Welfare Research CWG AHW

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Summary
Since January 2008, three European Research Area networks (ERAnets), were funded in the area of animal health under the European 7th Framework Programme (FP7), EMIDA, ANIHWA and STARIDAZ. These have been realised under the umbrella of the Collaborative Working Group on Animal Health and Welfare Research of the Standing Committee on Agriculture Research. Aim of these ERAnets was to improve the cooperation and ensure an effective coordination of national research programmes on farm animal health and welfare. As a tool for identifying research priorities in the field of animal health, foresights exercises were implemented within these ERAnets, and will be described in this conference. Experts with multidisciplinary background, from the academia, intergovernmental organisation and competent authorities, were gathered from different countries at global level to build a think tank in order to develop a Strategic Research Agenda (SRA) for animal health, guaranteeing a more rational balance of available funding.

Keywords
Animal health,
Foresight,
Research agenda,
Research coordination.

Riassunto
EMIDA, ANIHWA e STARIDAZ sono tutte azioni europee di coordinamento della Ricerca finanziate nell'ambito del 7° Programma Quadro a partire dal gennaio 2008. Queste in particolare, tutte nell'ambito della sanità animale, sono state realizzate sotto l'egida del Gruppo di lavoro in sanità e benessere animale (CWG AHW) del Comitato permanente di Ricerca in agricoltura della Commissione europea (SCAR). Gli Eranets sono strumenti sviluppati con lo scopo di migliorare le capacità di cooperazione e coordinamento dei programmi nazionali di ricerca; quelli qui in oggetto si riferiscono alla salute e al benessere animale delle specie allevate. Gli esercizi di foresight riportati in questa conferenza sono studi previsionali realizzati all'interno degli Eranets per focalizzare le necessità di ricerca in sanità animale. Sono stati utilizzati esperti in varie discipline, selezionati in settori diversi – accademia, organizzazioni governative e autorità centrali –, provenienti da diversi paesi. È stato così possibile dare vita ad un dibattito aperto e partecipativo finalizzato alla pianificazione strategica di un'agenda di ricerca in sanità animale che fornisca indicazioni preziose per un uso più coerente delle risorse disponibili.

The Standing Committee on Agriculture Research (SCAR) and its working groups exist with a mandate to produce durable and focused network of research funders from Member and Associated States of the EU. The Collaborative Working Group on Animal Health and Welfare Research (CWG AHW, Figure 1) is a long-lasting working group of SCAR, 12 years of activity, which provides a forum leading to improved collaboration on research prioritisation and procurement, creating the necessary critical mass and focus to deliver the research needs of our policy makers and at the European livestock and associated industries. It contributes to the preparedness for animal health control and to food safety and security for a future sustainable prosperity.

In the European Union (EU), animal health is funded by a number of national and transnational institutions, often operating independently and being based on local priorities. The vast majority of public research in Europe is still funded at national level (Könnölä and Haegeman 2012). As a

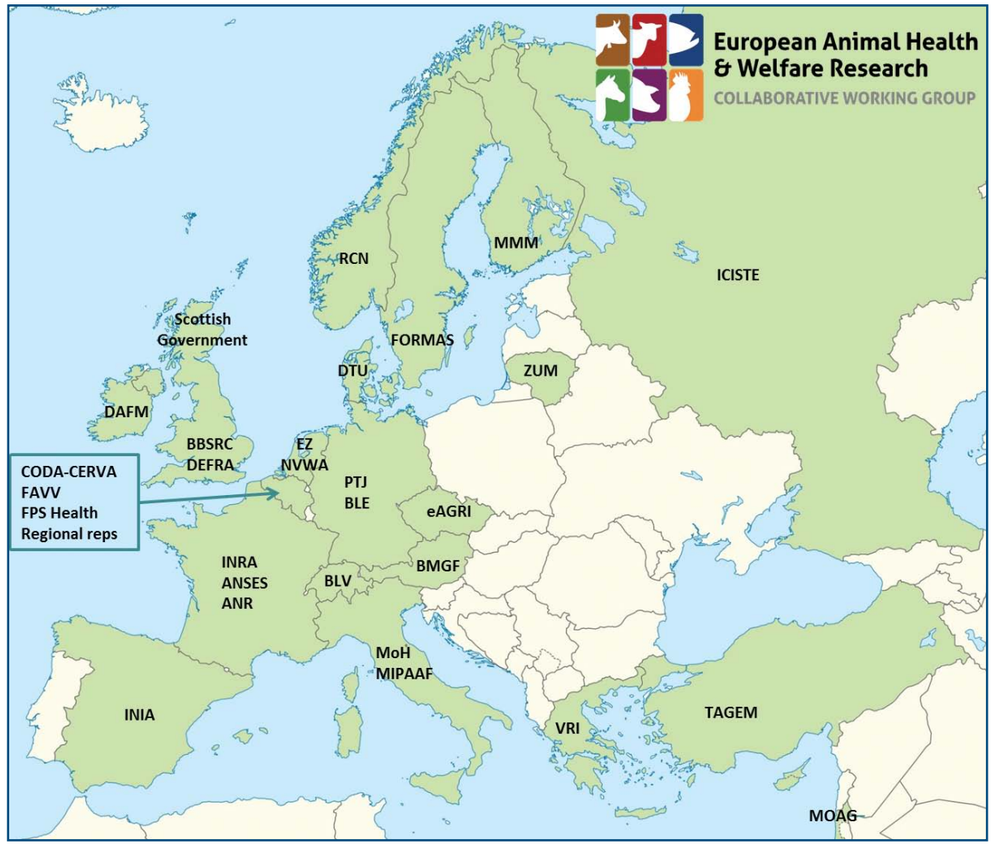

Figure 1. *CWG AHW partners' distribution.*

consequence, research duplication and incomplete availability of research outcomes are a common reality. Also, the lack of long-term planning stimulates the funding of researches to solve problems being already an issue, generating a gap in the availability of appropriate solutions and in facing future threats. The progressive restriction or reduction of public funding and the enhanced need of preparedness make research prioritisation and the prevention of unnecessary duplication fundamental (Gaunaud *et al.* 2012, Cox *et al.* 2012).

The enhanced movements of people and animals across countries, due to globalisation and easing of transport, make the circulation of diseases easier (Wentholt *et al.* 2012). In the whole Europe, and particular in the Mediterranean area, where mild climate represent a favourable habitat to a number of infectious disease and where the Sahara desert is no longer an efficient barrier for diseases coming from southern Africa, the need for a concerted approach to ensure human and animal health is paramount (Messori *et al.* 2017).

Consequently, several coordination actions (ERAnet) have been established in the EU in any field of research and under the umbrella of CWG AHW-SCAR for what concerns animal health and welfare research. This with the aim of improving transnational collaboration between researchers and creating the critical mass and efficient utilisation of expensive facilities necessary to improve competitiveness of European research and assist the creation of a coherent European Research Area. Among those, the EMIDA ERAnet (European Research Area Network on Emerging and Major Infectious DiseAses of livestock) was launched in 2008 and delivered a 10-15 years European Strategic Research Agenda (SRA) on animal health. This represented the first attempt to face the issue of defining research needs in the sector with a participative approach across large part of Europe (EMIDA 2011). Among the final recommendation of the agenda, the need of further updating and detailing of the identified needs was clearly stated.

A further action, the STAR-IDAZ (Global Strategic

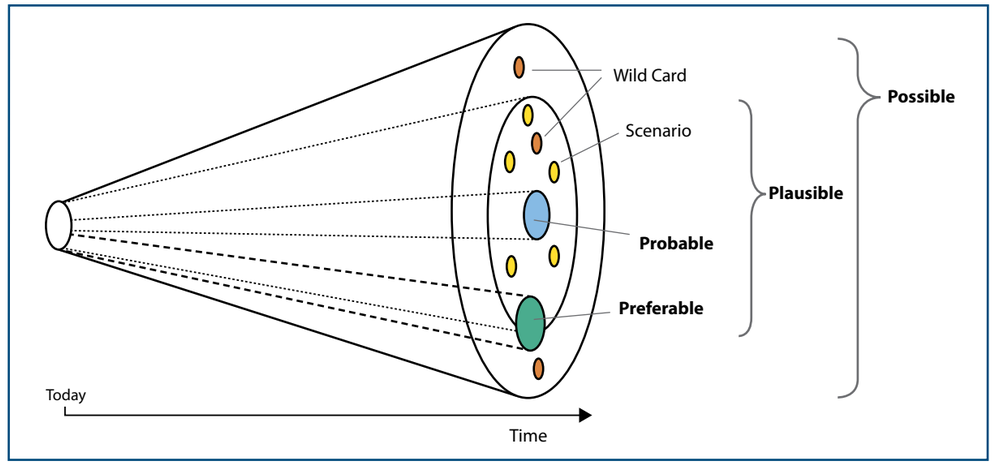

Figure 2. *Foresight studies: prediction is difficult, preparation is critical and possible.* Picture from "The future cone" Voros J. 2001.

Alliances for the Coordination of Research on the Major Infectious Diseases of Animals and Zoonoses) project, was launched in 2011 with the aim of improving coordination of research activities on the major infectious diseases of livestock and zoonoses, in order to help structuring animal health research globally. The building of a global SRA represented a starting point to reach this aim. To get to a common SRA, provisional studies are being gathered worldwide, were available, or designed and realised in a period of time included within 2012-2015.

In 2015, a further foresight exercise has been performed at Mediterranean level, the ForeMed (Foresight project for the Mediterranean area). This provisional study, promoted by the Italian Ministry of Health, aimed at defining research priorities on animal health, including production diseases and aquaculture, for the Mediterranean area as a whole. The SRA which was delivered by the ForeMed finally contributed to the STAR-IDAZ global research agenda (STAR-IDAZ 2015).

Foresights (Figure 2) are increasingly used for both short and long term research and development planning in several fields, supporting a more effective decision making process (Brummer *et al.* 2008, Könnölä and Haegeman 2012). The inclusion of foresights in the framework of ERA-Nets has been shown to support the overcoming of possible barriers, supporting priority setting and sustaining the building of a shared vision (Brummer *et al.* 2008). These effects appeared to be even more marked when these techniques are applied to transnational programming in a non-European context (Könnölä and Haegeman 2012).

The gathered information represents an important milestone to allow the planning of research activities for the next 15 years in the area. In the different studies emerged similar results but evidenced in a different scale of importance due to the geographical area and timespan used in the studies. It is possible to summarize that in any study three main research areas have been identified: Prevention and control, Fundamental Research and Development of disease control tools. Among diseases, vector borne diseases and antibiotic resistance appeared to be the one likely to represent a threat in the future and for which new control and surveillance tools still needed to be developed.

Moreover, the development of transversal researches, merging expertise in different fields, was seen as increasingly important in the future by all the experts. That is why, although the aim of the study was animal health, some research topics which are not classical veterinary topics were listed among the priorities. As an example, the think tank highlighted the increasing relevance of having developed, in addition to more sensitive and rapid methods for disease control, new alert, monitoring and communication systems, allowing faster responsiveness to new threats.

Interestingly, the development of new and harmonised traceability systems was identified as fundamental by several experts groups, highlighting the relevance of such systems for disease control purposes.

In the opinion of the experts, genetic research should still be a major research area in the next 15 years. In particular, the selection toward disease resistant animals (and vectors) and animal being more adaptable to new production systems (with regards to animal welfare as well), were often mentioned as priorities.

In general, all exercises highlighted the techno-economic gap among the northern and south-eastern areas of the World as far as for European and Mediterranean areas as a hurdle for the achievement of some of the expected results (e.g. traceability systems). Hence, an important area of research should target the development of cheap and scalable tools in the animal health field.

In order to maintain the SRA updated, modulation and further prioritisation actions should be implemented.

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Bluetongue virus: while changes do not always lead to improvement, all improvements require some change

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Summary
The title "While changes do not always lead to improvement, all improvements require some change" synthetizes the outcome of 15 years of national and international research on bluetongue. In all these years, we have learnt that bluetongue virus (BTV) is able to change its characteristic and behaviour and to adapt to new environments and episystems. Dealing with it implies, most of the time, to expect the unexpected and to continuously adapt our understanding. New serotypes and new potential vectors have been identified, an additional non-structural viral protein has been revealed, as well as the capability of some field strains/serotypes to be transmitted either vertically or horizontally, to alter their pathogenicity, host specificity, and capacity for spread. Furthermore, some serotypes/strains can cause severe clinical cases affecting different animal species and resulting in significant losses, whereas other infections are entirely asymptomatic with minimal or insignificant economic impact. Reassortments between field strains, vaccine strains, and between field and vaccine strains have generated and will continue to generate novel genotypes. The potential for these progeny strains to be transmitted more effectively and to have increased virulence poses significant additional risks for ruminant health. Thus, the continued evolution of BTV strains poses a substantial challenge to the research community. Within this context, it is then crucial to engage in a continuous dialogue among researchers, laboratories, and policy-makers in order to gain a deeper understanding of new developments, increase mutual knowledge, and share a unique and integrated strategic approach.

Keywords
Bluetongue,
Bluetongue virus,
Evolution,
Reassortment,
Control policy.

Riassunto
Il titolo dell'intervento "non sempre i cambiamenti portano ad un miglioramento, invece tutti i miglioramenti richiedono un cambiamento" sintetizza i risultati di 15 anni di ricerca nazionale e internazionale sulla bluetongue. In tutti questi anni abbiamo imparato che il virus della bluetongue (BTV) può mutare caratteristiche e comportamento, adattarsi a nuovi ambienti e episistemi. Lavorare con questo virus comporta, il più delle volte, essere pronti all'inaspettato e ad adattare continuamente le nostre conoscenze. Sono stati identificati nuovi serotipi e nuovi potenziali vettori, è stata rivelata una ulteriore preteina virale non strutturale come pure, per alcuni ceppi di campo/serotipi, è stata dimostrata la capacità di trasmettersi per via verticale o orizzontale, di alterare la propria patogenicità, la specificità dell'ospite e la capacità di diffondersi. Inoltre, alcuni serotipi/ceppi sono in grado di determinare gravi forme cliniche in diverse specie animali con ripercussioni economiche importanti mentre altre infezioni sono completamente asintomatiche con impatto economico minimo o insignificante. Il riassortimento tra ceppi di campo, ceppi vaccinali e ceppi di campo e vaccinali, ha originato e potrà originare nuovi genotipi più aggressivi e in grado di diffondersi più rapidamente con ulteriori aggravi per tutto il comparto zootecnico. Il continuo evolversi dei ceppi di BTV rappresenta perciò una notevole sfida per la comunità scientifica. In questo contesto, è quindi fondamentale impegnarsi in un dialogo continuo tra i ricercatori, laboratori e autorità al fine di ottenere una più profonda comprensione dei nuovi sviluppi, aumentare la conoscenza reciproca e condividere un approccio strategico unico e integrato.

Introduction

Bluetongue (BT) has been described as an infectious disease of ruminants and camelids (Vervoerd and Erasmus 2004), caused by Bluetongue virus (BTV) and transmitted by biting midges of the genus *Culicoides* (Mellor *et al.* 2000). Bluetongue virus is a RNA virus belonging to the Orbivirus genus in the Reoviridae family (Mertens *et al.* 2004). Its genome consists of 10 segments labeled 1 to 10 according to size, each encoding viral polypeptides divided into structural proteins (VP1-VP7) and non-structural proteins (NS1-NS4). Up to date, 27 immunologically distinct serotypes of BTV have been identified worldwide (Schwartz-Cornil *et al.* 2008, Chaignat *et al.* 2009, Mann *et al.* 2011, Zientara *et al.* 2014). Bluetongue has a significant economic impact, because of the direct effect of the disease on animals (morbidity, mortality, reproductive failure, reduction in milk yields, and weight gain), but also because of the ensuing ban on international trade of ruminants and their products between BTV infected and non-infected areas (Saegerman *et al.* 2010). Initially BT was considered a disease confined to the African continent, subsequently BTV and/or BT were noticed in much of Asia, including the Indian subcontinent, the Middle East, Southern and Norhern Europe, Australia, Central and South America, and the Caribbean Basin. Today, BTV is endemic in many tropical, sub-tropical, and temperate regions of the world (America, Australia, Africa, and some regions of Asia), between latitudes 40°S and 53°N. The title "While changes do not always lead to improvement, all improvements require some change" synthetizes the outcome of 15 years of National and International research on BT. In all these years we have learnt that the BTV is able to change its characteristic and behaviour and to adapt to new environments and episystems. Dealing with it implies, most of the time, to expect the unexpected, and to continuously adapt our understanding.

"While changes do not always lead to improvement"

In the last 20 years, BTV incursions have included unexpected epidemics in areas where it had not been recorded before or where it had not appeared for more than 10 years (e.g. BTV-4 in the mainland of the Balkan Peninsula in 2014); as well as also low-impact circulation of certain serotypes, some of them of unclear origin; incursions of new serotypes, vaccine incidents, and disease resurgence (BTV-8 in France in 2015). All these events, although posing new challenges, raised enormous concerns. In light of the recent disease evolution, the European Commission considers it necessary to review the overall BT policy at EU level and implement possible alternative methods to ensure safe trade of live animals from BT restricted zones.

However, BTV is in continuous evolution. Evolutionary dynamics, selection pressure, as well as the advent of improved diagnostic technology that has facilitated their identification favoured the emergence of new serotypes. Six new virus serotypes (BTV-25, BTV-26, BTV-27, Israeli new BTV, South African new BTV, Chinese new BTV) have been identified since 2008, 4 of them in the last 3 year only. Most of these viruses share novel common properties (Hofmann *et al.* 2008, Maan *et al.* 2011, Zientara *et al.* 2014, Wright 2014, Sun *et al.* 2016, Bombarov *et al.* 2016). BTV-25, BTV-26, BTV-27 and Chinese new BTV do not cause clinical signs in the infected animals and use goats as reservoir species. For at least 1 of them (BTV-26), horizontal transmission has also been demonstrated. Within a project sponsored by the Italian Ministry of Health in collaboration the ANSES Animal Health Laboratory (Maisons Alfort, France) and the Istituto Zooprofilattico Sperimentale della Sardegna, the National Reference Laboratory (NRL) for BT was able to identify a new serotype in Sardinia (BTVX ITL2015) with the same characteristics of those previously described (Savini *et al.* 2017). BTVX ITL2015 was found in healthy goats during the National Surveillance Plan for BT. Although, virologically and serologically distinct from the officially recognized BTV serotypes, the new strain gene segment 2 clustered with the other goat serotypes (BTV-25, BTV-26, and BTV-27). Unfortunately, virus isolation has been so far unsuccessful. Two projects aiming at investigating its distribution, biological cycle and reassortant capacity have been funded by the Italian Ministry of Health and the European Commission, respectively. The first will be carried out together with ANSES Animal Health Laboratory and the Istituto Zooprofilattico Sperimentale della Sardegna, the second is part of the Horizon 2020 project Call: H2020-SFS-2016-2017 with a consortium which involves 19 partners. Being it a segmented virus, reassortment phenomena in BTV are not a rare event. In these recent years, the numerous projects funded by the Italian Ministry of Health allowed us to build a solid network between the countries of the Mediterranean basin that led to sharing experiences, diagnostic reagents, and BTV strains. At the NRL we could sequence the entire genome of the BTV-4 (Greece 1999, Sardinia 2003, 2012, Tunisia 2013, Morocco 2014, Croatia 2014, Puglia 2014, Central Italy, Veneto, Sardinia 2016), which have recently circulated or are still circulating in Mediterranean countries. Of these, Sardinia 2012, Tunisia 2013, Morocco 2014, Croatia 2014, Puglia 2014, Central Italy, Veneto, Sardinia 2016 are reassortant strains with genome segments of 2 to 5 different serotypes. In particular, the 2016 BTV mainland Italian strains were almost identical to the Balkan BTV-4 except for

the Sardinian BTV-4. We also found that the 2016 BTV-4 incursion in Sardinia was due to a population of BTV-4 including at least the Balkan strain and a reassortant strain that has the segment 3 of BTV-16 Eastern strain instead of BTV-1. These findings clearly demonstrate that reassortment is common with BTV. Reassortments between field strains, vaccine strains, and between field and vaccine strains have generated and, thus may continue to generate, novel genotypes. The potential for these progeny strains to be transmitted more effectively (horizontally) and to have increased virulence poses significant additional risks for ruminant health.

"All improvements require some change"

In most of our projects, we address specific questions and try to find solutions that have direct applications to the field. In other words, most of our projects develop applied research. As repeatedly emphasized by European authorities, BT control policies need to be reviewed and updated.

Up to now, we have responded after BTV incursion and most of the time we have acted after the outbreaks. When BTV-4 first appeared in Puglia at the end of the 2014 vector season, only few herds of the Lecce province were infected. Had we reacted immediately, by vaccinating a buffer zone around the few infected herds, we would have saved national funding (only few hundred thousand of vaccine doses would have been necessary) and, probably, we would have stopped the virus avoiding its spreading across Italy in 2015 and 2016. On December 2013, a BTV-4 outbreak occurred in the province of Cagliari (Sardinia). An immediate vaccination campaign involving all animals (approximately 400,000) within the buffer zone drawn around the infected herd were conducted avoiding the further spread of the virus in the following year.

For this reason, NRL proposes to be proactive: it is time to stop responding and to anticipate BTV outbreaks. In Italy a capillary and very efficient National surveillance plan for BT, which is capable to detect BTV entrance and monitor its circulation in real time, and which can help us predicting and preventing BTV moves, is in place. In addition, the strong collaborations built in all these years with the Northern African countries give NRL the opportunity to monitor the BTV situation in this area. Considering how BTV strains have entered Italy in the recent years, this is crucial. Last November in collaboration with the Institut de la Recherche Vétérinaire de Tunisie, NRL has isolated a BTV-3 strain in Tunisia (Sghaier *et al.* 2017). The strain of sub-Saharan origin was able to cause disease in sheep. This is the first time that BTV-3 was detected in Northern Africa. As no inactivated vaccines but only a modified live vaccine is currently available on the market, particular efforts should be devoted to be prepared for its possible incursion by strengthening the National surveillance plan and prompting the production of inactivated vaccines.

In conclusion, at the NRL we believe that 3 keywords need to be kept in mind for controlling BT: collaboration, coordination, and flexibility. Strict collaboration among field, laboratories, and policy makers is crucial. Information on what occurs in the field (novel viruses and serotype) should be constantly updated and shared. At the same time, it should never be forgotten that any strategy is affordable only if perfectly coordinated. Finally, flexibility is of essence to be prepared for the unexpected and deploy effective measures able to counteract BTV as it evolves.

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New strategies and tools to improve knowledge on diagnosis and epidemiology of African Swine Fever

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Summary

African swine fever (ASF) is a devastating disease affecting swine caused by a complex virus, the only member of the *Asfarviridae* family. Disease transmission is maintained under different scenarios involving domestic and wild swine and arthropod vectors (soft ticks genus *Ornithodoros*). The complex epidemiology of ASF and the increasing mobility of people, animals and goods across the globe, emphasize the serious threat the disease can create to the growing pig farming sector in Africa and to the currently disease-free EU member states. In order to improve tools and strategies for the prevention and control of ASF, the work developed under the IZS-UM research projects aimed at fulfilling the following major objectives: (i) molecular characterization of field viruses in order to relate the possible occurrence of genome changes with new occurring forms of the disease and diagnosis problems; (ii) evaluation of the epidemiological situation of ASF in Sardinia to identify the risk factors; (iii) designing new control strategies including, among others, the role of ticks.

Keywords

African swine fever virus,
Fenetic characterization,
Risk factors,
Soft ticks.

Riassunto

La Peste suina africana (PSA) è una devastante malattia del suino causata da un virus appartenente alla famiglia *Asfarviridae*. Si conoscono diversi scenari della malattia che coinvolgono il suino domestico e selvatico ed artropodi vettori rappresentati da zecche molli (gen. *Ornithodoros*). La complessa situazione epidemiologica della PSA unita alla crescente movimentazione di persone, animali e beni di consumo a livello globale, costituiscono una seria minaccia sia per il settore suinicolo africano, sia per i Paesi europei ancora indenni. Al fine di migliorare gli strumenti e le strategie per la prevenzione ed il controllo della PSA, è stato intrapreso un percorso di ricerca i cui obiettivi più importanti sono: (i) caratterizzazione molecolare di stipiti di campo e relativa analisi genomica; (ii) valutazione dei fattori di rischio che condizionano la persistenza della PSA in Sardegna; (iii) indagine sulla presenza nel territorio di vettori in grado di trasmettere l'infezione.

Genetic characterisation of African swine fever viruses isolates from Sardinia

African swine fever virus (ASFV) is a double-stranded DNA virus classified within the *Asfarviridae* family, genus *Asfivirus*. Sequence analysis of variable genome regions has been extensively used for molecular epidemiological studies of ASFV isolates. The main aim of this study was to investigate by extensive sampling and genetic characterisation of ASFV isolates the relatedness of outbreaks occurred between 1978 and 2009 in Sardinia in order to contribute to the clarification of the epidemiological situation over years. For this purpose we applied genotyping to a wide range of field isolates by sequencing 3 single copy ASF genes. The *E183L* gene encoding the structural protein p54 and part of the gene encoding the p72 protein were used to delineate genotypes, prior to intra-genotypic resolution of viral relationships by analysis of the *B602L* gene. The Sardinian viruses did not show any significant variation in their p54 and p72 genome regions, in contrast the analysis of the *B602L* gene, revealed the presence of minor difference placing the Sardinian isolates into two clusters accordingly to their temporal distribution, namely sub-group III, comprising viruses collected up to 1990 and one isolate from 1998, and sub-group X which comprises all except one of the isolates collected from 1990 until 2009. In conclusion, the viruses responsible for the ASF outbreaks in Sardinia, showed a deletion of 12 or 13 tetramer repeats observed from the isolates obtained since 1990. This is consistent with the hypothesis that the ASF outbreaks were caused by very closely related, but mutated forms of the virus that had been circulating from the beginning, supporting the assumption of a single introduction of ASFV in Sardinia.

Evaluation of the risk factors contributing to the African swine fever persistence

In studies conducted in the 1990s it has been hypothesized that the ASF eradication program in Sardinia has failed because of a variety of risk factors that characterize the traditional pig management practices of farmers in the island. In the study here, Bayesian modeling was used to explore the nature and extent of the association between ASF occurrence in Sardinia from 1993 to 2009, and hypothesized risk factors for the disease. The exact causes of ASF disease persistence on the island remain unknown. The main explanations are likely related to sociologic and cultural factors, rather than pig susceptibility, environmental or biological reasons. For instance, the small and backyard farms represented by small pig holdings with typical structure keeping in very limited to absent biosecurity and unhygienic conditions. The traditional free range system, that is another important problem, which is deeply rooted in the culture of some parts of the island. Traditionally, these pigs were bred in communal areas where different animal species from different owners were brought together for several months of the year despite this practice is completely forbidden. The illegal trade of products and animal frequently occurs. The existence of unidentified pigs is still a common practice in certain areas. Piglets and products from these illegally raised pigs are sold and consumed, despite the lack of traceability and potential risk of the consumer. The vast woodlands allow for an abundance of wildlife species, including wild boar. This habitat makes the separation of metapopulations difficult and consequently facilitates the spread of disease between domestic and wild boar. In certain areas, where wild boars frequently contact free-range pigs, their role may be crucial. Hunting habits and practices can also greatly influence the spread of ASF.

Serological surveillance and direct field searching of Ornithodoros erraticus *ticks*

The negative results obtained from previous field studies led to the hypothesis that *Ornithodoros* ticks were absent from the island, and consequently, it was assumed that this tick species was not involved in the epizootic cycle of ASF in Sardinia. Nonetheless, given that no additional studies confirming this hypothesis have been performed since the 1980s, some uncertainty remains in the scientific community about the potential presence and role of *Ornithodoros* ticks in the ASFV persistence in Sardinia.

To clarify the presence and potential role of *Ornithodoros* ticks in Sardinia, a two-step study was developed during 2013-2014. Firstly, a serological assay was employed for screening the levels of immune response of the pig population (domestic and wild) in Sardinia against the salivary antigens of *Ornithodoros erraticus*. Once the serological survey was carried out, direct tick searching activities will be performed in those pig farms with positive serum samples to confirm the presence of *Ornithodoros* ticks in them. Serology was performed by ELISA using the soluble fraction of a salivary gland extract of *O. erraticus* as coating antigen. Due to the absence of positive serological results, the field tick searching activities were not linked with serological results, but exclusively performed in targeted premises where favourable habitats for *Ornithodoros* ticks were known to be present. During the summer of 2013, 6 farms/premises were examined for tick presence, and in the following summer, 3 of those farms were revisited and traps were placed again. Overall, more than 20 traps were placed in the 6 different holdings located in five different municipalities. Numerous insect specimens were collected from all the traps and founded in craves of the pig holdings. However, no *Ornithodoros* specimens were found in Sardinia during field collection activities confirming the results of the serology.

As a whole, all the about data confirm the hypothesis that *O. erraticus* is absent from Sardinia and that it does not participate in the epizootic pattern of ASF. Therefore, eradication efforts should not include additional control measures on ticks but focus on other factors present in the island that were clearly demonstrated to be involved in ASF in Sardinia.

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In vitro *study of the immune response against African swine fever virus in domestic and wild pigs*

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Keywords
African swine fever,
Immune response,
Macrophages,
Cytokine,
Pig,
Wild boar.

Summary
African swine fever (ASF) is a contagious and often fatal viral disease which is currently endemic in many sub-Saharan African countries, in some East European countries and in Sardinia. There is still neither vaccine nor treatment available and the disease control measures rely on stamping out and movement restrictions resulting in extreme losses for producers. African swine fever virus (ASFV) mainly targets cells of the myeloid lineage, especially monocytes and macrophages. Previous studies on genetic characterization of ASFV isolates collected in Sardinia revealed a deletion of 13 tetrameric repeats in the protein encoded by the B602L gene, found only in strains isolated from 1990 onwards. This study aimed to *in vitro* characterize the immunological response of monocyte and macrophages to two different ASFV Sardinian isolates, differing for a deletion in the B602L gene, both in domestic pig and wild boar.

Riassunto
La Peste Suina Africana (PSA) è una patologia emorragica acuta contagiosa dei suidi, attualmente presente in Sardegna, in Africa, nei paesi caucasici ed in alcuni paesi dell'Europa orientale. Il virus replica massivamente nelle cellule del sistema reticolo endoteliale. Non esistono terapie specifiche e profilassi vaccinale contro la PSA. Precedenti studi hanno evidenziato la delezione di 13 tetrameri nella proteina codificata dal gene B602L in quasi tutti i ceppi isolati in Sardegna dopo il 1990. Scopo di questo lavoro è stato quello di caratterizzare *in vitro* la risposta immunitaria di monociti e macrofagi di suino e cinghiale nei confronti di due isolati sardi che differiscono fra loro per una delezione nel gene B602L.

Introduzione

African swine fever (ASF) is a contagious infectious disease of domestic and wild pigs caused by an enveloped icosahedral dsDNA virus (ASF virus, ASFV). ASFV is the only member of the genus Asfivirus, family Asfarviridae, and the only DNA virus transmitted by arthropods of the genus Ornithodoros (Sanchez-Vizcaino 2006). The disease is present in Africa and since 1978, in Sardinia, where it is still endemic although control and eradication plans have been implemented (Mur *et al.* 2014). ASFV appeared in Georgia (2007), from where it spread to Russian Federation, Ukraine, Belarus arriving in eastern Europe in 2014, where is still present in Poland, Lithuania, Latvia, Estonia and Moldova.

African swine fever virus infection can occur as hyperacute, acute, subacute and chronic clinical forms, depending on the strain involved, the route and dose of infection and the host immunological status. ASFV replicates massively in the mononuclear phagocyte system cells (Gomez-Villamandos *et al.* 2013), largely targeting monocytes and macrophages, which are crucial for viral persistence and dissemination. Neither vaccine nor treatment is available and the disease control measures rely on stamping out and movement restrictions. Molecular characterization of Sardinian viral strains have allowed the subdivision of virus isolates into two groups on the base of the B602L gene analysis (Giammarioli *et al.* 2011). Almost all the strains isolated after 1990 show a deletion of 13 amino acid tetramer repeats in this genome region. The aim of this study was to in vitro characterize immunological response of monocyte and derived macrophages of two Sardinian ASFV isolates, differing for a deletion in the B602L gene, both in domestic pig and wild boar.

Materials and methods

Animals

Three healthy ASFV-naïve cross-bred pigs (*Sus scrofa*) and 3 wild boars, 6-18 months of age, were used in the study. The ASFV seronegative status of the animals was confirmed by a commercial ELISA test (Ingenasa, Madrid, Spain) and immunoblotting test (OIE 2012).

Virus strains

Two virulent Sardinian strains and one avirulent Spanish ASFV strain were selected for the study The virulent strains were 22653/14, characterized by the presence of a deletion of 13 amino acid tetramer repeats in the B602L gene and Nu81/2, without deletion. They were propagated *in vitro* by inoculation of sub-confluent monolayers of porcine monocytes/macrophages and titrated as already reported (Malmquist and Hay 1960). The avirulent ASFV BA71V strain, provided by CISA-INIA (Madrid, Spain), was propagated *in vitro* by inoculation of sub-confluent monolayers of Vero cells and titred as previously described (Carrascosa *et al.* 2011).

Differentiation of monocytes into macrophages

Peripheral Blood Monocytes Cells (PBMC) were obtained as previously described (Berg *et al.* 2013). Two methods to differentiate monocytes into macrophages were compared. Monocytes were cultured for 5 days at 37°C with 5% CO_2 in RPMI-1640 supplemented with 30% of autologous porcine plasma or with 50 ng/ml of recombinant human macrophage-colony stimulator factor (hM-CSF). The effect of both protocols on cell morphology and susceptibility to ASFV infection (p72 expression) with the attenuated BA71V and the virulent 22653/14 were analyzed in flow cytometry.

Characterization of immunological response of monocyte and macrophages against two Sardinian ASFV isolates

Monocytes and hM-CSF derived macrophages obtained from domestic pigs and wild boars were infected with the strains NU81/2 or 22653/14 [Multiplicity of infection (MOI) of 1], alongside with mock-infected controls. After 24 h, the susceptibility

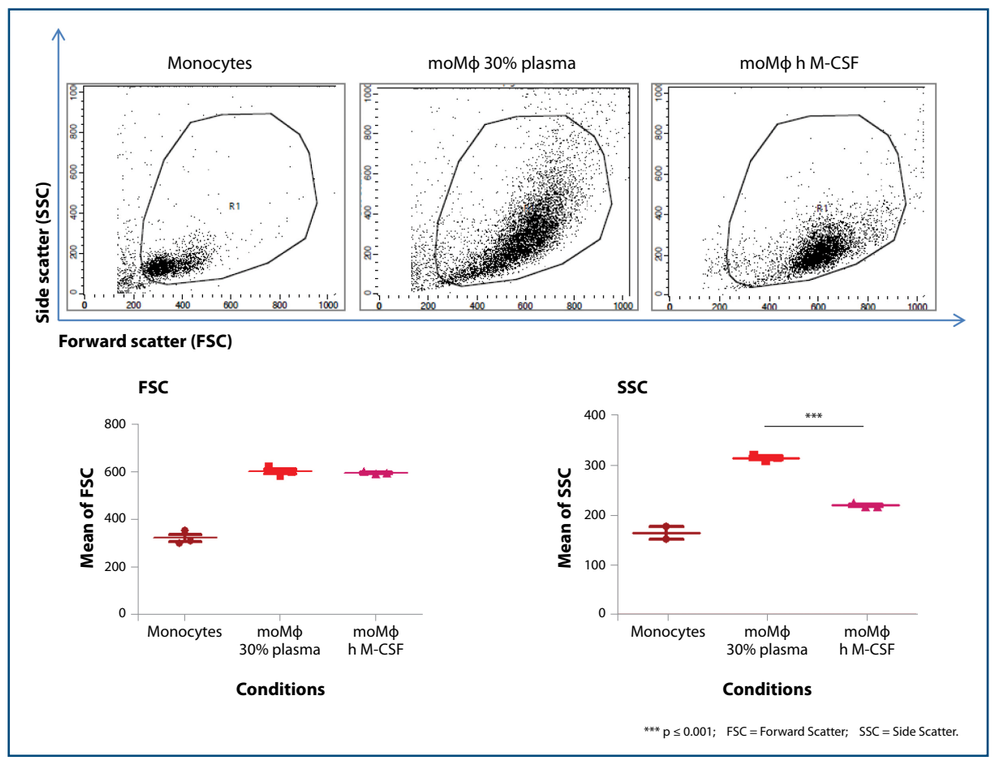

Figure 1. *Monocytes and macrophages differentiated with 30% autologous porcine plasma and hM-CSF.*

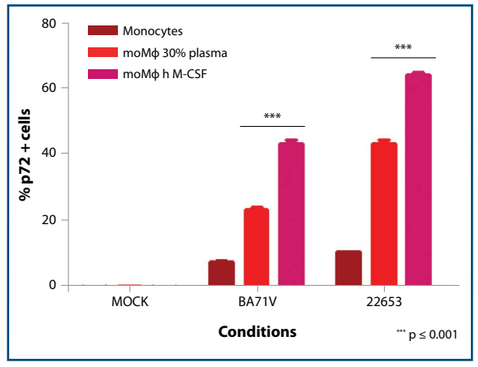

Figure 2. *Intracellular levels of p72 (ASFV late protein) in monocytes and macrophages after infection with 22653/14 and BA71V.*

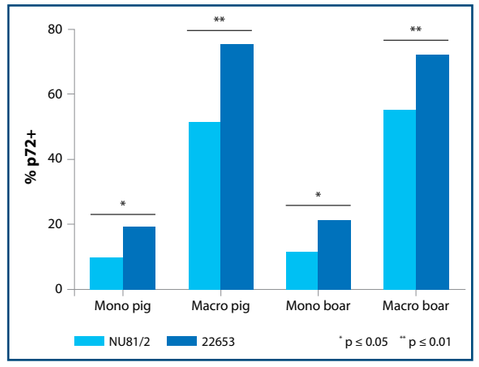

Figure 3. *Pig and wild board monocytes and macrophages infected with Nu81/2 and 22653: susceptibility to infection.*

to infection was determined with flow cytometry evaluating the p72 expression as already described (Franzoni *et al.* 2017a); the cytokine responses were assayed using a multiplex ELISA (Merck Millipore).

Results

Monocytes cultured with the two tested methods increased in size (FSC) and granularity (SSC), but SSC was greater in macrophages differentiated with porcine plasma (Figure 1). Macrophages were more susceptible to ASFV infection than monocytes, but hM-CSF differentiated macrophages showed a higher proportion of p72+ cells (Figure 2) and a lower phenotypic and functional inter-animal variability than those generated using autologous porcine plasma (Franzoni *et al.* 2017b).

In vitro infection performed with two Sardinian ASFV strains evidenced that the mutated strain 22653/14 was able to infect both monocytes and macrophages of domestic pigs and wild boars more efficiently than the unmutated strain NU81/2 (Figure 3). Macrophages were more susceptible to ASFV infection than monocytes while no differences were found in p72 expression between domestic pigs and wild boars. Furthermore, not deleted strain NU81/2 induced a greater cytokine response in domestic pig monocytes and macrophages with respect to 22653/14. Pig monocytes produced an increased cytokine production respect to what observed in macrophages following infection with both ASFV strains. IL10, IL12, IL18, IL1α, IL1ra e IL6 production revealed to be higher in pig than in boar following NU81/2 infection, whereas IL1β ed IL4 were higher in pig but after 22653/14 stimulation (Figure 4).

Discussion

Our results showed that the virus characterized by a deletion in the B602L gene seems to have a greater ability to infect cells and to induce a lower cytokines response in pigs. This deletion may have conferred a selective advantage to the ASFV allowing its spread from the 90s onwards. Moreover, despite our results have not highlighted differences in the ability of the two virulent strains to infect cells collected from wild boar and pig, in this latter we observed an higher cytokines production. We are aware that our *in vitro* model provided a partial view of the complex cell interactions that occur during natural infection; further studies are needed, in particular *in vivo* infection, in order to provide a better understanding of the immune response in domestic and wild pigs against ASFV.

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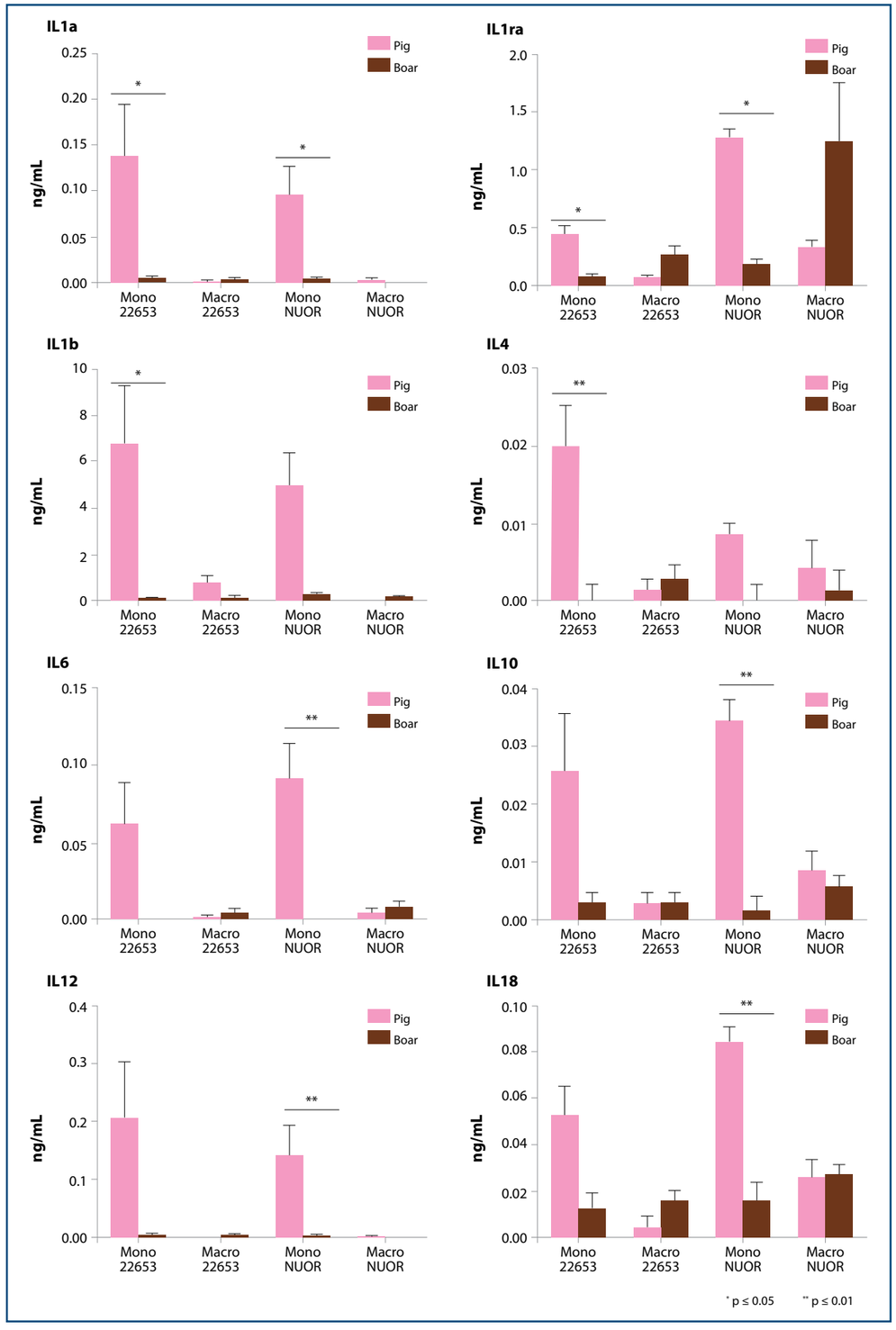

Figure 4. *Cytokines produced by monocytes and macrophages after infection with 22653/14 and NU81/2 (NUOR).*

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From foodborne botulism to the investigation on animal as source of infection: multidisciplinary network in AniBotNet project

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Keywords
Botulism,
Diagnosis,
Control,
Prevention,
Laboratory,
Epidemiology,
Networking.

Summary
Botulism is a severe neuro-paralytic disease affecting humans and animals. Human botulism is rare while the animal disease occurs with much higher frequency. In Italy, an ad hoc surveillance system cover only the human disease. Animal botulism outbreaks are only voluntary notified if laboratory confirmation is requested. Laboratory investigations are essentials for diagnosis confirmation and to mitigate the diffusion of the disease. In this respect, the National Reference Centre for Botulism and the network of the Istituti Zooprofilatici Sperimentali have a crucial role. To overcome the gaps in management of cases and outbreaks, several research projects have been performed at national and European level.

Riassunto
Il botulismo è una grave malattia neuro-paralitica che può colpire l'uomo e molte specie animali. Se la malattia umana è rara, il botulismo animale si verifica con una frequenza molto più alta. In Italia esiste un sistema di sorveglianza passiva che copre soltanto i casi e i focolai umani. I focolai di botulismo animale sono riportati soltanto volontariamente se viene richiesta la conferma di laboratorio. Le analisi di laboratorio sono fondamentali per la conferma diagnostica e per mitigare la diffusione della malattia. In questo senso l'attività svolta dal Centro Nazionale di Riferimento per il Botulismo e dalla rete degli Istituti Zooprofilattici Sperimentali è essenziale, così come è risultata essenziale la partecipazione degli stessi laboratori a progetti di ricerca nazionali e internazionali nella risoluzione di importanti problemi correlati alla gestione dei casi e dei focolai.

Introduction

Botulism is a severe neuro-paralytic disease that affects humans, all warm-blooded animals, and some fishes, consisting of a symmetric flaccid paralysis. The illness is due to the exposure to botulinum neurotoxins (BoNTs) that blocks the release of acetylcholine neurotransmitters from presynaptic neurons in the neuromuscular junctions (Sobel 2005, Anniballi *et al.* 2013). To date, seven antigenic variants of BoNTs (types A-G) are confirmed. A further potential eight serotype (type H) was described in 2013 (Peck *et al.* 2017). Because the high lethality of toxins and their potential use as biological weapon, botulism represents a global public health and security concern. For these reasons BoNTs are characterised as Tier One biowarfare/bioterrorism agents by the Centers for Disease Control and Prevention (Anniballi *et al.*, 2014).

BoNTs are produced by a heterogeneous group of Gram-positive, anaerobic, spore-forming bacteria belonging to the genus *Clostridium* and referred as BoNT-producing clostridia. To date, this group of organisms consists of *Clostridium botulinum* (capable of producing 7 types of BoNTs), *Clostridium argentinense* (previously recognised as *C. botulinum* type G, capable of producing BoNT/G), *Clostridium baratii* (capable of producing BoNT/F), and *Clostridium butyricum* (capable of producing BoNT/E). Based on their phenotypic and biochemical properties, BoNT-producing clostridia are divided in six different groups (I-VI), as extensively reported elsewhere (Hatheway *et al.* 1996, Anniballi *et al.* 2014, Peck *et al.* 2017). Group I, II, V, VI are mainly responsible for human botulism, whereas group III organisms are mainly associated to animal disease (Anniballi *et al.* 2013). Based on their genomic features, some strains capable of producing BoNT/B and previously classified as *C. botulinum*, seem to be neurotoxigenic strains of *Clostridium sporogenes* (Fillo *et al.* 2015, Weigand *et al.* 2015, Peck *et al.* 2017). Moreover, an unexpressed gene encoding for BoNT/B has been found in *Clostridium subterminale* (Franciosa *et al.* 1994). A further *bont*-related encoding gene has recently recovered in *Weissella oryzae* S25 strain (Mansfield *et al.* 2015, Zornetta *et al.* 2016). Generally, BoNT-producing clostridia can produce only one toxin, however, some strains can produce two active toxins or produce one toxin and carry a silent non-expressed gene (Anniballi *et al.* 2016). A strain harbouring three *bont* genes has been recovered (Dover *et al.* 2013). Genes encoding for BoNTs can be carried by the chromosome or by mobile genetic elements such as plasmids or bacteriophages. This latter express unstable lysogeny and are frequently lost during laboratory cultivation (Skarin *et al.* 2011).

Human disease does not differ in clinical features, diagnosis, supportive laboratory testing, management, or therapeutic measures from that seen in veterinary practice (Critchley 1991). For animal botulism, as well as for human botulism, the primary contamination route is the ingestion of preformed toxins in foods or feeds (foodborne botulism). In addition to this form of botulism, a second form is associated with the adsorption of BoNTs produced *in vivo* in the intestinal tract. In human disease, this latter is also known as intestinal toxaemia botulism and is distinguished as infant botulism (if it occurs in an infant under one year of age) or as adult intestinal colonisation botulism (if it occurs in young children or adults) (Sobel 2005). In animal disease is referred as visceral or chronic botulism (Böhnel *et al.* 2001). The third form, wound botulism, is due to the BoNT produced *in vivo* at the site of a wound. Wound botulism is common to human and animal disease. The latter form common to human and animal disease is due to intentional or accidental release of BoNTs (Sobel 2005, Anniballi *et al.* 2013).

Current status of human botulism in Italy

Each case of botulism represents an emergency due to the high potential impact of the disease on public health. According to the European legislation, human botulism is included among the communicable diseases to be progressively covered by epidemiological surveillance in the Community network. Based on the Directive 2003/99/EC, also known as "Zoonoses Directive", botulism has to be monitored by Member States according to their epidemiological situation. Although the human botulism is worldwide rare, the Italian incidence rate is one of the highest in Europe and the disease is statutory notified from 1975. From 1990, the reporting system requires for all suspected cases the immediate reporting (Squarcione *et al.* 1999).

The most frequent form of botulism recognized in Italy is foodborne botulism due to the consumption of improperly home-canned food, however, infant botulism cases account for about 50% of those notified in Europe. From 1986 to 2016 a total of 1338 suspected cases were notified to the surveillance system. Of them, 453 foodborne, 40 infant, 7 wound and 3 adult intestinal colonization botulism cases were laboratory confirmed.

The management of a botulism case is based on the synergy among the Public Health Authorities at both central and peripheral level. An early clinical suspicion is essential for a prompt clinical diagnosis and for a rapid treatment of patient avoiding un-useful and unadvisable therapies. Timely diagnosis is also essential to ensure the identification of the food vehicle and to prevent the diffusion of the disease. The rapid response is important especially if the food is an industrially canned product that can be widely distributed. Since at onset botulism symptoms and signs can be unspecific or confused with some other neurological disorders, clinical diagnosis has to be confirmed by laboratory investigations. In this scenario, laboratory investigations represent a crucial step in which the NRCB and the network of Istituti Zooprofilattici Sperimentali (IIZZSS) are involved.

Current status of animal botulism in Italy

Animal botulism represents a severe environmental and economic concern due to the high mortality observed during outbreaks (e.g. in cattle mortality rate is often higher than 90%). On a European level animal botulism is highly underreported because the limited knowledge among veterinary practitioners, the difficulties in laboratory diagnosis, the farmers' concerns related to the trade restrictions in case of botulism confirmation. Although these difficulties that hamper the gathering epidemiological situation, animal botulism is considered an emerging disease (Skarin *et al.* 2013). In Italy, wild birds are the species most frequently involved in botulism outbreaks and mortality rate usually increase during torrid summer. The disease is laboratory confirmed in wild ducks, pochards, mallards, coots, moorhens, egrets, peewit gulls, herons, gooses, cormorants. Among farmed birds, the disease is mainly observed in pheasants, chickens, turkeys. Beef and cattle are also involved in outbreaks reported in Italy.

Because animal botulism is not included in the list of compulsorily notifiable diseases its prevalence is

highly underestimated. The absence of a national surveillance system does not make possible a reliable estimation of the number and geographical distribution of outbreaks. The NRCB, as well as the network of IIZZSS, receive voluntary notifications only if the laboratory confirmation of suspected cases is requested by veterinary practitioners (if cases or outbreaks occurs in livestock), or if Environmental Police or environmental non-governmental organizations/associations find and collect died wild birds. In some circumstances, the NRCB get information on suspected cases and outbreaks looking local newspapers and/or mass-media.

Strengths and weakness of Italian botulism surveillance system

Human botulism surveillance system demonstrates to be capable of properly controlling botulism in Italy, combining strong epidemiological and laboratory capacity to detect botulism vehicles and agents as well as providing effective emergency managing procedures. However, new efforts have to be made to improve awareness among front-line medical professionals and advance in consumers' communication with the aim to promote the correct home-canning practices. In fact, foodborne botulism due to traditional home-canned food still represents a public health challenge especially in southern regions. Respect to infant botulism, although Italy reported half the cases notified in Europe, these are concentrated in few hospitals in which physicians have acquired high awareness on this form of botulism and promptly formulate the clinical suspicion.

In terms of laboratory investigations, despite *in vitro* diagnostic method have been developed, the detection of BoNTs are still performed by mouse bioassay. Conversely, for the detection of BoNT-producing clostridia mouse bioassay has been replaced the multiplex real-time PCR methods developed and validated by the NRCB and adopted by the network of IIZZSS.

About animal botulism, as also emerged during workshop held in Uppsala, Sweden in 2012, there is a need for a surveillance system. In this respect animal botulism should be included in the B list of zoonoses and zoonotic agents to be monitored according to the epidemiologic situation. Although several improvements in terms of diagnostic methods have been made during the research project AniBioThreat (funded by Directorate General Home Affairs under the programme "Prevention of and Fight Against Crime" of the European Commission) several drawbacks remain (Skarin *et al.* 2013). Questions related to diagnosis, epidemiology, prevention and control plans must be elucidated to improve surveillance and management strategies.

Conclusion

Thanks to national and international research projects in the field of botulism, there are now accurate *in vitro* diagnostic methods and awareness on animal botulism.

To overcome the remaining problems a consortium composed of ten research groups from six European countries (Finland, France, Germany, Italy, Sweden, and The Netherland) with expertise in the field of human and veterinary medicine, molecular biology, animal and public health, mass spectrometry, epidemiology, and animal experiments, are involved in a research project funded within the European Animal Health and Welfare ERA-net coordination actions. The project named "Animal botulism: innovative tools for diagnosis, prevention, control and epidemiological investigation" comprises five work packages mainly focused on: mechanism of *C. botulinum* neurotoxin production; tool for detection and genotyping of etiologic agents; epidemiology; evaluation of measures to prevent and control the animal disease.

The expected results are addressed to improve understanding of the epidemiology and the ecology of etiologic agents. The development of diagnostic and surveillance tools, as well as the control strategies will improve the overall knowledge on the factors affecting neurotoxin expression, genomic diversity of strains and their distribution through Europe, contamination and predisposing factors.

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Poster

EAV control by virus characterisation

Equine arteritis (EAV): international disease control by improved methods of virus detection and characterisation – FAIR CT_98

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EAV is world-wide distributed and is a threat to the horse industry, due to horses movements among countries and to use of uncontrolled infective semen. Infection of stallions often results in a persistent infection of the reproductive tract. Timely detection of infected animals is a cornerstone in prevention of spread of EAV. The overall aim of this project was: develop and harmonise detection of EAV, characterise EAV strains and study pathogenetic and immunological aspects of infections. A highly specific and sensitive TaqMan PCR for viral detection in semen and clinical samples, amplifying part of the ORF7 gene was developed.
The study of genetic stability in shedder stallions indicated that EAV ORFs 5, 6 and 7 are in general genetically stable in semen and the ORF5 gene analysis confirmed its suitability as tool for tracing EAV routes of spread.
Using an infectious cDNA clone as a backbone, five chimeric clones of EAV comprising segments of the virulent and avirulent phenotypes were tested by experimental infections, to assess the genome region(s) involved in determining virulence. The clinical signs indicated that the virulence properties of the parent virulent strain were not transferred via the structural proteins to the recombinant viruses.

FMD networking for diagnosis and control

FMD: technical international network for performing diagnosis and control of the disease in endemic countries

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The National Reference Centre for FMD with the Biotechnology Laboratory has acquired wide expertise in the production and characterization of monoclonal antibodies (mAbs) and their use in the development of diagnostic immunoassays.
Through consecutive National Grants and EU-funded Consortia projects, extending over 30 years, more than 500 well-characterized mAbs were produced against the seven serotypes and multiple variants of FMDV, and the corresponding hybridoma are stored in the IZSLER Biobank.
MAbs-based in-house diagnostic ELISAs were initially developed, followed by a significant improvement derived from their conversion in stabilized, ready-to-use kits. The IZSLER portfolio includes a complete spectrum of kits for FMD viruses and antibodies detection and serotyping. In addition, a collaborative study led to the development of the rapid test for field diagnosis (LFD). All these products are being currently used in many endemic countries in Africa, Asia and Middle East, where they enable confirmation of suspect cases with identification of FMDV serotypes, country serosurvey to define infection prevalence, post-vaccination monitoring.
In addition to this established research branch, further potent tools like NGS were implemented for phylogenetic reconstruction of recent outbreaks in North Africa.
Future perspectives include continuous improvement of FMD diagnostics and strengthening relationships with endemic regions.

OTC distribution in ovine milk during cheese-making

The fate of oxytetracycline in spiked sheep's milk during cheese-making and cheese ripening

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This paper presents the results of a study on the effects and the distribution of oxytetracycline (OTC), added to ovine milk at MRL concentration, during cheese-making and cheese ripening.
The evidences currently available concern partition of antiparasitic drugs from milk to milk-derivatives and only a few studies investigated the technological effects related to low doses of antibiotics on cheese-making processes.
The OTC distribution was assessed through the cheese-making process starting from both raw and thermized milk.
OTC caused a dose dependent delay in pH lowering of the curd. Despite that, no effects on the total microbial count or on cheese composition and ripening were observed.
Similarly, MRL OTC did not influence mass balance or the recovery of fat and protein in cheese.
OTC added to raw whole ovine milk was mainly recovered in the 1-day-old cheese (60% and 80% in cheese from raw and thermized milk, respectively).
The absolute OTC amount decreased in the cheese during ripening, leading to suppose the contribution of degradation phenomena.

The authors report published data and partial data that will be available for potential future publications.

Comparative genomics of *Listeria monocytogenes* strains

Comparative genomics of Listeria monocytogenes *strains from an Italian human listeriosis outbreak and strains from different European countries showing the same sequence type*

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An increasing number of severe human listeriosis has been recorded in Marche region, Italy, since May 2015. The circulating strains were subtyped by different molecular methods: PFGE, MLST and NGS. MLST and PFGE identified a large group of strains exhibiting ST7 and a pulsotype never detected before. Following an official inquiry to the EURL and EU MS laboratories addressing to share the collected dataset, the novel Italian pulsotype was compared to those present in the international databases. Austria, France and Netherland provided 27 strains belonging or not to the same pulsotype but within the same ST. The phylogenetic analysis NGS based displayed the cluster nature of the Italian isolates confirming the previous MLST/PFGE typing. Moreover, the analysis excluded from the Italian outbreak all the isolates from the other European countries, namely those from Netherlands characterized by the same pulsotype. A suspected source of infection was hypothesized in January 2016 with the isolation of a food strain from a Headcheese locally produced. The matching with the human strains was based on molecular typing including NGS analysis. A concrete cooperation between EURL and MS laboratories is encouraged due to the international dimension of food trade and related pathogens. The comparative genomics approach represents a precious tool in course of outbreak investigation.

Clostridium difficile in food of animal origin

Food as source of toxigenic Clostridium difficile *strains*

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Clostridium difficile (Cd)is an emerging enteric pathogen involved in several outbreaks of infection over the last decade worldwide. The aim of our studies was to assess the occurrence of Cd in food samples of animal origin and to characterize the toxinotype and ribotype of the isolates. Nine-hundred seventy-eight out of the 1,110 samples analyzed (88%) were shellfishes (*M. galloprovincialis* 945; *T. philippinarum* 32; *V. verrucosum* 1) while the remaining 132 samples (11.9%) were cow milk. Overall, 80 Cd strains were recovered. The higher prevalence was highlighted in *T. philippinarum* (26/32) followed by *M. galloprovincialis* (49/945) and the milk samples (5/132).
The non toxigenic strains were 41 (49%) and 39 isolates (48%) were found to be toxigenic. The most prevalent toxinotype was $A^+B^+CDT^-$ (21/39), followed by $A^+B^+CDT^+$ (15/39) and $A^-B^+CDT^-$ (1/39). Several ribotypes were detected, most were grouped into riboprofiles previously recognized (001, 002, 003, 010, 012, 014/020, 018, 045, 070, 078, 106, and 126), whereas 10 additional isolates were grouped in 8 new profile (SLO 002, SLO 038, SLO 063, SLO 084, SLO 121, SLO 122, SLO 123, and SLO 124). This investigation draw attention to the possible route of exposure to Cd represented by food.

Lagomorphs and lagoviruses

From "X disease" to RHDV2: over 25 years of research on lagoviruses

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When we started to work on "X disease" of rabbits on 1986, its aetiology of was not yet defined. We contributed to firstly describe the viral aetiology and to classify such virus. Thereafter, in the new genus Lagovirus of the family Caliciviridae, with RHDv as prototype, some other agents were included as results of our studies i.e. the European Brown Hare Syndrome Virus (EBHSV) on 1988, the first consistent antigenic variant RHDVa on 1996 and the first non-pathogenic lagovirus (Rabbit calicivirus - RCV) on 1997. These important achievements were made possible by the set up and extensive use in field surveys of innovative serological and virological tests, mainly based on specific reagents, including MAbs, produced and characterized in IZSLER. Thanks to these consolidated expertise and the recognition as OIE Reference Lab, multiple collaboration were established worldwide. When the new serotype RHDV2 was firstly reported in France on 2010, the enforced epidemiological surveillance on domestic and wild lagomorphs and the availability of diagnostic methods permitted to quickly detect the same virus in Italy on 2011, and then to better define its antigenic, genomic and pathological characteristics, including the capacity to infect other lagomorph species of the Lepus genus. In addition, on 2014 we found another non-pathogenic lagovirus in hares named Hare Calicivirus (HaCV).

One Health and mosquito-borne diseases

Mosquito-Borne Disease and One Health: multidisciplinary surveillance networks as a tool for "early detection"

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Mosquito-Borne Diseases (MBDs) represent an emerging threat. Given the complex epidemiology multidisciplinary surveillance is needed, according to the One Health concept.
West Nile virus (WNV) National Plan is an excellent example of One Health approach, integrating human and veterinary surveillance in order to early detect virus circulation and to quickly apply control measures aimed at reducing the risk of transmission trough blood and blood components.
IZSPLV is actually involved in Regional (IPLA, SEREMI, ASL2TO, Regional Blood Center) and National (Regions of the Po river plain, National Blood Center) surveillance networks and participated in a transboundary one ("REDLAV" projects).
Thanks to entomological surveillance, Usutu virus was detected in Piedmont since 2011. WNV was detected in 2014 in Piedmont and Liguria and in 2015/2016 only in Piedmont with an expansion of its activity. Since 2014, 80597 bags were tested and no-one resulted positive. For the first time *Aedes albopictus* was detected in Valle d'Aosta in 2013 and *Aedes koreicus* in Liguria in 2016.
We'll investigate the vector competence of mosquito populations in Piemonte, Liguria and Valle d'Aosta, develop innovative PCR assays for mosquito-borne viruses detection, create new partnerships and strengthen networking activities at national and international level.

Milk microbiota of water buffalo

Milk microbiota of water buffalo

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Mastitis is an important disease in the water buffalo dairy industry. Microbial pathogens identified as causative agents in mastitis are traditionally diagnosed by bacterial culture. This study used a target metagenomic approach considering the 16S rRNA gene as target, in order to investigate bacterial DNA diversity in milk samples of mastitic and healthy water buffaloes. One hundred and thirty-eight milk samples from water buffaloes with clinical mastitis, sub-clinical mastitis and healthy animals were analysed. Moreover, eleven samples obtained from the washing water of teat surface, in order to assess environmental microbiota, were included. Bacterial DNA was isolated from the same milk samples and the hypervariable V1-V2 regions of16S rRNA gene were individually amplified and sequenced using the semiconductor sequencing strategy. Results demonstrate that healthy status seems more correlated with the microbiome rather than to the environment.

Dietary Exposure to Elements/Radionuclides in Apulia

Assessment of dietary exposure to inorganic contaminants in the Apulian population

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Dietary exposure of the Apulian population to toxic trace elements and species (inorganic arsenic, cadmium, lead, methyl-mercury, inorganic mercury, aluminium, nickel and thallium) and radionuclides (radioisotopes of caesium, uranium and potassium-40) has been targeted.
A Total Diet Study (TDS) approach was used, entailing representative sampling of food items, their preparation as consumed, and pooling into food groups. Exposure assessment consisted in the estimation of the intake of trace elements and the effective dose of radionuclides.
Based on previous studies, six food groups significantly contributing to the exposure of the local population (i.e. leafy vegetables, other fresh vegetables, potatoes, fish, crustaceans and molluscs, tap water) were selected, whereas exposure arising from the other food groups of the total diet was accounted for relying on the results of the Italian national TDS 2012-2014.
Based on the size of the resident population and geo-anthropogenic characteristics, four geographic areas were selected for the sampling plan. After preparation according to local culinary habits and pooling, analytical samples were submitted to the determination of trace elements (ICP-Mass Spectrometry), inorganic-As by (HPLC-ICP-MS), and radionuclides (gamma-spectrometry).
Occurrence data were combined with individual consumption data to estimate mean dietary exposure and the latter compared with the reference points for risk characterization.

Anisakis in fish products and allergy potential

Evaluation of Anisakis *presence in fish products of Sicily and survey on the efficiency of storage methods on the viability and allergy potential*

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The research aimed to evaluate the prevalence, geographical distribution and seasonal occurrence of Anisakidae nematodes infestation in fish products marketed in Sicily. A study on mercury detection was also conducted in order to evaluate a difference in the accumulation between parasitized and not parasitized fishes. We examined 1419 fish samples for Anisakidae larvae detection by visual inspection and digestion method. The collected larvae were identified at genus level by optical microscopy and at species level by molecular methods (PCR-RFLP and multiplex- PCR). A Real Time PCR was carried out for the assessment of DNA of Anisakidae presence in 23 transformed fish products. The method showed high sensitivity and specificity for the scope. The results obtained revealed the presence of Anisakidae larve in 463 fish samples (32,6%) and of Anisakidae DNA in 3 trasformed fish products (13%).
All the larvae detected in transformed fish products were dead, with absence of motility until 24 h. Molecular analysis confirm *Anisakis pegreffii* as the prevalent species of Mediterranean Sea. Furthermore, the presence of *A. simplex s.s., A. physeteris, A. pegreffii/A. simplex s.s.* hybrid forms and *Hysterothylacium fabri* was verified with lower prevalence values.
A significant correlation was found between Anisakidae infestation and presence of heavy metals (Hg) levels ($p<0.05$).

Hidden food allergens and consumers protection

Allergeni nascosti negli alimenti: implementazione delle strategie di rilevazione per una maggiore tutela del consumatore allergico

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Food allergy indicates an adverse immune response to food. About 2% of the adult population and more than 8% of the pediatric population are allergic to one or more foods. The EC Regulation 1169/2011 defines the right of the consumer to be informed on the presence of the 14 substances at higher allergenic risk. About 2-3% of food placed on the market may be potentially contaminated with allergens not declared on the label. This project wanted to assess the analytical tests for the detection of hidden allergens in food and to develop new tests for the detection of so-called "minor allergens" for which no ready for use test are now available. A new method for the detection of pine nuts in food was developed. An isoform of pistachio LTP was identified and characterized and the "may contain" sampling plan confirmed the abuse of voluntary food labelling by food operators.

***Yersinia enterocolitica*: risk evaluation in pork**

Molecular approaches for evaluating the risk of pathogenic Yersinia enterocolitica *along the pork production chain*

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Enteropathogenic *Yersinia* is the third most common cause of bacterial enteritis in European countries and infection is most often acquired by raw or undercooked pig meat. Slaughtered pigs are considered the principal animal reservoir for pathogenic strains of *Y. enterocolitica* (*Ye*). The presence of *Ye* in pork production chain was detected by ISO Real-time PCR method. In order to evaluate the pathogenetic variability of *Ye*, different virulence genes were detected using molecular platforms with simultaneously detection. The platforms have been used to evaluate 153 strains of *Ye* and 130 samples taken along the pork chain. The isolated strains, showed the presence of ail and *ystA* genes only in pathogenic bio-serotypes and in a strain 1A, isolated from human case. The *ystB* locus, has been identified in four non-pathogenic bio-serotype, fes and *fepD* genes in most of the non-pathogenic bio-serotypes, and *virF* only in serotype O:3.
The results obtained from the analysis of samples, showed the absence of *ail*, *ystA* and *virF* genes, and the detection of *ystB, fes* and *fepD* in about the 45% of the samples.
The developed methods allow the rapid identification and characterization of *Ye*, in order to estimate the microbiological risk along the pork chain.

***Lactococcus lactis* treatment for mastitis**

Lactic acid bacteria as an alternative to antibiotics for the treatment of mastatis in ewes : from in vitro *assays to* in vivo *studies.*

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The intramammary infusion of lactic acid bacteria has recently emerged as a potential new alternative to antibiotics for preventing and treating bovine mastitis.
In this study we first assessed in vitro the probiotic potential of *Lactococcus lactis* LMG 7930. We then evaluated, in two trials, the efficacy and safety of the intramammary infusion of a live overnight culture of *L. lactis* for the treatment of subclinical and clinical mastitis in lactating ewes. Finally, we in-depth investigated the mammary gland immune response induced by *L. lactis* intramammary inoculum using the mouse mastitis model.
We found that *L. lactis* showed *in vitro* probiotic properties. *In vivo, L. lactis* rapidly activated the mammary innate immune system. This led to a transient clearance of the pathogen in the gland, but also caused both mild and moderate inflammations in ewes. In the mouse mammary gland, *L. lactis* inoculum caused variable histological features, ranging from undamaged tissue to severe inflammation.
In conclusion, our results suggest that *L. lactis* can act as mastitis-causing pathogens when inoculated at high density into the mammary glands of mammals. The use of bacterial formulations as alternatives for the treatment of mastitis in ruminants remains in need of further experimentation.
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Inorganic arsenic in mussels from Campania

Occurrence, origin and seasonal variations of inorganic arsenic in bivalve molluscs from Campania

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Inorganic arsenic (iAs) is ubiquitous in the environment as AsIII and AsV compounds. Chronic oral exposure to iAs, depending on its duration and magnitude, is associated with cancer and a wide range of other adverse health effects.
The diet is the main exposure source for humans but accurate assessment of the dietary iAs intake is a challenge since arsenic is present in food also as a variety of organic species, which however have negligible to low toxicity. Especially in fish and seafood, organoarsenic species occurs at very high levels and thus speciation analysis for the selective determination of iAs is needed to assess potential risks for human health.
Bivalve molluscs, primarily mussels, accumulate substantial amounts of iAs and may represent a significant exposure source for humans. In Campania, mussel farming is an important economic activity with a long-standing tradition in the Gulf of Naples. In this area, a number of active volcanic systems exist and may contribute to arsenic environmental levels as a consequence of the emerging fluids at many sites in the coastal plane. The project addresses the issue of potential contamination of mussels by iAs from the standpoint of food safety. Analytical determination of iAs by HPLC-ICP-MS and of multi-element fingerprints will provide evidence for such an undertaking.

Applying the 3R principle in AAI

The 3R principle: A reflection on its application in AAI

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The Three Rs principle (Replacement, Reduction, and Refinement) was introduced in 1959 by Russell and Burch to improve systematic organization and to achieve ethically acceptable methods for animal research. The potential application of the 3R principle in the context of animal assisted interventions (AAI) is based on the deep commitment of animals in AAI and the importance of considering all aspects related to animal health, welfare and behaviour. In the last half century, there has been a significant development of AAI worldwide, but whether bioethical aspects, particularly from the animal's point of view, are considered thoroughly is in doubt. This poster summarises the essential features of the 3R principle in animal experimentation, then it outlines the potential transposition of the principle in AAI. The analysis of the conditions for said transposition is accompanied by suggestions to guide research on AAI in favour of animal welfare, including assessments related to the environmental conditions and the animal's sense of pleasure. Finally a fourth R, Relationship, is proposed to distinguish the AAI context, on the basis that the effectiveness of the interventions is highly dependent on the human-animal relationship.

CSF: new tools for new strategies

Antiviral treatment and DIVA vaccination: novel tools for the prevention and control of Classical Swine Fever

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Classical Swine Fever (CSF) is a highly contagious viral disease of domestic and wild pigs, clinically related to the African Swine Fever (ASF). Both these infections represent the most important transboundary diseases of pig sector. After the epidemic waves registered during the '90s, the CSF has been practically eradicated from European Union, but the viral circulation is still notified in several countries at the eastern European borders, so the threat of virus re-introduction is not negligible.
The Italian Reference Laboratory for Swine Fevers is involved in different activities to improve the diagnosis of the disease, assure the maintenance of free status for the Italian pig herds and provide useful instruments to prevent/front eventual new outbreaks. The research projects were founded by the Italian Ministry of Health otherwise by European Commission; it is relevant that these studies were conducted cooperating with other laboratories at national and international level. Until now, the gained results permitted to identify new important tools useful to increase the knowledge regarding the genetic structure of the CSF virus and the immunological dynamic in the hosts; the next goal remain to develop new and better strategies to prevent the spread of the disease.

Monitoring of European swine influenza virus

Swine influenza A virus surveillance in Europe 2010-2015: monitoring of the antigenic and genetic evolution of swine isolates also in comparison to seasonal human influenza virus

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Influenza A virus (IAV) circulates actively in the Italian pig population and subtypes H1avN1, H1huN2, H3N2, A(H1N1) pdm09 are detected. Monitoring of circulation of swine influenza virus (sIAV) was conducted in Italy in 2010-2015 and the incidence of 14% of sIAV infection was observed. Whole genome sequences were obtained from 104 sIAV isolates and from 75 human influenza viruses. Phylogenetic analysis was performed to assign each gene segment to its origin. Human H1N1 strains belonged to A(H1N1)pdm09 lineage whereas H1N1 pig strains were mainly (80%) avian-like swine H1N1. Sporadically (12%) A(H1N1)pdm09 or its derived reassortant strains (8%) were observed, moreover gene reassortment events (14%) between H1huN2 strains and A(H1N1)pdm09 strains were observed.
A large-scale genomic characterization of 290 sIAVs collected from 14 European countries between 2009 and 2013 is reported. A total of 23 distinct genotypes were identified, with the 7 most common comprising 82% of the incidence. H1huN2, showed multiple long-lived geographically isolated lineages, while H3N2 had short-lived geographically diffuse lineages. Many transmission events have resulted in A(H1N1)pdm09. The high number of reassortant genotypes observed in European swine, underlines the importance of continued surveillance for the purposes of maintaining public health.

Combating *Campylobacter* in broiler chickens

Effect of a synbiotic product for the control of Campylobacter in the broilers chickens

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Campylobacter infections are the leading cause of human bacterial gastroenteritis in EU. Broiler chickens are potential reservoirs for *Campylobacter* and contaminated retail chicken meat is believed to be the source of most of the clinical cases. Interventions at the farm level focused on reducing the pathogen load in the animal gastrointestinal tract should be considered in the overall control strategies. In this research, laboratory and in field trials on broiler chickens were carried out. The animals were fed with regular and supplemented feed to evaluate the effectiveness of a modified diet in reducing the *C. jejuni* load at caecum level. The supplement was a symbiotic compound composed by the probiotic bacterium *Bifidobacterium longum* PCB133 and a xylooligosaccharide (XOS). In the laboratory trial, the results proved a significant decrease of *Campylobacter* in the animal receiving the symbiotic compound in which we observed an unusual microbiota. The in-field trial with naturally contaminated broilers confirmed the effectiveness of the product in combatting the *Campylobacter* colonization. These findings suggest the use of the symbiotic compound in the broiler diet for a new and more sustainable control of *Campylobacter*. The use of the synbiotic product together with effective biosecurity measures can be an adequate approach, to enhance the safety of poultry meat.

Genome diversity of *Brucella melitensis* and *B. suis* biovar 2

Origins and spread of brucellosis in wildlife and domestic animals in Europe

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Brucellosis is a zoonotic disease that causes high concern to public health and significant economic impact on livestock production. *Brucella melitensis* (Bm) and *Brucella suis* biovar 2 (Bs2) are currently the most epidemiologically important species in Europe. Determining which lineages are present throughout Europe is paramount for surveillance and epidemic preparedness. Previous molecular typing techniques were unable to assess the population structure accurately. The aims of this study were to evaluate the genetic diversity of Bm and Bs2 and to discover their phylogenetic place in the global context. We applied whole genome sequencing to 102 isolates. Genomes were sequenced with paired-end Illumina sequencing and compared with public available genomes. Single nucleotide polymorphisms (SNPs) were detected in read alignments and whole genomes, while maximum parsimony and Bayesian analyses were used to determine the phylogeny. *Brucella* analyzed depicted the known global diversity. Significant phylogeographic structure was observed at country level with Bm organized into three main distinct clades and Bs2 featured into six clades suggesting a European origin. Our results indicate a complex history for *Brucella* and suggest a host associated evolution.

Molecular surveillance of listeriosis in Italy

The importance of the European Molecular Surveillance Service for identifying cluster of Listeria monocytogenes*: the Italian experience during an outbreak in the Marche region in 2015-2016*

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The European Surveillance System (TESSy) Molecular Surveillance Service (MSS), hosted by ECDC, allows EU Member States to upload molecular typing data for *Salmonella, Listeria* and *E. coli* STEC in the MSS database, through the nominated operational Contact Points. The objective of the new service is to improve: (i) speed of detection of dispersed international outbreaks, (ii) trace-back of the source of an outbreak and identify risk factors, (iii) investigation of transmission chains across the EU and globally, (iv) response to outbreaks.
This surveillance system played a central role during an outbreak due to *Listeria monocytogenes* serotype 1/2a, occurred in Marche region from January 2015 to March 2016, identifying a pulsotype never isolated before in Europe.
According with the ECDC nomenclature, twenty-two out of 37 clinical isolates showed identical PFGE genetic profile. Both epidemiological and molecular investigation identified, as a source of infection, a cooked pork meat sausage.
The positive management of this outbreak was possible only through the collaboration among ISS, NRL for L.m., IZSUM and the Regional Veterinary and Health Authorities. Particularly, the communication and cooperation among all the parts involved were crucial to identify the sources of the outbreak and to implement appropriate control measures.

Wildlife disease surveillance in Europe

APHAEA – harmonized Approaches in monitoring wildlife Population Health And Ecology and Abundance

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Wildlife disease surveillance requires knowledge of the population sizes of wild animals and of changes in population size and in geographical distribution over time. Such knowledge is necessary to design appropriate sampling protocols for disease surveys, to develop disease contingency plans, to assess the risk of pathogen transmission to other species, and to guide wildlife management strategies in general. Despite the performance of both wildlife disease surveillance and estimation of wildlife population abundance in Europe, there is a considerable lack of homogeneity regarding sampling and diagnosis protocols for target surveillance programs and regarding methods for estimation of wildlife abundance, which prevents a good comparison of results between and even within countries.
The project "Harmonised Approaches in Monitoring Wildlife Population Health, And Ecology and Abundance" aims at establishing a european wildlife disease surveillance network that was capable of providing reliable estimates of abundance of wildlife species and of pathogen distribution in key wildlife species.
The main objectives of this project is concerned to develop harmonized methods for collecting and analyzing samples to diagnose key wildlife pathogens in Europe anddevelop a European wildlife disease network in which harmonized methods are adopted for estimating species abundance and for diagnosing pathogens in key wildlife host species and key wildlife pathogens in Europe.

Food safety: molecular diagnostic

From monitoring to innovation: molecular diagnostics in food safety

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Molecular diagnostics have the potential to revolutionise quality testing in food safety and offer a faster and more sensitive alternative to traditional immunoassays and culture techniques.
Scientific Research and Development laboratory, by a collaborative network with Italian Universities (UNIBA, UNIFG) and public institutions (ISS, IIZZSS), provides to the development and optimisation of novel alternatives methods for the monitoring, characterisation and enumeration of food-borne pathogens, a key aspect in food microbiology. The working group is developing new analytical methods based on Droplet Digital PCR (ddPCR) in order to improve food-borne pathogens detection as enteric viruses (e.g. NoV and HAV) and bacteria, to detect traces of DNA for species fraud and product mislabelling in processed food. The aim of these researches is to provide a useful strategy for high-throughput screening of microorganisms to ensure the quality and safety of food products, increasing analytical specificity and sensibility with minor costs. These research activities are supported by the Italian Ministry of Health (Rome) (RC IZSPB 06/11, RC IZSPB 02/13, RC IZSPB 03/14, RC IZSPB 07/15).

Bovine Besnoitiosis: emerging disease

Besnoitiosis: understanding an emerging disease

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Bovine Besnoitiosis is a serious and debilitating protozoan disease. (Diesing *et al.*, 1988), most frequently in an asymptomatic form particularly difficult to diagnose and responsible for spread of the parasite (EFSA, 2010; Lepri *et al.*, 2011). In spite of the recognized large occurrence of this disease at international level, there is still a lack of data regarding Italy. For this reason a research project, funded by Ministry of Health, has been carried out in 2011-2012, aiming to investigate the presence of *B. besnoitii* in Italian cattle. The project included several national and international scientific partners, in order to take advantage of the multiple expertise available. Blood serum samples were collected from 991 subjects, belonging to 37 cattle farms located in in central Italy, sharing a common area for pasture. Indirect ELISA (PrioCHECK®Besnoitia) was used for testing samples; 504 animals revealed antibodies against *Besnoitia*, showing an individual prevalence rate of 52% and a farm prevalence of 94,6%. The in-herd prevalence ranged from 2,13% to 91,67%. A clinical observation in each farm was also included in the diagnostic protocol. During the whole project, 12 clinically symptomatic cattle were observed. The high seroprevalence confirmed a wide distribution of infection in cattle population in the area under study. However, there is still a lack of reliable epidemiological data both at national and international level: Moreover, the diagnostic protocols are still mainly based only on clinical diagnosis. For this reasons, surveillance plans are strongly recommended aiming to better clarify the occurrence of this emerging disease, its etio-pathology and the possible role of other species as potential asymptomatic reservoir.

Monitoring of TB in Alpine wildlife

Monitoring of TB in Alpine wildlife

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TB (caused by *Mycobacterium bovis, Mycobacterium caprae*) has one of the broadest host ranges of any known zoonotic pathogen. Under certain conditions, wildlife can play a role as reservoir and source of infection for domestic animals. Evidence is increasing that *M. caprae* is emerging in free-ranging red deer and cattle in the Alps.
To estimate the prevalence of TB in wildlife in the Alps, 1655 hunted red deer of both sexes and different ages were investigated in Austria, Germany, Switzerland and Italy during the 2009-2012 hunting seasons. A transnational harmonized approach was used for sampling activities, collection of lymph nodes, culture isolation, PCR detection and genotyping of isolates.
M. caprae was isolated from 59 red deer: 55 in Austria, 3 in Germany and 1 in Italy. Most of the strains were characterized by genotype Lechtal. Statistical analysis revealed the presence of evident spatial cluster of *M. caprae* positive red deer in Alpine region (hot spot region) mainly located in Austria.
Our data indicate a localized TB problem in wildlife in the Alps related to wildlife management strategies based on supplementary feeding with consequent increase of spatial aggregation and risk of TB infection transmission and maintenance.

European Network of GMO Laboratories

The European Network of GMO Laboratories for EU risk management and research programmes

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The European Network of GMO Laboratories (ENGL) is involved in the development, harmonization and standardization of methods for sampling, detection, identification and quantification of Genetically Modified Organisms (GMOs). The network was launched on December 2002, and it currently consists of 95 national enforcement laboratories, from EU Member States plus Norway, Switzerland and Turkey. The Italian ENGL members are IZS LT and ISS, involved in GM food and feed analytical control, and CREA, focused on seed testing.
ENGL is coordinated by the EU Reference Laboratory for GM Food and Feed (EU-RL GMFF), established at the Joint Research Centre (JRC) of the European Commission.
The main mandate of the ENGL is to respond to the technical and scientific challenges that European enforcement laboratories face in the field of GMO analysis in the frame of the EU legislation. This is pursued through the organization of meetings and training events for exchanging experiences and scientists, and promoting knowledge and technology transfer within the network. The main output of the ENGL is the elaboration of guidelines and technical documents produced by ad hoc working groups on topical issues.
Networking activities are also the basis for the development of innovative analytical methods, new analytical strategies and project ideas stimulating interests in promoting co-operative research among EU Member States involving also research groups of third countries.

BPRACTICES project, an international experience

New indicators and on-farm practices to control honeybee transmissible diseases: the international network B-PRACTICES

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European beekeeping suffers significant regional differences in colony losses due to climate change and prevalence of diseases (EPILOBEE, 2014). The spread of the new honeybees parasite *Aethina tumida* found in 2014 in Italy will get worse the honeybee-health status, together with other important honeybee diseases.
The international network established with the "NEW INDICATORS AND ON-FARM PRACTICES TO IMPROVE HONEYBEE HEALTH IN THE AETHINA TUMIDA ERA IN EUROPE" project (BPRACTICES) aims to develop new management practices adopting new clinical methods, biomechanical and innovative biomolecular techniques.
The Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" is the coordinator of the consortium represented by research Institutes (University of Namik Kemal - Turkey, Agricultural Institute of Slovenia, Centro de Investigación Apícola y Agroambiental de Marchamalo - Spain, Austrian Agency for Health & Food Safety, Mississippi State University – USA, Istituto Zooprofilattico Sperimentale delle Venezie, University of Genova) and the International Federation of Beekeepers' Associations (APIMONDIA). Moreover, the project is carried out in collaboration with FAO and the EU Reference Laboratory for Bee Health.
The multi-actor and multi-disciplinary competencies of the partners will guarantee a holistic approach to find the best and most suitable innovative solutions to monitor and control the honeybee diseases.

Male hypofertility in Bluetongue virus infection

Sheep and Bluetongue virus: an animal model for studying the pathogenesis of hypofertility caused by Arboviruses

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Herein, Bluetongue virus (BTV) in sheep was used as an animal model for studying the pathological consequences of Arbovirus infection on male reproductive tract.
By using blood samples collected from naturally BTV serotype 1-infected sheep in the 2006 and 2013 epidemic waves, two different groups of fertile rams were subcutaneously infected and serially euthanized. Real time RT-PCR (OIE Manual, 2014) detected BTV-1 RNA in the testicle, epididymis, bulbourethral gland, seminal vesicles and prostate. By in-depth immunohistochemistry and confocal immunofluorescence, NS2 BTV protein was exclusively found in the endothelial cells of blood vessels in the intertubular space of the testicle and epididymis as well as in the prostate, between 5 and 11 days post-infection.
Histologically, the testicle displayed a severe degeneration of the germinative epithelium leading to azoospermia.
Vimentin, MX-1 interferon-induced protein, α-inhibin and β-hydroxysteroid dehydrogenase expression profile demonstrated that BTV infection causes infertility by impairing testosterone synthesis, similarly to what observed in mouse model experimentally infected with Zika virus and Mumps virus. Our results clearly underline the potential pathological effect of endotheliotropic RNA viruses on male fertility.

Metagenomic approach for tick's population

Analysis of tick's microbial populations to identify known and emerging pathogens: a metagenomic approach

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Ixodes ricinus is an important vector of pathogens, which are constantly evolving. This study aimed to characterize microbiota of *I.ricinus* collected in north-eastern Italy, by using targeted amplicon sequencing. The V1-V2 and V3-V4 hypervariable regions of 16SrDNA gene were amplified for single adult (n=17) and 10 pooled nymphs (n=40) and sequenced by Illumina MiSeq platform. Overall, 308 genera belonging to 20 bacteria phyla were found. Proteobacteria was the dominant phylum, followed by Actinobacteria, Firmicutes and Bacteroidetes. Results revealed complex microbial communities, included endemic pathogens, such as A*naplasma, Ehrlichia, Rickettsia* and *Borrelia*, as well as endosymbiotic and environmental bacteria.
Both regions (V1-V2; V3-V4) performed well in taxa identification. V3-V4 identified a larger number of species than V1-V2. Higher heterogeneity of taxa was identified from nymphs than adult specimens. V1-V2 and V3-V4 regions of 16SrDNA showed to be effective genetic targets to reveal a high bacterial diversity including rare taxa. As taxa results using the two regions were complementary rather than overlapping, the investigations of both regions are recommended.
How the microbial community dynamic of *I. ricinus*, as well as other arthropods, can modulate the vector susceptibility to pathogens and their transmission is a future scientific challenge.

Social epidemiology study on poultry biosecurity

Biosecurity in poultry farms. A pilot study of social epidemiology (RC 18/13)

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In the past two decades several avian influenza (AI) epidemics occurred in Italy. AI outbreaks took place mainly in the regions with high density of poultry farms (DPPA).
The project aimed to identify the major constraints that limit the continuous application of biosecurity measures at a farms level, combining both epidemiological and social research methods.
A representative sample of meat-turkey farms was selected in the province of Verona, which has the highest concentration of turkey farms in Italy. The holdings at higher risk of AI due to biosecurity issues were identified according to structural and managerial parameters. Then, turkey farmers' daily practices were analysed in order to discover routine behaviors that do not comply with the correct application of regulations and guidelines. The managerial, economical and psychological reasons underlying the lack in implementing biosecurity measures were highlighted.
These results set the basis for the study and implementation of preventive actions and for the definition of communication and training strategies useful to improve the application of appropriate biosecurity measures in poultry farms. The integration of veterinary expertise and social research methodologies was crucial to the understanding of both the turkey farmers' point of view and the organization of poultry farms in a DPPA.

A check list for welfare-biosecurity in dairy sheep

Welfare and biosecurity risk assessment in dairy sheep: development of a check list

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Starting from methodology EFSA (Welfare Quality project), the National Reference Centre for Animal Welfare has perfected a new system for the welfare assessment, based both on management-based measures (MBM) and animal-based measures (ABM). Ruminant Welfare Project, funded by the Ministry of Health, has the purpose to identify the main welfare risks for semi-extensive systems of dairy sheep through the development of a check list. It was decided to identify production categories (lactating sheep, dry sheep, weaned lambs, lambs up to 30 days, rams). Check list includes some macro areas: Area A – management and personnel procedures; Area B - facilities and equipment; Area C animal based measures, Area D bio-security, Area E great risks and alarm systems. Risk threshold (above which there is evidence of adverse effect) has been identified, as well as adverse effects on animal and risk measurement.
The check list has been tested in 36 dairy sheep farms of Sardinia and Sicily; the most severe identified risk factors have been in water availability (absence), inadequate dimensions of the troughs, structural deficiencies in facilities.

Sustainable shellfish aquaculture in the Mediterranean

From sea to fork: applied research for a sustainable shellfish aquaculture in the Mediterranean

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Bivalves, during the natural intense filtration activity, retain in their organism not only plankton necessary to their metabolism, but also bacteria, viruses and chemical contaminants, that may be present in the environment.
Contaminants eventually present in bivalves could be transmitted to humans by food consumption, causing a large variety of gastro-enteric syndromes, of varying extent. Food-borne diseases are currently a major public health problem and related diseases can spread and become epidemic.
Given the current global food marketing system, they can cause considerable economic damages.
Food illnesses resulting from bivalve mollusc consumption and caused by *Vibrio* (*V. parahaemolyticus, V. vulnificus,* etc), Hepatitis A virus or Norovirus are in constant increase.
In this view, applied research for a sustainable shellfish aquaculture in the Mediterranean is a strategic question of public health.
To reach the best "Consumers safety", It is important to study the environment (Shellfish water quality and ecological status for production areas), bivalve resources and interaction with new pathogens and contaminants.

STEC survey in cattle livestock

An integrated epidemiological strategy for the Shiga toxin-producing Escherichia coli: risk factors and clusters from a survey in cattle livestock

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The aim of the project was to understand the causal web related to the environmental contamination and dissemination of pre-harvest Shiga toxin-producing *Escherichia coli* (STEC) in cattle livestocks. We conducted a cross sectional study and we combined diagnostic, molecular, spatial and epidemiological tools. In the 94 randomly selected farms, we collected pooled faecal samples, soil and vegetables from crop field and vegetable garden. They have been analysed to detect and strain type STEC. To identify potential risk factors we carried out a survey administering 4 questionnaires to collect information about the managerial and structural features. Finally, the results served also to carry out a spatial analysis using SaTScan and a molecular cluster analysis based on pulsed-field gel electrophoresis . Our results highlight that the areas where human cases of Hemolytic-Uremic Syndrome have occurred in the last 10 years, overlap with those of STEC circulation in cattle livestock. Moreover some managerial routines and structural features are statistically associated with the risk of presence of STEC. We found that alive STEC are not transmitted into the field or garden by aged manure; therefore, the risk to human or animals from self-producted food or feed can be considered low. Finally molecular and spatial clusters allowed the detection of high risk areas.

Effects of contaminants on zebrafish's genome

Effects of contaminants detected in animal biological indicators of Sicily on the zebrafish's genome

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The research aimed to obtain data on the presence of toxic substances in dairy surrounding the Sicilian risk areas and to analyse the in vivo effects of these toxic agents in embryos and larvae of zebrafish. We examined 368 milk samples from cattle and sheep farms (50 for dioxins and 318 for heavy metals detection, respectively). The analysis of heavy metals has provided the development of an ICP-MS method. The dioxins levels were assessed by a HRGC/HRMS method. The results on milk samples revealed a significantly greater presence of dioxins in sheep milk, with average values near the limit of the Reg. EC 1259/2011. Ten samples that came from farms close to Bellolampo dump have detected concentrations greater than EC Reg. 1259/2011 limits. Only six samples of sheep milk have detected Pb concentrations over the LOD but below the limits imposed by the EC Reg. 1881/2006. The highest concentrations of dioxin detected in milk samples studied lead to a decrease in the survival of the larvae and malformations of the skeletal and circulatory systems.
Furthermore, an over-expression of genes involved in phenomena of environmental stress such as *hsp70, gadd45b, ATF3 CYP1A* was found. The data obtained in this study confirm the close relationship between environmental factors and health effects of the population.

Exosome secretion by *L. infantum*

Exosome secretion by Leishmania infantum: modulate innate and adaptive immune responses and create an environment permissive for early infection

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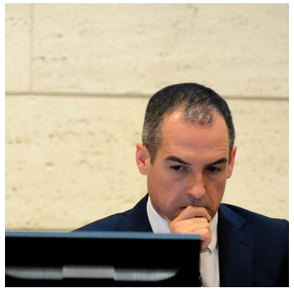
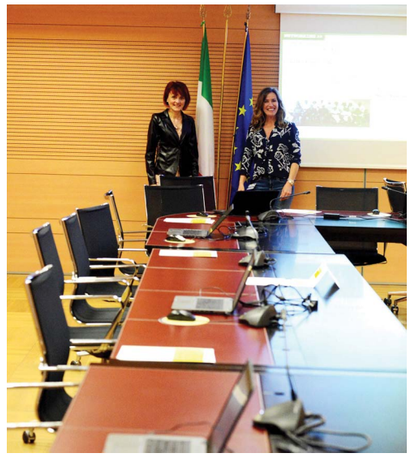
Several studies demonstrated the role of exosomes in intercellular communications, however, the mechanisms involved in *Leishmania* remained elusive. Exosomes were collected by *L. infantum*-conditioned medium by ultracentrifugation. IL10, IFNγ, IL1α, IL1β, IL12, IL4 and IL18 secretion were evaluated by ELISA kit. We first purified extracellular vesicles shed by *L. infantum* on a sucrose gradient and later we characterized these extracellular vesicles for HSP70, HSP83/90 and acetylcholinesterase by Western blot assay. NanoSight nanoparticle tracking analysis revealed an average of the mode value of 76±5 nm for promastigotes and 94±5 nm for amastigotes exosomes. Data demonstrated that *L. infantum* released exosomes. The treatment of U937 cells with 10 µg/ml of promastigote and amastigote exosomes showed an increase in motility. We showed also an overproduction of IL10 by macrophages after treatment with exosomes that support parasite persistence and disease establishment, while exosomes limited the production of IFN-γ and IL12 that block the parasite killing and host protection, as well as the production of IL1α, IL1β and IL4. Regarding IL18 production, that is involved in the Th1-type immune responses, exosomes determined a reduction in the production of IL18 in monocyte cultures. We demonstrated that exosomes can regulate the immune system and exacerbate disease outcome.





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