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Chairperson: S. Jutzi, FAO

TOPIC: Setting the Scene

A vision of gene-based technologies for the livestock industries in the third millennium

E.P. Cunningham

Trinity College Dublin, Ireland

E-mail: epcnnghm@tcd.ie

While knowledge of DNA structure is now fifty years old, technologies for intervening in genetic structure effectively date back just a decade. From small beginnings with restriction enzymes, we have in the last ten years had an explosion of techniques for isolating, amplifying, reading and inserting DNA. These techniques have been scaled up and automated to make possible mass genotyping. Coupled with developments in information management, this wealth of data facilitates prediction of genetic structure and function, and thus accelerates the pace of knowledge accumulation. This explosion in knowledge has been compared in significance to the development of the Periodic Table one hundred years ago, and to the expansion of horizons to encompass the whole globe in the 16th century [1,2].

The first complete genome sequence of an organism was for yeast in 1996. Since then, the much larger task of doing a complete human sequence has been completed. Those of all domestic animals are following rapidly. It will always be impossible to foresee the full potential, both positive and negative, of such an explosion in knowledge. However, already aspects of gene-based technologies are beginning to have an impact in the livestock sector.

The first, and most obvious, concerns the feed supply, which constitutes 50–75% of total costs in many livestock systems. Production costs for corn and soya bean are being reduced by genetic modification of the crop for herbicide and insect resistance. Corn has also been modified with the effect of reducing phosphorous and nitrogen excretion in swine and poultry, and also to provide more valuable amino acid balance.

Genetic modification of the animal is also possible. Most dramatically, the insertion of the grown hormone DNA in fish accelerates growth. However, in this and all other cases the genetic modification of animals had produced profound physiological disturbances. On the other hand, the administration of GM-produced growth hormone to dairy cows in now routine in the US and several other countries. This is not permitted in Europe, where the attitude to all GM technologies has been much more cautious.

Control of disease in animals using GM technologies is much less contentious. As pressure to reduce antibiotic and drug use increases, genetically modified vaccines with improved specificity and distinguishable from natural infections are already in use. DNA typing is helping with rapid and precise diagnosis. In addition, the interaction of some pathogens (e.g. scrapie) with the genotype of the animal calls for the application of DNA technologies.

Following the BSE epidemic in Europe, safety of livestock-derived foods is high on the research and regulatory agendas. DNA techniques are already in use for tracking of sources of salmonella and E.coli outbreaks, as well as for traceability of product in the food chain.

The possibilities of genetically improving animals for disease resistance or production traits is being pursued along two parallel tracks: using marker technology to augment normal selection programmes, and using functional genomics to target DNA sequences of known or suspected function.

Finally, animals have been genetically modified to contribute directly to human health through the production of therapeutic proteins in their milk, or to produce compatible tissues for human transplants.

Not all of these developments will find their place in livestock production systems, for reasons of cost, consistency, ethics or public acceptability. However, they present such an array of possible gains in health and productivity that we can speak of a revolution in livestock technology.

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TOPIC: Setting the Scene

Challenges and opportunities for controlling and preventing animal diseases in developing countries through gene-based technologies

M.H. Jeggo

Australian Animal Health Laboratory, CSIRO Livestock Industries, PMB 24, Geelong,Vic 3220, Australia

E-mail: Martyn.Jeggo@csiro.au

The livestock revolution so robustly and frequently described in the past five years, is argued to provide a real opportunity for the rural livestock keeper in developing countries to escape the poverty trap, move away from subsistence farming and enter the more rewarding areas of farm enterprise and income generation. To do so though, will require more than merely acknowledging this marketing opportunity. It will be essential to address the many constraints and critical risks that constantly face rural farming in developing countries. Of these, livestock disease rates as one of the most challenging. However, for effective participation in the livestock revolution it will be essential that livestock disease is either controlled or prevented.

For the livestock producer in developing countries, many of the life threatening diseases that have been eradicated from the developed world area are ever present and the extent and range of production-limiting diseases are considerable. The situation is further compounded since in many cases veterinary services and other animal health delivery systems are either non-existent or ineffective. For some time donor organisations have been driving countries in transition to privatise services such as animal health delivery. The current situation is the virtual elimination of functioning State veterinary services without replacement by a private system – and certainly not in rural areas.

The elimination of the major killer diseases of livestock in the developed world was achieved, for the most part, through considerable State investment, extensive veterinary input and a large share of public money. Such resources are certainly not available today in most developing countries. No wonder therefore that diseases such as Contagious Bovine Pleuropneumonia, African Swine Fever ad Foot and Mouth Disease continue to exist endemically in most poorer regions of Africa and elsewhere.

In terms of the production limiting diseases, control of these in most developed countries is through a mixture of management and therapy. The former requires knowledge and considerable local understanding and the latter resources and supplies. Both of these are limited in the developing country situation, particularly in a rural setting.

Finally but of equal importance, is the considerable negative impact of the presence of disease on trade. Those trading internationally in livestock and livestock products are demanding a clear demonstration of freedom from an ever increasing list of diseases. Participation within national markets is increasingly constrained by the need to provide safe products to urban communities. But in the absence of effective surveillance it is often not possible to determine what disease is present, let alone meet the rigours of demonstration of freedom from a particular disease or infectious agent.

Given this complex of challenges, can gene-based technologies really make a difference to the management of livestock disease for the producer in developing countries? To be effective in the developing country situation, any intervention must be relatively simple, cost effective, sustainable and convincing. Can this be delivered? Perhaps an insight can be gained from an appreciation of the fundamental nature of gene-based technologies. Inherent in the approach is the recognition that the gene is the basic building block of biology. Management and manipulation of the gene therefore enables us to design and direct an endless array of precise solutions, whether this be designer livestock, genetically engineered biological products or genetically altered organisms.

Without doubt, the availability of livestock resistant to disease, or at least one or two of the major diseases affecting livestock in a particular region, is a simple and applicable solution to the developing country situation. Attempts to understand the genetic basis of trypanotolerence are still on-going but if successful would enable livestock production in large areas of Africa currently restricted by the presence of trypanosomosis. Another example would be the demonstration of resistance to internal parasites by certain breeds of sheep. Locating the genetic basis of this could be revolutionary in the management of this particular disease risk. As work starts on sequencing both the bovine and the ovine genome, the future opportunities for designing livestock resistant or tolerant to a range of diseases looks highly promising.

Looking at the causative agents of livestock disease, the ability to exquisitely alter these to better understand the way they cause disease is providing a fast track to developing ways of control or eradication. For example identifying the gene coding for a protein that allows cell attachment would permit genetic engineering to delete this gene from a particular disease causing organism. This could then form the basis of a vaccine that is safe but highly efficacious. Even more exquisite is the incorporation of this particular gene into another carrier such as harmless virus, bacteria or other similar organism. The expression of the protein can then be used to evoke an immune response in a susceptible host without any risk of disease. Many groups around the world are currently exploring these concepts for a wide range of causative agents and a variety of different expression systems.

All the above is already feasible but not without problems. Firstly and perhaps foremost, the acceptance of genetically modified organisms (GMOs) by consumers is far from complete. Considerable debate has taken place in the plant industry and whilst partially applicable to animals, the issues are dissimilar in many areas. In developed countries a great deal of research is taking place in this area but it is still unclear how well the consumer will accept genetically modified animals as a food source, or products from animals protected by genetically engineered vaccines. This debate needs to urgently take place. Secondly, this technology is not without considerable cost. If it is to be harnessed for those diseases that most affect rural livestock producers in developing countries then a new paradigm of global research partnerships and funding will be required. There will need to be a recognition that those diseases that continue to affect livestock in the developing countries, will continue to pose a risk to all livestock. Their eradication or control in the developing world will be an advantage to all livestock producers. Their continued presence in certain regions might seem to be an advantage to producers free of disease but recent outbreaks of Foot and Mouth Disease in Europe clearly demonstrate the fallacy of this approach.

Gene-based technologies have the potential to deliver workable solutions to the management of animal diseases and these can be considered particularly applicable to the developing country situation. Success though will depend on consumer acceptance of this approach and the use of innovative global partnerships to undertake the enabling research.

SESSION I: GENE-BASED TECHNOLOGIES APPLIED TO LIVESTOCK GENETICS AND BREEDING

Chairperson: J. Gibson, ILRI, Kenya

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Molecular genetics and livestock selection: Approaches, opportunities and risks

J.L. Williams

Department of Genomics and Bioinformatics Roslin Institute, Roslin Midlothian EH25 9PS, UK

E-mail: john.williams@bbsrc.as.uk

There are over 1,200 million cattle worldwide that provide a source of food, motive power and clothing. Cattle were first domesticated about 12,000 years ago with both the archaeological [1] and molecular evidence [2,3] suggesting that this occurred in the Near East and that domesticated cattle then spread to Africa and Europe. Traditionally breeding was carried out at a local level, often using a limited number of shared bulls. The selection of individuals with particular characteristics suited to local environments, needs and preferences led to the emergence of distinct breeds with characteristic phenotypes. In 1993 there were 783 cattle breeds worldwide [4], although the definition of a breed is often vague.

With the introduction of artificial insemination (AI) in the more developed countries during 1950s particular bulls with desirable characteristics were more widely used in preference to local bulls. The use of AI, coupled with improvements in management in Europe and North America, allowed rapid progress to be made in the improvement of simple production traits. Breed improvement has been further enhanced by the development of statistical methods to maximize genetic gain achieved by selection on traits that can be readily measured. Consequently, where the economic environment supports high input agriculture, there has been a dramatic increase in milk yield and meat produced from the improved stock. The unfortunate consequence of intensive selection in these areas has been the reduction of genetic diversity, both within the selected breeds, as the superior individuals within these breeds have been used as breeding stock, and also through the replacement of traditional breeds. While the use of improved breeds in areas advantaged by good environmental conditions and a favourable economic climate has allowed the increase in production, all-be-it with the penalty of lost diversity and damage to the environment occasioned by intensive farming practices, in less developed and environmentally less favoured areas the use of these breeds presents a greater cause for concern. Local breeds are usually adapted to survive in their local environments eg with increased tolerance of extremes in temperature or in the face of particular disease or parasite challenge. Attempts at the inappropriate and/or unmanaged introduction of improved dairy breed into some areas has met with disastrous consequences. In 1993, 112 of the 783 cattle breeds worldwide were at risk of extinction. The greatest risk is the replacement of local stock that are adapted for survival in the face of disease challenge with disease sensitive stock in areas where standards and resources to provide extensive veterinary care are not available.

Much work has been carried out over the past 10 or so years to produce genetic and physical maps of the bovine genome [5,6,7]. In the first instance these maps were composed predominantly of anonymous markers, but more recently genes, and expressed sequence tags (ESTs) have been added to the genome maps of cattle. Use of genetic maps together with other molecular genetic approaches, like micro-array technology to examine gene expression, will enable the genes having a major influence on a wide range of traits to be identified. Most

production-associated traits are under the control of several genes, which have varying levels of effect on the trait, and are generally referred to as Quantitative Trait Loci. To date considerable success has been reported in localising QTL for a wide range of traits [8,9], however two notable successes have identified the major genes involved in increased muscling [10,11] and milk production [12].

Knowledge of the loci controlling individual traits will allow the direct selection for favourable alleles at these loci. In the first instance this can be done by marker-assisted selection with markers linked to the gene involved in the trait. However, ultimately, knowledge of the allelic variation within that gene will allow more efficient selection to be carried out. There are several advantages of using markers in selection programmes, rather than relying on phenotype based selection. In using markers it will be possible to introgress favourable alleles for particular traits from one breed into another, taking advantage of specialised characteristics of different breeds, for example to maintain disease resistance while increasing production. By using information on the markers spanning the genome, as well and the genes under selection, it will also be possible to maintain the widest possible genetic diversity within breeds. Thus considered and well-managed use of molecular information will help preserve the genetic diversity of cattle populations.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

First report on the state of the world's animal genetic resources: Views on biotechnologies as expressed in country reports

R. Cardellino, I. Hoffmann, K.A. Tempelman

Animal Production and Health Division, FAO, 00100 Rome Italy

E-mail: Ricardo.Cardellino@fao.org

The Food and Agriculture Organization of the United Nations (FAO) has been requested by its member countries to develop and implement the Global Strategy for the Management of Farm Animal Genetic Resources. The global livestock sector is faced with the challenge of the fast increasing demand for animal products in developing countries. FAO has estimated that demand for meat will double by 2030 (2000 basis) and demand for milk will more than double in this 30-year period. On the other hand, animal genetic resources worldwide are disappearing rapidly. Over the past 15 years, 300 out of 6000 breeds identified by FAO have become extinct. Successful genetic improvement programs in adapted indigenous breeds are less than a handful. Although in many developing countries there have been considerable efforts in training professionals in animal genetics, breeding programs applied to livestock under low input farming systems have largely failed. As part of this country-driven strategy for the management of farm animal genetic resources, FAO has invited 188 countries to participate in the First Report on the State of the World's Animal Genetic Resources, to be completed before 2006. To date 145 countries have accepted to submit country reports. The drafting of the country report is under way, with target date August 2003. It must be stressed that the national reports are basically strategic policy documents, and as such the FAO-provided blueprint, approved by FAO's governing bodies, is designed to answer three questions regarding the countries animal genetic resources: where the country is now, where it needs to be, and how to get there (FAO, Animal Genetic Resources Information, 30, 2001; www.fao.org/DAD-IS). The country reports should reflect problems, needs and opportunities. They are organized in five parts. Part 1 reports on the state of genetic resources in the farm animal sector covering both *in-situ* and *ex-situ* conservation aspects, as well state of the art of techniques being used, in the context of production systems and socio-economic conditions of each country. Part 2 should describe the changing demands on the farm animal sector and the implications for future national policies in conservation and utilization of animal genetic resources. Part 3 is a review of the state of national capacities related to farm animal genetic resources and an overall assessment of capacity building requirements. Part 4 should identify national priorities covering diverse fields of activity, animal species and breeds, as well as short and long term needs for institution-building, research, information systems, policy, legislation and regulations. Part 5 deals with recommendations for international cooperation, indicating the areas, levels and mode of cooperation which the country wishes to follow, and proposed contributions and requirements. It is expected that countries will include views on biotechnologies in relation to farm animal genetic resources in their reports, within the context of the recommended report structure, particularly in parts 1 and 4. The analysis of country reports may also serve to estimate the gaps in biotechnology application between developed and developing countries.

In September 2003, 41 country reports had officially been submitted to FAO. For this paper, 30 country reports representing all regions were analysed with regard to information on

biotechnologies used in animal breeding and reproduction, in conservation of animal genetic resources and for commercial uses. In addition, the information gained from discussions in regional workshops in Latin and Central America, covering 20 countries, was included.

The West Africa region was represented by 12 country reports. With few exceptions, all use AI (artificial insemination), mostly in cattle, but at a very low percentage. No ET (embryo transfer) is used, and limited molecular characterization has been carried out, mainly as part of international development projects. Priorities were expressed in capacity building and training on AI and ET in the context of performance and genetic evaluations of livestock, and also in molecular techniques for the characterization of local animal genetic resources. Major constraints to reach priorities are financial resources and the lack of skilled human resources to undertake in-country training.

Eastern Europe was represented by seven country reports. AI is widely used for several species, mainly cattle, and often connected to national AI programmes and activities of breeders associations. ET is used in a limited number of cases, or it is in national plans. Many countries have legal instruments to regulate AI and ET. Most countries undertake some research on molecular characterization of local breeds. Some countries have gene banks, which often still need further development. Priorities identified are: gene banks, technical expertise in AI, ET and cryoconservation, and the major limiting factor mentioned is availability of financial resources.

Countries of Western Europe, represented by eight country reports, have national AI programmes in place and AI is used widely throughout the farming sector. There are national and private gene banks in all countries for both commercial and conservation purposes. Priorities expressed are in cryoconservation of genetic material, expansion of gene bank activities, breed characterisation (phenotypic and molecular), and adaptations required in national and international policies on the use and conservation of genetic material.

The Near East was represented by only one country report. AI is mostly used in cattle, no ET is used, and work is in progress regarding regulation on the use of GMOs (genetically modified organisms). Priorities were expressed on creation of ET facilities, training in new biotechnological methods and establishment of gene banks. Major constraints are funding and the lack of skilled human resources.

Two countries in Asia reported that AI is used but no ET facilities exist. Priorities were expressed in training, expanding the national gene pool, and updating existing regulations for conservation AnGR. China reported use of AI and ET, and microsatellite DNA technology. No specific priorities were mentioned but the country is implementing plans for a centre for AnGR germplasm.

As a result of a regional meeting on country report preparation, national and regional priorities, ten countries of Central America and the Caribbean, and Mexico identified AI as means of diffusion of genetic improvement, and some mentioned ET as rarely used but of interest. Emphasis, both as needs and actions was put on molecular characterization and cryoconservation of local breeds, especially criollo cattle, sheep and goats. All countries identified the need for national programmes in conservation and utilization of AnGR, including development of biotechnology and updated legislation. A regional meeting with ten South American countries identified similar interests and priorities in biotechnology.

From country reports analyzed to date by FAO, it is concluded that AI is the most common biotechnology used developing countries and needs are expressed for training and expansion.

Often AI is introduced without proper planning and is seen as a potential threat to the conservation of local breeds. Although ET use is mentioned and the desire for its introduction or expansion expressed, no clear objectives for this technique are mentioned. All countries have expressed a wish for the introduction and development of molecular techniques, often as a complement to phenotypic breed characterization. Cryoconservation was identified as a priority by all countries and gene banks were recommended, but at the same time funding remains a major constraint. When GMOs are mentioned it is mainly to express the lack of proper regulations and guidelines for their eventual production, use and exchange. It is, however, not clear in all cases whether the technologies used are a sensible part of an overall genetic improvement strategy.

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Development of germline manipulation technologies in livestock

B. Whitelaw

Department of Genomics and Bioinformatics Roslin Institute, Roslin Midlothian EH25 9PS, UK

E-mail: bruce.whitelaw@bbsrc.ac.uk

Breeding, based on conventional selection, has been the mainstay for livestock genetic improvement for more than 70 years, and is still so today. Sophisticated statistical and computing tools now enhance conventional genetic selection, nevertheless traits such as fertility and disease resistance have still proved difficult to improve. Gene transfer technology (transgenesis) offers the potential, as yet unproven, to modify these types of traits.

A transgenic animal carries integrated DNA sequences in its genome. The introduced DNA can be derived from species other than the host and can be modified *in vitro* prior to being introduced into the germline. Therefore, transgenic livestock overcome some of the limitations of classical animal breeding regimes, where importation of genes by crossbreeding is limited to those traits already present within a given species.

The most used method for introducing genes into the germline of animals involves the direct microinjection of DNA into the pronuclei of fertilised eggs. Pronuclear microinjection, although conceptually simple - a fine needle is used to pierce the pronucleus and the DNA is injected - requires special equipment and considerable dexterity on behalf of the person involved. By adapting the techniques employed for gene transfer in mice, pronuclear microinjection has been used to generate transgenic farm animals.

The first attempts to genetically modify livestock owe much to pioneering experiments in mice, where the introduction of growth hormone gene dramatically increased the growth rate and final size of the animals. By contrast, the same approaches in livestock did not prove successful. Indeed, in terms of modifying livestock for agricultural purposes, most of the early expectations were not realised. Rather, it has been the development of novel uses of livestock, particularly for human medicine, that has led the way and advanced this technology. For example, targeting the expression of human proteins to milk and generating animals for organ transplantation (xenotranplantation).

The majority of transgenic livestock have been produced using this method but it only allows gene addition. For gene removal the integration of the introduced transgene has to be targeted to the gene of interest. This requires a relatively high frequency of homologous recombination that occurs in embryonic stem (ES) cells; it does occur in somatic cells but is substantially less efficient. ES cells once introduced into an embryo can contribute to all cells types of the adult animal. Thus the desired genetic modification can be identified and selected for while the cells are grown in culture. This enables vastly more sophisticated genetic changes to be engineered, including gene knock-out. Unfortunately, ES cells have only been isolated for mice and even in this species there are only a few permissive strains.

The lack of methods for gene knock-out in livestock was the driving force leading to the development of nuclear transfer technology. This technique was made famous through the generation of ‘Dolly’. It is fair to say that although catching both the scientific and media in a

frenzy of cloning issues, perhaps the greatest legacy of nuclear transfer will be the development of cell based therapeutic strategies based on stem and somatic cells to treat human genetic diseases. Although 'Dolly' is not herself transgenic, this technique does offer the potential to make transgenic animals more efficiently than by using the pronuclear microinjection method. This is primarily because all founder animals are transgenic and a flock/herd of clonal animals can be produced within one generation. More importantly, nuclear transfer uses cells grown in culture therefore, for the first time, allows precise changes to the germline of ruminants to be attempted.

This has now been shown to be possible, a sheep carrying a disruption of the PrP gene, a targeted insertion into the collagen gene and pigs that have a deletion of the α(1,3)galactosyltransferase gene having been produced. However, the generation of knock-out transgenic livestock is a hugely demanding technical and financial undertaking. First, the techniques utilised efficiently in mice, do work in livestock cells but are considerably less effective. Second, the stringent selection and extended *in vitro* culture required for targeting somatic cells (the target for the genetic modification prior to nuclear transfer) reduces their developmental potential, compounding the high cost and low efficiency of nuclear transfer. Finally, nuclear transfer in livestock is beset by the losses *in utero* and after birth, having both a welfare and economic cost. In summary, yes we can generate gene knock-out livestock using nuclear transfer but unless there is a conceptual leap in our understanding of the technique it will not become common place.

At the beginning of last year a new approach to transgenesis was reported for the generation of transgenic mice and rats. Two groups demonstrated that lentivirus (specialised retrovirus) vectors can be used to efficiently introduce foreign DNA into the germline. There appears two dramatic advantages to this technology which make it very appealing for use in livestock. Only a fraction of the resources needed for conventional pro-nuclear injection would be required, given the DNA transfer efficiencies reported in the mouse. Even more appealing is the simplicity of delivery, abrogating the need for specialised equipment. If this method is applicable to livestock – and there is no reason to think it will not be – then previously only dreamed of transgenic applications may become reality, creating tremendous opportunities for the genetic modification of livestock.

Perhaps the most exciting goal envisaged is the engineering of resistance to infectious disease, through combining the efficiency offered by lentiviral vectors with the emerging molecular tool of RNA interference (RNAi which is based on siRNA molecules). We anticipate the generation of transgenic animals that constitutively express RNAi vectors targeting knockdown of a pathogenic virus and/or its transcription products, thereby engineering cellular resistance to infection. These animals will be of great value in dissecting disease progression, the challenge will be to evaluate their potential in commercial breeding regimes.

It is over 18 years since the first demonstration that transgenic livestock can be produced. Subsequently, the development of nuclear transfer technology was set to revolutionise this area of biotechnology, since it overcame the lack of livestock ES cells. Certainly it does enable gene-targeting approaches to the generation of transgenic livestock to be performed; although this produces a recessive mutation in the first instance. Both pronuclear injection and nuclear transfer are inefficient methods for modifying the germline of animals. The recent development of new methods of transgenesis based on viral vectors again offers an avenue to overcome the current restricted application of transgenesis in livestock. Perhaps we are now at the start of a new era in livestock transgenesis?

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Polymorphism in Sahiwal breed of zebu cattle revealed using synthetic oligonucleotide markers

Shashikanth, B.R. Yadav

Livestock Genome Analysis Laboratory, National Dairy Research Institute, Karnal – 132 001, India

E-mail: bry@ndri.hry.nic.in

Livestock improvement greatly depends on the exploitation of DNA level polymorphisms. Specific sequences of DNA are being used as genetic markers to identify loci responsible for expression of complex traits both in man and animals. Presently several classes of markers are available namely RFLPs, AFLPs, VNTRs, STRs, SNPs etc. DNA Sequences with basic repeat motifs of two to six nucleotides can be synthesized and hybridized to genomic sequences from a variety of species to produce multilocus band patterns. Several such oligonucleotide sequences have been reported to be useful in producing highly polymorphic DNA fingerprints in a variety of species. These markers have short-range uses such as parentage determination, individual identification, detection of twin zygosity, etc., and long-range applications such as gene mapping and marker assisted selection

The degree of polymorphism elucidated from a probe or a marker may differ from species to species depending on probe-species combination. It is important to screen DNA markers for their informativeness and polymorphism for various domestic species of animals before considering them for further use. In literature several synthetic probes having the core sequences of (AT) (GT), (GC), (CAC), (GAA), (GGAT), (GACA), (TGG), and (GATA) have been reported for DNA fingerprinting of a variety of species of animals. However, the indigenous Zebu cattle, which constitute major proportion of Indian cattle population has poorly been explored with DNA-based markers. In this study, four different oligonucleotide markers were screened for their usefulness as markers in Zebu cattle.

The investigations were carried out on genomic DNA of randomly selected unrelated (15 animals) and from two sire families (11 animals) of Sahiwal breed of Zebu cattle maintained in a herd at National Dairy Research Institute, Karnal. Oligonucleotide probes were custom synthesised and used after radio-isotopic labelling with (γ^{32}P) dATP ^{32}P using the enzyme polynucleotide kinase by the standard procedure. Hybridization of labelled oligonucleotide probes to genomic DNA on Nylon membranes was carried out at 45°C for probes $(GTG)_5$ and $(TCC)_5$, 43°C for $(GT)_8$ and 65°C for $(GT)_{12}$. Post-hybridization treatments and autoradiography were carried out and size of each fragment on X-ray film, i.e. DNA fingerprint, was estimated using computer software GelBase (UVP, UK). Number of total bands and shared bands in the fingerprints of each individual were recorded in the range of 2.5 to 23.0 KB. Number of bands, average band sharing rate (BS), mean allelic frequencies (a) and heterozygosity (h) level were calculated.

All four probes used produced multilocus fingerprints with differing levels of polymorphism. Means of number of bands per individual, band sharing rate, allele frequencies and heterozygosity was calculated. The probes $(GT)_8$, $(GT)_{12}$ and $(TCC)_5$ produced fingerprinting patterns of medium to low polymorphism whereas the probe $(GTG)_5$ produced highly polymorphic pattern. The probe $(GT)_8$ probe produced as many as 32 bands in resolvable

portion of the gel. However, nearly 40% of the bands were shared by all the individuals hence, the average bands sharing rate was found to be high. High band sharing rate in this study indicate that the animals examined might be genetically more homogeneous with respect to $(GT)_n$ sequences. Comparison of average number of bands obtained between different probes reveal that the probe GT_8 hybridized to more number of fragments than the other probes. This result indicates that GT_n are more abundant in zebu cattle genome compared to other sequences studied. The probe $(GT)_{12}$ produced a multilocus fingerprints with lower level of polymorphism in comparison with $(GT)_8$ fingerprints. Mean number of bands and polymorphism were low as compared to $(GT)_8$ fingerprints. Variation in the nucleotide constitution of repeat sequences and differences in hybridization and stringency conditions could be the reason for variation in banding pattern of same core sequences of differing length. The probe TCC_5 produced multilocus polymorphic fingerprints. The level of polymorphism was low as revealed by high mean band sharing values of 0.75. The reason for this deviation from the present observation could be the variation between genome of *Bos taurus and Bos indicus.* Alternatively it is possible that H*inf*I restriction sites are adjacent to TCC_n sequences are conserved while there may be variation in H*ae*III restriction sites. The probe GTG_5 produced highly polymorphic DNA fingerprints. The number of bands ranged between 9-17 with average band sharing of 0.48. The probe GTG_5 or its complementary sequences CAC_5 produced highly polymorphic fingerprints. High heterozygosity level obtained in this study and low level of mutation rate associated with the sequences indicate that the probe can be used for analyzing population structure, parentage verification and as a marker to identify loci controlling quantitative traits, disease resistance, fertility etc.

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Genetic diversity and differentiation of Mongolian indigenous cattle populations

B. Lkhagva[a,b], J.W. Ochieng[a], D.H. Yoon[c], O. Hanotte[a], H. Jianlin[a]

[a] International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi, Kenya

[b] Mongolian State Agricultural University, Zaisan, Ulaanbaatar 210153, Mongolia

[c] National Livestock Research Institute, RDA, 441-350, Suwon, Korea

E-mail: sukhbaatar@yahoo.com

Livestock production plays an important role in Mongolian economy. Over the last decade it has contributed to around 80–90% of the gross domestic agricultural products and to 30% of the revenues generated from exportations [1]. Cattle is one of the five tranditional and most important livestock species of Mongolia together with horse, sheep, goat and camel. Out of a total of 1.57 millions Mongolian cattle, 1.55 millions supposedly belong to three indigenous *Bos taurus* cattle breeds, namely Mongol, Selenge and Khalkhun Golun, all herded under extensive pastoral systems. Indigenous Mongolian cattle are generally small but look sturdy and strong. They have a well-off coat of hair, solid forward looking shoulders and short stubby snouts, and they are used for meat, milk and transport. Beef production contributes to 30% of the total meat supply in Mongolia. The Mongol breed is by the far the commonest with 1.53 million animals and it is found almost throughout the country. The Selenge breed, found in Selenge province and numbering 9000 heads, was developed in middle of the 20th century by crossing the Kazakh Whiteheaded with the local Mongol cattle. The Khalkhun Golun breed was developed from local Mongol cattle and it is distributed in Eastern and Sühbaatar provinces with about 10,000 heads [2] Until now, to the best of our knowledge, only a single population of Mongolian cattle has been studied with microsatellite DNA markers [3] and no information is available on the genetic relationship between the Mongolian indigenous cattle breeds.

In this study, we collected samples from two populations of the Mongol cattle (sampled at Ikhtamir soum in North Hangay province and Tsogt soum in Govĭ Altay province) and one population of the Khalkhun Golun cattle (sampled at Tumentsogt soum in Sühbaatar province). Samples were characterised with nine microsatellite markers *MGTG*4B*, ILSTS*005*, ILSTS*006*, ILSTS*008*, ILSTS*023*, ILSTS*028*, ILSTS*036*, ILSTS*050 and *ILSTS*103. To assess the genetic diversity and relationship of Mongolian cattle populations with breeds from neighboring countries and exotic breeds, data from the ILRI cattle genotyping database were included. More particularly, we used previously obtained data from Asian taurine (Hanwoo, Yanbian and Japanese Black), two European taurine (Friesian and Charolais), two African taurine (Baoulé and N'Dama) and two zebu breeds (Sahiwal and Ongole). For each breed, observed (*Ho*) and expected (*He*) heterozygosities as well as the mean number of alleles (MNA) across the nine loci were calculated (TABLE I) [4] between pairs of populations were also estimated and a UPGMA tree was constructed (Figure 1).

The heterozygosities (*Ho* and *He*) in Mongolian cattle populations are similar to those obtained in Northeast Asian taurine breeds but the values are higher compared to the ones obtained for the European and African taurine breeds. The Mongol cattle in North Hangay has

the highest corrected MNA value (all animals or 28 animals only). The UPGMA tree, built with the Reynolds' genetic distances, shows all six Northeast Asian cattle populations clustering into one group linked to the two European taurine breed. Interestingly, the two populations of the Mongol cattle are not closely related to each other. However, bootstrap values between the Northeast Asian taurine breeds, with the exception of the bootstrap value between Yanbian and Hanwoo, are relatively low, therefore the relationship between the Northeast Asian populations should be taken with caution. *Fst* values between the three Mongolian cattle populations are significant ($P < 0.01$), with the Govĭ Altay population being more differentiated from the North Hangay population than from the Khalkhun Golun breed (data not shown). Our data suggest that the traditional classification of Govĭ Altay and North Hangay populations as one breed, the Mongol cattle, should be revisited.

TABLE I. HO, HE AND THE MNA ACROSS THE NINE LOCI

Populations	Countries	Number of samples	Ho ± SD	He ± SD	MNA ± SD (all animals)	MNA ± SD[1] (28 animals)
North Hangay	Mongolia	44	0.726 ± 0.032	0.641 ± 0.026	7.78 ± 2.28	7.09 ± 2.14
Govĭ Altay	Mongolia	40	0.639 ± 0.056	0.619 ± 0.026	6.11 ± 2.52	5.63 ± 2.37
Khalkhun Golun	Mongolia	40	0.673 ± 0.038	0.658 ± 0.026	6.44 ± 2.13	6.03 ± 2.09
Hanwoo	Korea	77	0.635 ± 0.051	0.699 ± 0.020	7.00 ± 2.45	5.48 ± 2.07
Japanese Black	Japan	30	0.673 ± 0.037	0.644 ± 0.031	6.56 ± 2.01	6.38 ± 1.95
Yanbian	Northeast China	30	0.635 ± 0.041	0.639 ± 0.032	6.00 ± 2.24	5.85 ± 2.20
Friesian	Netherlands	35	0.644 ± 0.036	0.632 ± 0.028	5.78 ± 2.33	5.61 ± 2.29
Charolais	France	33	0.604 ± 0.035	0.594 ± 0.029	4.67 ± 1.94	4.57 ± 1.90
Baoulé	Burkina-Faso	35	0.547 ± 0.047	0.506 ± 0.029	4.56 ± 1.88	4.34 ± 1.82
N'Dama	Guinea	35	0.509 ± 0.053	0.535 ± 0.029	3.89 ± 1.45	3.77 ± 1.39
Sahiwal	Pakistan[2]	35	0.695 ± 0.037	0.656 ± 0.027	6.11 ± 1.27	5.89 ± 1.28
Ongole	India	28	0.752 ± 0.026	0.657 ± 0.031	5.78 ± 1.39	5.78 ± 1.39

[1] Mean value after 250 re-sampling; [2] Sampled in Kenya.

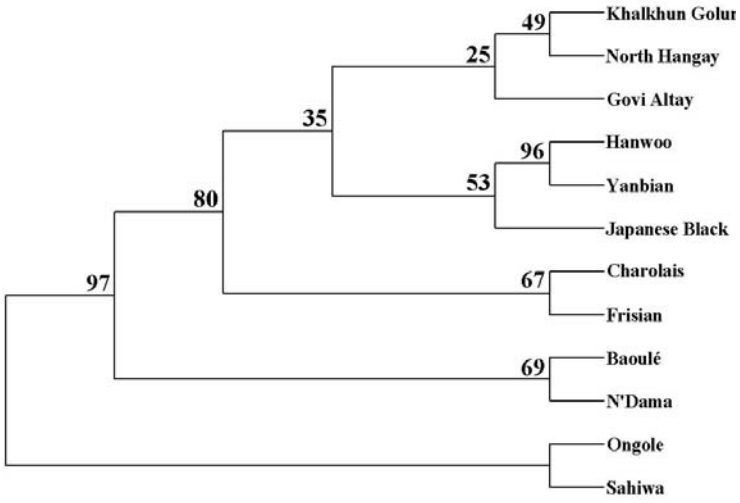

FIG.1. UPGMA tree, constructed with the Reynolds' Fst genetic distances, showing the relationships among the 12 cattle populations. Numbers indicated bootstrap values in percentage after 1000 resampling. Sahiwal and Ongole were used as outgroup.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Genetic diversity and relationships of Vietnamese and European pig breeds

E. Melchinger[a], T.D.T. Nguyen[b], A.W. Kuss[a], T. Peischl[a], H. Bartenschlager[a], V.C. Nguyen[b], H. Geldermann[a]

[a] Department of Animal Breeding and Biotechnology, Institute for Animal Husbandry and Animal Breeding, University of Hohenheim, D-70593 Stuttgart, Germany

[b] Institute of Biotechnology (IBT), National Center for Natural Science & Technology,18-Hoang Quoc Viet Rd, Cau Giay, Hanoi, Vietnam

E-mail: tzunihoh@uni-hohenheim.de

East Asia contains more than 50% of the world's pig population and Europe about 30% (according to FAO inventory [1]). Both indigenous resources were domesticated from different sub-species and are assumed to be the basis of the world-wide genetic diversity in pig. Indigenous resources of Asia, however, are less defined and only rarely compared with European breeds. Taking advantage of DNA diagnostics, animals within as well as between breeds from Vietnam and Europe were analysed for numerous well defined markers in order to gain more knowledge about pig genetic biodiversity. The main objective was to investigate indigenous Vietnamese pig breeds from different local geographic regions.

A set of pig breeds was chosen for this study of genetic diversity: five indigenous breeds from Vietnam (Mong Cai, Muong Khuong, Co, Meo, Tap Na), two exotic breeds kept in Vietnam (Large White, Landrace), three European commercial breeds (Pietrain, Landrace, Large White), and European Wild Boar. Samples and data from 317 animals (17 to 32 unrelated animals per breed) were collected. A panel of 27 polymorphic microsatellite loci was chosen according to FAO recommendations for diversity analyses and genetic distance studies. The loci were distributed evenly over the porcine genome with additional loci linked to immunological relevant genes (MHC, IFNG). Moreover, a few Type I loci (RYR1, FSH) were genotyped. DNA was isolated and PCR fragment lengths analysis were carried out on an ALF DNA sequencer (Pharmacia, Freiburg, Germany). Some of the RFLPs were analysed by agarose gel electrophoresis. Selected microsatellite alleles of equal lengths were sequenced for animals of different breeds.

Within-breed diversity estimated heterozygosities and tests for Hardy-Weinberg equilibrium by taking into account sample sizes, tests per locus and breed as well as breed-locus combinations. Calculations were performed using the BIOSYS-1 software package [2]. Breed differentiation was evaluated by the fixation indices of Wright [3]. Genetic distances between breeds were estimated on the basis of allelic frequencies of the loci in each breed using different measures, e.g the standard Nei's distances. Distances between breeds were further analysed according to the neighbour-joining algorithm of Nei [4] and the bootstrapping procedure of Felsenstein [5].

In average of the marker loci, heterozygous genotypes occurred more frequently than expected, but this was, not statistically significant. Heterozygosity was higher in indigenous Vietnamese breeds than in the other breeds.

Breed differentiation was shown which allowed grouping of all individuals in clusters corresponding to the breeds. Herein the Vietnamese indigenous breeds form a distinct cluster with considerable genetic distance to the European breeds. Vietnamese exotic breeds were similar to the breeds in Europe. European Wild Boar displayed closer relation with commercial breeds of European origin than with the indigenous Vietnamese breeds.

The microsatellite loci which are closely linked to functional genes of immune response showed differences between breeds. This finding may indicate adaptation to local geographic conditions. Type I loci revealed considerable differences between Vietnamese and European breeds which are partly due to breeding influences.

The comparative DNA sequencing showed differences between microsatellite alleles of equal lengths. About 30% of these alleles displayed length independent variants in at least one nucleotide position. Between the genetic diverse breeds, like those from Vietnam and Europe, DNA sequences between alleles differed more often. Their relevance is discussed in view of the use of microsatellite polymorphisms.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Combining gene-based methods and reproductive technologies to enhance genetic improvement of livestock in developing countries

J.H.J. van der Werf, K. Marshall

Animal Genetics, School of Rural Science and Agriculture, University of New England, Armidale, NSW 2351, Australia

E-mail:jvanderw@edu.au

The advent of molecular markers allows determination of actual genotype at gene loci, without error due to random and non-random environmental effects. In the ideal situation we can directly identify genotypes at loci containing genes with substantial effects on quantitative traits (QTL). When selection is on indirect markers there is no guarantee of QTL genotype as marker alleles linked to the preferred QTL allele can be different in different families. In such a case information about linkage phase needs to be accumulated based on phenotypic and pedigree information (e.g. a progeny test). Selection based on DNA markers, either direct markers: Genotype Assisted Selection (GAS) or indirect markers: Marker Assisted Selection (MAS), is most useful for traits that are hard to measure and have low heritability. Selection of animals based on (most probable) QTL genotype will allow earlier and more accurate selection, increasing short and medium term selection response and may aid in targeting genotypes for specific production environments or markets.

Novel reproductive technologies boost reproductive rates of breeding males (through artificial insemination – AI) and of females (through multiple ovulation and embryo transfer – MOET- or harvesting of oocytes in juveniles followed by *in vitro* fertilization - JIVET). The benefit arises from increased selection intensity, as well as from increased selection accuracy (larger families) and decreased generation interval (higher reproductive rates result in optimal designs with younger breeding animals). Increased reproductive rates potentially decrease effective population size, and therefore increase inbreeding. Selection for increased merit needs to be balanced against maintenance of sufficient effective population size. Therefore, selection needs to be optimized such that contributions from selected parents are optimal not only with respect to the next generation, but to future generations as well. Extra benefit from scenarios with unlimited use of reproductive technologies are restricted by the need to maintain genetic diversity (and sufficient effective population size).

Benefits for selection based on genotypic information is potentially higher in breeding programs that use technologies to boost reproductive rates, as the value of providing information about genotype is more beneficial for early selection. Moreover, GAS would provide information about within family variation, which has extra value as response to early selection based on between family differences is limited in breeding programs where loss of genetic diversity is to be controlled. Therefore, reproductive technologies potentially might provide the 'selection space' [2] which can be exploited when using genotype information.

Under optimal selection strategies, i.e. effectively under similar inbreeding scenarios, the additional response resulting from increased reproductive performance is constrained by maintaining sufficient effective population size. However, compared with natural mating strategies, response (after 10 years of selection) could be increased significantly, e.g. by about 20% and 35% for MOET and JIVET schemes, respectively [1]. Use of GAS can increase

genetic response initially but on the longer term (10 years) the advantage is much smaller even if major gene and polygenic response are optimally balanced. The initial benefit from applying GAS is lost later on because of loss of response from under utilizing the remaining polygenic part of the genetic variance. In the so-called 'juvenile schemes' where first selection occurs before the first phenotype has been measured, response based on phenotypic selection is difficult and GAS provides significantly more benefit. In this case, GAS can easily double initial response and even after 10 years of optimal selection the superiority of GAS over non-GAS selection can be in the order of 40% for MOET and JIVET schemes and about 20% for natural mating schemes [1]. Therefore, the combined application of reproductive and gene technologies in breeding programs work synergistically. In general, however, use of genotypic information in breeding programs for within breed selection will generally have limited extra benefit, unless selection based on phenotype is difficult or advanced reproductive technologies are used.

Most breeding programs in both developed and developing countries struggle to obtain rates of genetic response that are anywhere near to what might be expected based on theoretical considerations. The discrepancy is often due to the lack of control of selection decisions, which are often inefficient and uncoordinated. Implementation of advanced genetic and reproductive technologies may therefore not be the first steps needed in genetic improvement. However, when the gains in response can be significant, they should not be avoided either. Such larger benefits from gene technologies maybe expected when exploiting variation across populations, as described below.

Rather than exploiting existing QTL in within breed selection, a more likely scenario is that valuable QTL will be introgressed into other populations. Either indigenous breeds may contains valuable QTL, but could benefit from upgrading from crossing with superior breeds, or valuable QTL could be introgressed from exogenous breeds. Examples are the Booroola gene in the Garole breed (having a moderate and desirable effect on number of lambs weaned), and a number of genes affecting resistance to endemic local diseases. Furthermore, there are many cases of QTL found in crosses of extreme breeds, and a number of those will be a candidate for introgression. Use of genotype information is likely going to be more useful in marker assisted introgression (MAI) compared with selection within breeds. Also in the case of MAI, reproductive technologies will be beneficial because they can help increase the number of animals with the desired genotype. Again, optimal strategies will have to consider genetic diversity as well as risk. No studies have looked at MAI scenarios in a complete framework for livestock production, but such studies would be warranted, as they would likely form an important basis for the use of genetic technologies in genetic improvement programs in developing countries.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Evaluation of the utility of the FecB gene to improve the productivity of Deccani sheep in Maharashtra, India

C. Nimbkar[a,b], V.C. Pardeshi[c]

[a] Nimbkar Agricultural Research Institute, Phaltan, Maharashtra, India

[b] University of New England, Armidale, NSW, Australia

[c] National Chemical Laboratory, Pune, India

E-mail: cnimbka2@une.edu.au

The Booroola fecundity gene (*FecB*) is an autosomal gene in sheep with a large effect on ovulation rate and consequently, litter size. The Nimbkar Agricultural Research Institute (NARI) at Phaltan, Maharashtra, India (latitude 18^{o}N and longitude 74^{o}E) has embarked upon a breeding program to introgress the *FecB* gene from the Garole breed of Sunderban, West Bengal, into the local Deccani breed. Garole sheep, the probable original source of the *FecB* gene, are small-sized (average adult live weight 15 kg) and adapted to hot humid conditions. The Deccani are the native sheep of the semi-arid Deccan plateau and adult ewes weigh about 27 kg.

The reproductive performance of 188 ewes with ¼ Garole genotype (progeny of Deccani ewes mated to Garole x Deccani F1 rams), 97 of which were heterozygote carriers of the *FecB* gene (*FecB*$^{B+}$) and 91 were non-carriers (*FecB*$^{++}$), was analyzed to quantify the advantage in lamb production conferred by the gene. The percentage of abortions/stillbirths among maiden ewes was compared between ewes of the two FecB genotypes using a Z test. Other traits analysed were litter size (lambs born alive) and lambs weaned per lambing (weaning age being 120 days) as traits of the ewe. Fixed effects fitted for both traits were *FecB* genotype of ewe, birth year of ewe, year and season of lambing with age of ewe at lambing as a covariable. The interaction of ewe genotype and litter size was also fitted for lambs weaned. In addition, another model was fitted for the trait lambs weaned per lambing, with litter size instead of ewe genotype as a fixed effect. A random sire effect was fitted for both traits. A repeated measures analysis was done for both traits, using data from 1–3 lambings per ewe. Sire variance was found to be very low for both traits.

The proportion of abortions/stillbirths among heterozygote maiden ewes (0.21) was significantly higher ($P<0.05$) than that among non-carrier ewes (0.11). At the second and third parities, the proportion of abortions (0.04 and 0 respectively) was similar in both groups. Birth year of ewe, year-season of lambing and age of ewe at lambing were not significant for litter size or lambs weaned. *FecB* genotype of ewe had a highly significant ($P<0.001$) influence on litter size, as expected. The least squares mean litter size of non-carrier ewes was 1.00 and that of heterozygote ewes increased from 1.44 at the first lambing to 1.88 at the third lambing. These results are similar to those reported earlier [1] for a larger dataset including the ewes considered here.

Of the lambing ewes carrying one copy of the *FecB* gene, 54.5% , 44.4% and 25% had single lambs at the first, second and third lambings respectively (Table I). One heterozygous ewe at the second lambing and three heterozygous ewes at the third lambing had triplets. One non-

carrier ewe had twins at the first and second lambing while none had twins at the third lambing. The interaction between litter size and *FecB* genotype was significant for lambs weaned per lambing (P=0.03 at first lambing, P<0.01 at subsequent lambings). Table I below shows that lamb production of ewes bearing twin lambs is 65 to 112% higher than those bearing singles while heterozygote ewes produce 37 to 45% more weaned lambs than non-carrier ewes.

TABLE I: Least squares mean number of lambs weaned per lambing (LW) by 25% Garole ewes for the fixed effects of litter size and FecB genotype

Fixed effect	First lambing			Second lambing			Third lambing		
Litter size	No. of ewes	LW	S.E.	No. of ewes	LW	S.E.	No. of ewes	LW	S.E.
Single	123	0.66	0.11	74	0.82	0.11	26	0.96	0.12
Twin	35	1.09	0.19	29	1.74	0.19	15	1.71	0.17
Triplet	No triplets born			1	2.99	0.47	3	2.87	0.33
FecB status									
$FecB^{B+}$	77	0.90	0.05	54	1.33	0.07	24	1.45	0.11
$FecB^{++}$	81	0.90	0.05	50	0.97	0.08	20	1.00	0.12

There are indications that the proportion of heterozygote ewes bearing twins is likely to increase substantially at the third and later parities when they reach peak production and they are therefore likely to produce significantly higher number of weaned lambs than non-carrier ewes. Lamb production performance of heterozygote ewes will be evaluated in local shepherds' flocks in the next two years.

Further reduction in the proportion of Garole genes beyond 25% is likely to yield additional benefits since Deccani sheep have been observed to have higher milk production and consequently better ability to rear lambs compared to Garole sheep. These preliminary results of performance of ewes on NARI's farm suggest that the introduction of the *FecB* gene into the Deccani under the more efficient management of native shepherds may prove successful and lead to an increase in lamb production. However, this will depend to some extent on the reproductive performance of Deccani ewes homozygous for the *FecB* gene, and this is an issue we are currently investigating.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Effect of pregnancy on sex steroid receptor mRNA endometrial expression and on prostaglandin $F_{2\alpha}$ metabolite concentrations in heifers

A. Meikle[a], D. Cavestany[a], L. Sahlin[b], W.W. Thatcher[c], E.G. Garófalo[a], H. Kindahl[d], M. Forsberg[d]

[a]Veterinary Faculty, Uruguay

[b]Karolinska Institutet, Sweden

[c]Animal Sciences, University of Florida, USA

[d] Swedish University of Agricultural Sciences, Sweden

E-mail: anamei@adinet.com.uy

The aim of this study was to investigate the effects of pregnancy on mRNA expression of estrogen and progesterone receptor (ERα, PR) in endometrial biopsy samples. It was also tested whether uterine biopsy provokes $PGF_{2\alpha}$ release and induces luteolysis or allows pregnancy to be maintained. Twenty nine heifers in heat (Day 0) were inseminated (n=21) or not inseminated (control, n=8). Blood samples for progesterone (P_4) determination were taken daily from Days –1 to 25. On Day 17 endometrial samples ipsilateral to the corpus luteum were taken by transcervical biopsies for solution hybridisation assay determinations of ERα and PR mRNA. In 12 heifers (4 controls and 8 inseminated) hourly bleedings were performed from 5 h before to 12 h after the biopsy to determine 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ (PGFM), P_4, and cortisol patterns by RIA. One of these heifers had already low concentrations of P_4 on Day 17 and was excluded. Pregnancy was determined by ultrasonography on day 35 after estrus. Data were analysed by Mixed Models analysis of SAS.

At Day 35, 6 out of 21 inseminated heifers were diagnosed as pregnant. Inseminated non-pregnant cows (n=15) were classified in two groups according to P_4 concentrations at Days 21-25: luteal (P_4> 18 nmol/L, AI non-pregnant A, n=2) or basal (P_4< 3 nmol/L, AI non-pregnant B, n=13), Figure 1. Heifers with luteal concentrations of P_4 at Day 25 (AI non-pregnant A) may have suffered early embryonic mortality and were possibly pregnant at Day 17, thus, they were considered as pregnant for mRNA analysis (pregnant, n=8).

The concentrations of ERα mRNA in pregnant heifers tend to be lower than in controls, P=0.10, but no differences were found in PR mRNA concentrations (data not shown). Recently, it has been demonstrated that pregnancy inhibits ERα mRNA expression as well as other components of the luteolytic cascade [1].

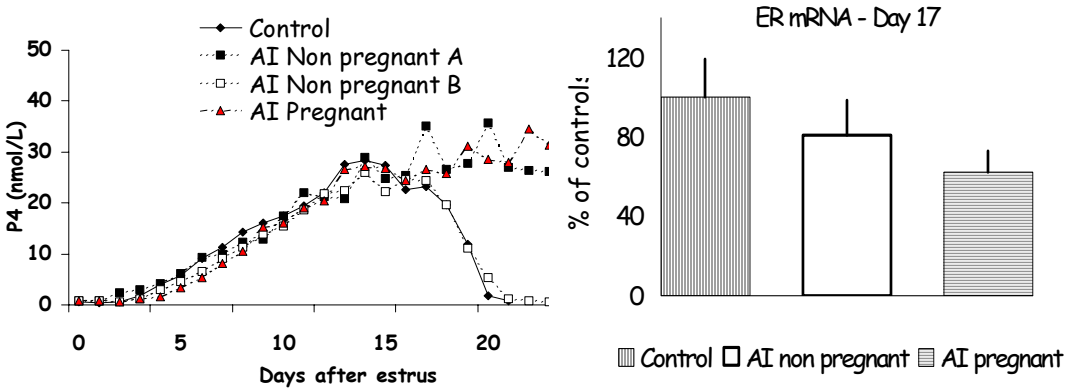

FIG.1. Left panel: Mean concentrations of progesterone in control, pregnant, non-pregnant cows with luteal (A) or basal (B) concentrations of P4 at Day 25 postestrus. Right panel: Concentrations of mRNA of estrogen receptor α (ER mRNA) in the endometrial biopsies in control (n=8), pregnant (n=8), non-pregnant (n=13) cows on Day 17. The results are presented as percentages of control heifers.

PGFM concentrations increased after the biopsy and remained high for the following 2 to 4 h (Figure 2, left panel). Pregnant heifers had lower concentrations of PGFM, in agreement with ER mRNA data showing that the embryo signal –interferon-τ inhibits $PGF_{2\alpha}$ secretion and the corpus luteum is maintained.

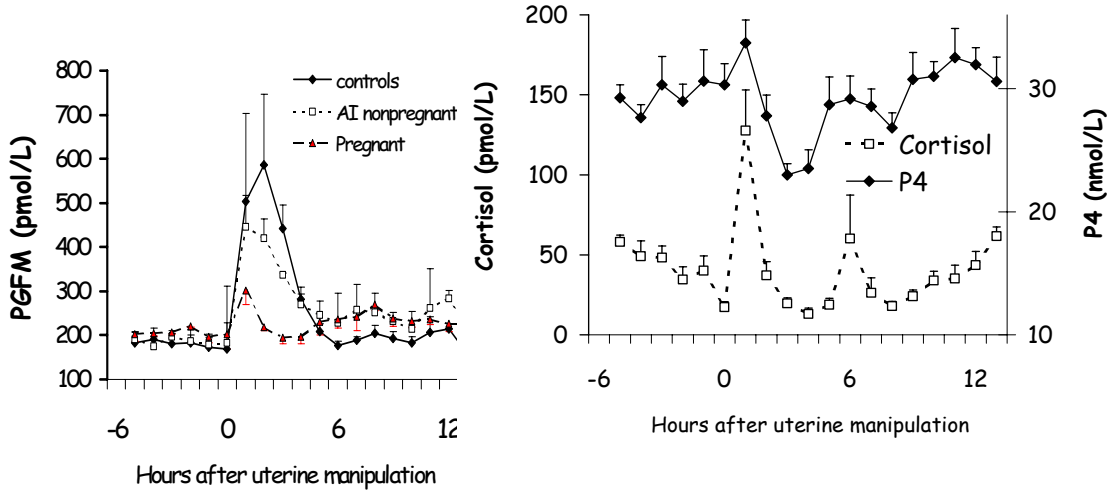

FIG 2. Mean (± SEM) concentrations of PGFM (left panel) in controls (n=4), inseminated non-pregnant (n=4) and pregnant (n=3) heifers and progesterone and cortisol (right panel) in all (n=11) cows before and after uterine biopsy.

Progesterone concentrations increased in the first blood sample after the biopsy, and decreased 2 to 4 h later in a temporal pattern consistent with the increase in PGFM peak, with both returning to normal soon thereafter. No statistical differences in P_4 or cortisol concentrations between groups were detected. The P_4 increase in the first bleeding after the biopsy was coincident with the cortisol peak at that moment (Figure 2), showing that the procedure provoked a stress response. Progesterone and cortisol were correlated ($r=0.21$, $P<0.01$), as reported previously [2] and may be due to the stress of the uterine biopsy since both P_4, a precursor to cortisol, and cortisol respond to ACTH stimulation. Uterine biopsies in

cattle may be used for clinical diagnosis of endometritis, and the measurement of plasmatic PGFM has been suggested to aid in the diagnosis of endometritis [3].

In this study, we have shown that pregnancy affects endometrial expression of ERα mRNA and plasma concentrations of PGFM. The uterine biopsy induces a temporal release in $PGF_{2\alpha}$, which is followed by a transient decrease in P_4 concentrations, but this procedure does not provoke luteolysis. Thus, it can be used for uterine sampling for studies on endometrial gene expression in pregnant cows.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

West African cattle breeds characterizations: Review of CIRDES genetic works

D.M.A. Belemsaga[a], K. Moazami-Goudarzi[b], S. Thevenon[a,c], S. Sylla[a]

[a] Centre international de Recherche-Développement sur l'Elevaga en zone Subhumide (CIRDES), Bobo-Dioulasso, Burkina Faso

[b] Institut National de la Recherche Agronomique (INRA), Centre de Recherche de Jouy-en-josas, France

[c] Centre International en Recherche Agronomique pour le Développement (CIRAD), Montpelier, France

E-mail: belemsaga.dma@coraf.org

The improvement of domestical animal breeds productivity or the animal genetic diversity maintenance to allow breeders to select animals or to create new breeds in order to adapt to environmental modifications, new diseases and societies needs, requires first a detailed inventory and, secondly, a genetic characterization of domestic animal breeds. Indeed, in developing countries, the notion of breed is not clear; visual parameters are often used even if these procedures are subjective. So it is necessary to complete this phenotypic approach by a genomic one in order to contribute to an efficient characterization. At CIRDES, a regional center for subhumid livestock research and development, these studies have been conducted during the past ten years. They permitted (i) to describe the cattle phenotypic traits and their geographical localization and to highlight the breeds threatened with extinction, (ii) to determine zebu introgression level in taurine trypanotolerant cattle (iii) to identify specific alleles of different cattle breeds, and (iv) to quantify the importance of Robertson translocation in livestock production. Data collection has been realized using a bibliography study, completed by investigations in seven countries of West Africa. Blood collection has been also done for an analysis of 4 categories of genome markers (11 blood group systems, 3 blood protein loci, microsatellites and chromosomes).

According to phenotypic description and to the conceptions of autochtone human population, 13 local cattle breeds have been identified: Ndama, Kouri, the group Baoule-Somba, the group Lagoon cattle, zebu Azawak, zebu Maure, zebu Touareg, zebu Goudali, zebu Bororo, zebu White Fulani, zebu Djelli, zebu peuhl soudanien, zebu Gobra, and their crossbreds (Zebu x Ndama and Zebu x West African Shorthorns). Nine exotic breeds have been also identified: American Brahman, Gir, Girolando, Droughtmaster, Santa Gertrudis, Holstein, Montbéliarde, Jersey and Brown Swiss; and five exotic crossbreds (Holstein x Goudali, Montbéliarde x Goudali, Holstein x Azawak, Brown Swiss x Azawak and Brown Swiss x Zebu peuhl soudanien). From this initial investigation, a map of cattle distribution in each country has been realized. The areas of heavy concentration of stock and the most important breeds have been described. In addition to that, it has been revealed that Benin Pabli breed has disappeared, that Lagoon cattle and Kouri breed are threatened with extinction, that the group Somba-Baoule is subjected to an absorption by Borgou and zebu breeds. The taurine cattle proportion is decreasing, compared with the total number of cattle. But the size of Borgou population and zebu breeds is increasing considerably in the zones where trypanosomosis risk is high.

On the other hand, the analysis of genome markers polymorphism has permitted to identify specific alleles for cattle characterization and to identify the genetic reasons of some trypanotolerant cattle declining. So, some alleles were found to be significantly ($p<0.01$) correlated to breeds (107 bp of HEL1 locus and 191 bp of ETH151 locus to zebu; 197 bp of ETH152 locus to Lagoon and 139 bp of locus ETH225 to Somba). These allele frequencies in the Borgou population were roughly intermediate between those in zebu and taurine breeds. The results of statistical analysis have permitted to define the rate of zebu gene crossing in trypanotolerant cattle (21.5% in Somba cattle and 1.2% in Lagoon cattle); and to determine the degree of zebu gene introgression in Somba cattle (1.5% and 21.5% according to blood groups aspect and to hemoglobin and albumin combination). Y chromosome morphology has been found to be accrocentric in zebu cattle but submetacentric or metacentric in taurine cattle. Robertson's translocation has been observed and its prevalence has been calculated in Lagoon cattle (0.0%), in Somba cattle (10.4%) and in Borgou cattle (18.3%). These observations ought to be take into account for a regional programme development aiming at preserving and conserving the endangered breeds to maintain the biodiversity.

PANEL DISCUSSION 1: WHICH GENE-BASED TECHNOLOGIES ARE MOST LIKELY TO SUCCEED IN ENHANCING ANIMAL PRODUCTIVITY IN DEVELOPING COUNTRIES?

Moderator: J. Donelson, USA

TOPIC: Which gene-based technologies are most likely to succeed in enhancing animal productivity in developing countries?

Application of gene-based technologies directed at commensal gut bacteria to solve animal productivity constraints in developing countries

R.I. Mackie, I.K.O. Cann

Department of Animal Sciences, Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801, USA

E-mail: r-mackie@ux1.cso.uiuc.edu

Animals of a wide range of orders and classes have a portion of their digestive tract adapted to accommodate a fermentation which assists in digestion as well as providing a variety of other benefits. Advances in our understanding of fermentative digestion have tended to obscure the vital role that the gastrointestinal microbiota plays in the physiological, immunological and protective functions of the host animal. Indeed it is estimated that microbial cells outnumber host cells by a factor of 10 and that animals and humans contain 90% of their total cells as prokaryotic cells. The association of microbes with tissues of the gastrointestinal tract of animals during evolution has resulted in a balanced relationship between resident microbes and host. Numerous biochemical, physiological and immunological features that are considered intrinsic characteristics of animal species are actually responses by the animal to the physical presence and metabolic activities of the normal indigenous microbiota. This microbial challenge has modified the course of evolution in animals resulting in the selection of animal microbe relationships which are dynamic, complex and vary tremendously ranging from competition to cooperation. In fact, we can consider the gut microbial population to be the most metabolically active and rapidly renewable organ of the body.

The microbial community inhabiting the gastrointestinal tract is represented by all major groups of microbes and is characterized by high population density, wide diversity and complexity of interactions. Our current knowledge of gut microbial diversity and ecology is largely based on classical anaerobic culture techniques, phenotypic characterization of culturable isolates as well as light and electron microscopic examination. However, despite this vast amount of knowledge, microscopic and culture based enumeration and classification schemes of microbial community members have tremendous limitations. The two major problems faced by microbial ecologists studying the gut ecosystem are the inevitable bias introduced by culture-based enumeration and characterization techniques, and the lack of a phylogenetically based classification scheme. These limitations can be overcome by the application of modern molecular techniques based on sequence comparisons of nucleic acids and used to provide genotypic characterization while at the same time providing a classification scheme which predicts natural evolutionary relationships. Importantly this molecular approach does not rely on cultivation but rather the analysis of community DNA, representing all resident microbes, extracted from intestinal samples. The powerful combination of molecular biology and Woese's new phylogeny has created the now recognized field of molecular microbial ecology. This is defined as the application of molecular technology, usually based on comparative nucleic acid sequence information, to identify specific microorganisms in the gut environment, to assign functional roles to these specific microorganisms, and to assess their significance or contribution to specific metabolic

and physiological processes in the gastrointestinal tract. The advantages and limitations of various techniques employed for molecular microbial ecology studies will be reviewed in an effort to identify which gene-based technologies directed at commensal gut bacteria are most relevant and applicable to solving animal productivity constraints in developing countries. The use of these techniques has lead to major advances in our knowledge and will provide the first complete description of the gastrointestinal ecosystem.

The latest and most powerful technologies revolutionizing microbiology are based on genomics – the mapping and sequencing of genomes and analysis of gene and genome function. Genomics refers to a suite of functional and comparative methods that capitalize on the availability of entire or high coverage draft sequence. More than 100 microbial genome sequences have been completed providing information on more than 300,000 predicted genes with approximately half of these being of unknown function and potentially novel. These novel genes represent exciting new opportunities for future research and potential sources of biological resources for exploration and exploitation. The development of microarray technology is the first step in the experimental use of whole genome sequences and enables a thorough analysis of gene expression patterns in different environmental conditions. In this approach, individual DNA probes are arrayed on a small glass surface and labeled cDNA is hybridized onto the array. The amount of fluorescence at each DNA probe spot correlates with the abundance of specific mRNA transcript in the cell. This approach enables characterization of transcriptionally regulated pathways at a genomic scale. Comparison of closely related genomes can be achieved cheaply using DNA microarrays based on one completely sequenced representative. DNA microarray analyses will only provide information on which genes present in an arrayed and sequenced genome are absent from probe genomes, not which additional genes may be present in the probe. Genomic subtraction methods can provide this reciprocal information. An improved and more sensitive PCR-based method suppressive subtractive hybridization has been developed for this purpose. Finally, cloning large fragments of DNA isolated directly from microbes in the gut environment provides access to community DNA – the metagenome. Large insert Bacterial Artificial Chromosome (BAC) libraries can be used to detect gene expression from poorly studied difficult to manipulate or as yet uncultivated species. Importantly the metagenomic approach enables the linkage of specific functions with the phylogenetic group responsible for them.

TOPIC: Which gene-based technologies are most likely to succeed in enhancing animal productivity in developing countries?

Animal breeding in developing countries based on gene-based selections

J.P. Gibson

International Livestock Research Institute, Nairobi, Kenya

E- mail: J.Gibson@cgiar.org

Background information on the use of molecular genetic markers to detect and select for genes controlling genetic variation (quantitative trait loci, QTL) is provided by Williams and van der Werf (these proceedings). Use of QTL is predicted to be most beneficial for traits that have low heritability or are difficult, expensive or impossible to record during a breeding program. These conditions apply to disease resistance and adaptation traits in the low to medium inputs systems of the developing world and use of QTL could therefore be expected to be particularly beneficial here. The failure to use QTL information in the developing world reflects a lack of investment in QTL mapping. Such investment is needed not only to detect QTL that could be useful in genetic improvement programs, but also to design improvement programs utilising QTL information that would be sustainable under developing world conditions. A strategy for use of molecular markers that is hypothesis driven and has clear goals and routes to impact poor farmers is outlined here. The strategy is presented in greater detail by Gibson [1].

Population genetics theory predicts that natural selection will fix different genetic solutions in populations that are isolated from each other. Selection acts stochastically on the variation available, and this variation will differ in nature and extent between populations. The more genetically distinct are any two populations, the greater the likelihood they will contain distinct genetic polymorphisms and the greater the chance that selection will lead to fixation of different genetic solutions to the same problem in the two populations. Experimental support for this theory exists in model species and most recently also for the case of trypanosomosis tolerance in livestock.

While there is enormous functional diversity in the characteristics of livestock breeds, there are few cases where any breed has achieved a perfect solution to a given problem. Trypanotolerance in cattle and gastrointestinal helminth resistance in sheep are good examples, where breeds exist that are able to survive and produce under disease challenge, but such breeds still perform better in the absence of the disease. It would be desirable to produce animals with even higher resistance to disease, which would be able to thrive under the highest challenge in the absence of other disease control measures. There are well documented examples of several distinct breeds of a given species having evolved partial resistance to a given disease. A good example is gastrointestinal helminth resistance in sheep, with at least 8 breeds of sheep having been recorded as having some degree of resistance. Given the general lack of information on the characteristics of livestock breeds there are probably many more undocumented examples.

In order to identify the best possible genotype for each of a range of production environments, the ideal situation would be to test all breeds with potentially useful characteristics globally, along with all their crosses in each production environment. In practice such testing is not

feasible, due to economic and logistical limitations. It would be feasible in many cases to undertake testing of just two breeds from two different countries (or regions). Many countries would see the advantage of a reciprocal exchange of germplasm with another country, which could overcome concerns related to benefit sharing in many cases. The critical question is which two breeds would maximise the probability of being able to develop a better genotype than currently exists? Obviously choice of breeds will involve careful examination of existing data on breed performance and the environments under which they evolved. Where it is desired that a particular trait be further improved one consideration would be the likelihood that two breeds have evolved different mechanisms of adaptation, such that a higher level of adaptation (and/or performance and/or resistance) could readily be developed from a cross between them. In this case one would seek breeds that have suitable phenotypes in the targeted environment, yet are as genetically distant from each other as possible. Genetic distances among existing breeds can be estimated using molecular genetic markers and a global survey of distances among all the breeds of each species need be completed only once.

Having selected two breeds it will be important to test the hypothesis that they carry different genetic mechanisms controlling the desirable traits, before proceeding with a breeding program. A suitable method for testing that hypothesis is to perform a genome-wide interval mapping for QTL based on anonymous genetic markers in an F2 and/or backcrosses between the two breeds. Based on whether or not the hypothesis is confirmed, the size of the QTL detected and the performance of the pure breeds and the F2 or backcrosses, an informed decision can then be taken on a suitable genetic improvement program. The outcome might be to utilise one of the purebreds, or to develop a crossbreeding program, or to develop a new breed through selection from a crossbred or backcross population, or to introgress QTL from one breed to the other. In many cases the population used for testing the QTL hypothesis can also be used as the base population of a breeding program. An informed decision can be taken on whether or not the genetic improvement program should incorporate marker-based selection. This decision will depend not just on the potential value of the marker information, but also the cost and logistics of collecting and using the marker information in the genetic improvement program.

The steps outlined above, from initial mapping of global livestock diversity through to hypothesis testing and possible use of markers in selection forms a coherent strategy for detecting useful genetic variation between different populations, with a clear route to utilizing such variation using molecular genetic information.

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TOPIC: Which gene-based technologies are most likely to succeed in enhancing animal productivity in developing countries?

Gene-based vaccine development for improving animal production in developing countries

J.R. Egerton

Faculty of Veterinary Science, University of Sydney, Camden, Australia

E-mail: johne@camden.usyd.edu.au

The cloning and expression of microbial genes in alternate hosts to enhance production of antigens for animal vaccines against all disease is theoretically achievable. It is essential, however, that antigens expressed in this way are known to be protective. Many years of costly research usually precedes the identification of such antigens or combinations of antigens. Thus, while conventional vaccines based on living, attenuated or inactivated microorganisms may be effective, the protective components contained in them i.e. the candidates for cloning, have yet to be found. The principal protective antigen in vaccines against foot rot of sheep and goats is fimbrial protein of *Dichelobacter nodosus*. Recombinant vaccines against this infection are ineffective if the protein subunits are not assembled and presented to the host in a manner morphologically indistinguishable from those of the natural fimbriae [1].

Availability of recombinant antigen does not necessarily avoid the need for the use of adjuvants to potentiate response. Oil emulsion vaccines, while enhancing immune response, almost inevitably cause a marked reaction at the site of injection. Livestock owners in developing countries are as likely as those elsewhere to object to these reactions. The need to find an acceptable and effective formulation adds to the cost of recombinant vaccines and their application in countries with limited resources for disease control. Another costly feature of recombinant vaccines has been the patenting of processes involving gene technology and licencing agreements for production under the protection of these patents.

In some systems antigenic competition between similar and disparate antigens limits the usefulness of even recombinant antigens that, administered individually, are highly potent [2]. In the case of programs for the control and eventual eradication of footrot in sheep and goats in Nepal this problem was overcome by the prior identification of causal serotypes and production of vaccines that were strain specific [3]. These provided duration of immunity not achievable with multivalent preparations. Wider application of this approach to vaccinology is inhibited by the requirement for rapid and accurate identification of strains of pathogens involved either in regions or in particular epidemics and the preference of commercial vaccine manufacturers for general purpose products.

The usefulness and effectiveness of vaccines based on gene technology is dependent on their inherent quality and also on the veterinary infrastructure in the countries where they are being used. This infrastructure includes knowledge of the epidemiology of target diseases and the role of vaccines in their management in the physical and social environment in different countries. Successful use of vaccines depends on having the resources to employ people to undertake vaccination and re-vaccination programs at the appropriate time or the successful completion of extension and training which together result in farmers' use of vaccines consistent with national programs. Where defined outcomes are part of national programs, assessment of response to vaccination is another component of infra-structural support. It may

also be that vaccination programs need the support of disease control legislation similar to that which exists in most parts of the developed world. Where the losses from animal disease are dramatic and obvious compliance with vaccination schemes is more readily achieved. Where losses are more insidious and related to production loss rather than deaths,veterinary service providers may need legislative support.

Cheap and effective vaccines prepared by exploiting gene technology have the potential to enhance animal production throughout the developing world. While the benefits of this technology are many it is possible that the vocal opposition, in some more developed communities, to genetically modified products will also be heard in other parts of the world. We should not assume that they are acceptable, without question, everywhere.

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SESSION II: GENE-BASED TECHNOLOGIES APPLIED TO PATHOGENS AND HOST-PATHOGEN INTERACTIONS

Chairperson: P.P. Pastoret, UK

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Current and future developments in nucleic acid-based diagnostics

G.J. Viljoen, M. Romito, P. Kara

Biotechnology Division, Onderstepoort Veterinary Institute, South Africa

E-mail: gerrit@moon.ovi.ac.za

The detection and characterization of specific nucleic acids of protozoa, rickettsia, bacteria and viruses have proven to be particularly useful for detecting pathogens of human and veterinary importance. It is also proving an invaluable tool for surveillance purposes and as a means of ensuring food security. Previous approaches towards pathogen isolation have often been tedious or even impossible. PCR, first conceived by Mullis in 1983, has proven to be a revolutionary technique for the rapid and accurate detection of numerous pathogens. The discovery and cloning of thermostable DNA polymerases has further contributed to this technology. Many additional developments, based on the basic principles of PCR, have been described e.g. RT-PCR, NASBA, RAPD, AFLP, LCR, PCR ELISA, strand displacement amplification (SDA), transcription-mediated amplification (TMA), branched DNA (bDNA), hybrid capture, immunocapture PCR. This list continues to expand with new variations on basic PCR principles.

Improvements in thermocyclers involve the development of integrated amplification and signal detection systems, including on-line real-time devices. In addition, rugged portable instruments have been designed for field use. These are particularly useful as systems for early warning in detecting biowarfare agents and outbreaks of cross-boundary and other pathogens. Fluorophores, utilising principles of fluorescence resonance energy transfer, are used as labels for probes in such real-time assays. Molecular beacon technology also utilises such mechanisms. Real-time thermocyclers allow the monitoring of amplified DNA as well as establishing sequence characteristics based on melting or hybridisation curves. Taqman chemistry makes use of such a system. Stem-loop DNA probes have been designed to have increased specificity for target recognition and include molecular beacon methodologies, suppression PCR approaches and hairpin probes in DNA microarrays. Automated sample processing or robotic devices are now also commercially available and have the advantages of greater efficiency and reduced contamination associated with conventional procedures for nucleic acid extraction.

The distinction or typing of protozoal, rickettsial, bacterial or viral strains can be done using DNA fingerprinting: including PFGE, ribotyping, genomic RFLP analysis, mitochondrial RFLP, RAPD, repetitive element-based PCR (rep-PCR) or post amplification sequencing. Sequencing of nucleic acids has proved invaluable in this regard, especially for the subtyping of viral and bacterial strains. Automated sequencers have facilitated this process and technological developments in this field can be expected to make this approach even more accessible. High-throughput DNA sequencers have played a major role in elucidating the genomes of many organisms. Pyrosequencing utilises real-time light emission during the DNA polymerisation process. Other envisaged developments include miniaturisation, thereby reducing sample size and reaction times. Prototype chip sequencers perform both PCR amplification and capillary electrophoresis. Sequencing of single molecules using

exonucleases is also now described. Further developments in the field include new dyes, polymeric matrix materials and alternative formats for capillary electrophoresis.

Reverse hybridisation uses sequence-specific linear array probes attached to nitrocellulose membranes that bind to amplicons generated by PCR. It is useful for genotyping of related organisms. Detection of single nucleotide polymorphisms, the most common stable genetic variation, is now being made using mass spectrometric applications e.g. matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) on silicon chips.

Previously detection methods were frequently based on radioactively labelled molecules, now increasingly alternative approaches are being used e.g. fluorescence resonance energy transfer (FRET) as used in real-time detection devices, surface plasmon resonance (SPR) and mass spectrometry e.g. MALDI-TOF MS.

Bioinformatics has also considerable contribution to make in the design of suitable probes, analysis of sequence data, generation of phylogenetic trees and molecular epidemiological studies. The developments of improved software programmes and extensive data banks are contributing to this process. The detection of unique DNA signatures of specific organisms has been greatly facilitated by pair-ways comparisons apart from suppressive subtractive hybridization (SSH).

Microfabrication technologies are playing an important role in the construction of microchips. Contributing areas include microfluidics, materials sciences, generation of new biorecognition elements, integrated circuits. DNA microarrays with large numbers of oligonucleotide probes, allow the detection of a variety of different pathogens simultaneously. Detection technologies would include fluorescence labelling but developments in the biosensor field also have a direct application. Particularly interesting are developments in the fields of microfabrication and nanotechnology. Lab-on-a-chip devices will not only process sample materials but also perform the detection assay. Quantum dots have the potential of giving a vast repertoire of labels to molecules that would greatly facilitate the ability to distinguish between different agents. Numerous biosensor technologies utilising a variety of transducers together with biological receptors as well as nucleic acids are described. Hybridisation with target nucleic acid generates a signal detected by the transducer that can include amperometric and potentiometric electrodes, field-effect transistors, thermistors, piezoelectrical crystals, cantilevers and various optical and opto-electronical devices. The development of dip-stick or hand-held devices utilising nucleic acid detection technologies for field, patient- or crush-site use can be envisaged.

Nanotechnology promises to make many exciting contributions. More versatile labelling methods include quantum dots, gold beads and magnetic nanoparticles. Nanosensors and nanoarrays, which are a thousand fold smaller than microarrays and millions of times denser, are also likely to make significant impacts. Lab-on-a-chip devices and other integrated technologies hold particular promise in this regard. A goal for an ideal device is one capable of a broad spectrum or even universal detection of pathogens that can yield ‘yes-no’ answers. Dip-stick or hand-held devices for patient-side or on-farm use are also highly desirable.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Reverse genetics with animal viruses

T. Mebatsion

Department of Virology, Intervet International B.V., 5830 AA Boxmeer, The Netherlands

E-mail: teshome.mebatsion@intervet.com

Reverse genetics of negative-strand RNA viruses (NSV), which allows generation of recombinant viruses entirely from cloned cDNA, has progressed rapidly in the past decade. NSV are a large and diverse group of enveloped viruses of both medical and veterinary importance. They differ widely in morphology, genome structure and host interactions. The first NSV that was completely amenable to genetic manipulation is the neurotropathogenic rabies virus of the rhabdovirus family [1]. In subsequent years, vesicular stomatitis virus and a number of viruses belonging to the family Paramyxoviridae, including viruses causing important animal diseases such as rinderpest virus, canine distemper virus, bovine respiratory syncytial virus, bovine parainfluenza virus and Newcastle disease virus (NDV), succumbed to genetic engineering.

The ability to genetically manipulate NSV opens a wide range of possibilities to study the virus biology and develop improved vaccines. Identification and analysis of attenuating mutations using the recombinant system could lead to generation of safe vaccine strains.

Introduction of one of the previously studied mutation into an infectious rabies virus (RV) clone by replacing the arginine at position 333 of RV glycoprotein (G-protein) by an aspartic acid resulted in a dramatic attenuation. Combination of this mutation with a deletion that eliminates the interaction between RV P-protein and the cytoplasmic dynein light chain (LC8), which is presumably involved in retrograde transport of RV, further attenuates the rabies virus by 30-fold after intramuscular inoculation [2]. Since extreme attenuation may adversely affect immunogenicity, reverse genetics was used to introduce an additional G-protein to the step-wise attenuated RV to increase its effectiveness. The resultant recombinant virus may be helpful in developing a highly safe and effective live RV vaccine for oral immunizations of animals.

Reverse genetics of NSV has also helped in providing important insights into viral pathogenesis. The roles played by many accessory proteins, including V, C and NS proteins of Paramyxoviridae and influenza viruses as interferon antagonists were studied in detail using infectious clones. Since interferon antagonists are important virulence factors, their identification and modification by knocking them out or reducing their expression should provide opportunities to generate safe attenuated vaccine strains. Like other members of Paramyxovirinae, NDV produces the accessory V protein from the P gene by a process called RNA editing. Introduction of mutation into the editing site resulted in reduction of the editing frequency and as a result, V was expressed at a 20-fold lower level than the wild type NDV and was highly attenuated in chicken embryos [3]. Administration of the recombinant NDV with an editing site mutation to 18-day-old chicken embryos did not affect hatchability. Hatched chickens developed high levels of NDV specific antibodies and were fully protected against lethal challenge, demonstrating the potential use of editing-defective recombinant NDV as a safe embryo vaccine.

The ability to manipulate the genomes of animal viruses has also important implications in designing and developing marker vaccines. Different approaches can be used in designing marker vaccines. One approache that we employed for generating a marked NDV was first localizing a conserved B-cell immunodominant epitope (IDE) on the nucleoprotein (NP) gene and then successfully recovering a recombinant NDV lacking the IDE by reverse genetics [4]. In addition, a B-cell epitope of the S2 glycoprotein of murine hepatitis virus (MHV) was inserted in-frame to replace the IDE. Recombinant viruses properly expressing the introduced MHV epitope were successfully generated, demonstrating that the IDE is not only dispensable for virus replication, but can also be replaced by foreign sequences. Chickens immunised with the hybrid recombinants produced specific antibodies against the S2 glycoprotein of MHV and lacked antibodies directed against the IDE. These marked-NDV recombinants, in conjunction with a diagnostic test enable serological differentiation of vaccinated from infected animals and may be useful tools in ND eradication programs. NSV accommodates not only small epitopes, but also large foreign genes in their envelopes to be able to induce broad-spectrum immune responses. Exchange of surface proteins is also realizable with a number of NSV, demonstrating the manifold possibilities of reverse genetics in designing and developing novel vaccines.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Viral subversion of the immune system

A. Vanderplasschen

Faculty of Veterinary Medicine, B43b, University of Liège, Belgium

E-mail: a.vdplasschen@ulg.ac.be

The continuous interactions between hosts and viruses during their coevolution have not only shaped the immune system but also the counter measures used by viruses. Studies of the last decade have described the diverse array of pathways and molecular targets used by viruses to elude immune detection and destruction. These include targeting of pathways for major histocompatibility complex restricted antigen presentation; natural killer cell recognition, apoptosis, cytokine signalling, humoral immune responses and complement activation. In this presentation, an overview of the immune-evasion mechanisms described for viruses to date, emphasizing on the importance in understanding the interaction between viruses and the immune system to improve our ability to manipulate and exploit viruses will be given.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

The molecular basis of livestock diseases in developing countries as illustrated by African trypanosomosis

J.E. Donelson

Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242, USA

E-mail: *john-donelson@uiowa.edu*

Viral, bacterial, protozoan and helminthic diseases of domestic livestock continue to be serious impediments to the agricultural economies of most developing countries. Many of these livestock pathogens have evolved sophisticated molecular mechanisms for evading or circumventing the mammalian immune system. These same pathogens frequently acquire resistance to drugs that are initially effective. African trypanosomes are protozoan parasites that cause the fatal diseases of ngana in cattle, surra in camels and horses and sleeping sickness in humans. They are the paradigm for a livestock pathogen in developing countries for which much is now known, yet little has been achieved in controlling or eliminating the disease.

African trypanosomes were identified as the cause of trypanosomosis more than 100 years ago and in many ways are ideal pathogens to study in the laboratory. From the perspective of research on the parasites themselves, excellent laboratory rodent models for their infection exist. They can be readily grown *in vitro* in culture flasks. Their mechanism of immune evasion is known. The completed DNA sequence of their genome is nearly determined. They can be manipulated genetically in the laboratory – genes can be mutated and deleted from, or inserted into, their genome. They contain unique organelles and metabolic pathways not found in mammals that could potentially be exploited for new drug development. They are pathogens of humans as well as livestock, so they attract the interests and enormous resources of the medical research community. Advantages also exist from the standpoint of experiments on their animal hosts. Breeds of domestic cattle that are either “trypanotolerant” or trypanosome-sensitive are well known and animals of each type have been cross-bred. The molecular basis of trypanosome-tolerance in at least one indigenous wild animal species (the Cape buffalo) has been elucidated. The reason some African trypanosome species are killed by human serum, but not by livestock serum, is understood.

Despite the extensive molecular characterizations of African trypanosomes and their interactions with livestock hosts during the past century, African trypanosomosis remains ranked among the top 10 livestock diseases impacting negatively on developing countries in Africa, Asia and South America. The many unique molecular properties of African trypanosomes will be described and discussed in the context of the other main livestock pathogens of developing countries. The reasons this information has not translated into vaccines or better drugs against trypanosomes will be presented and prospects for more successful applications of gene-based approaches against livestock diseases of developing countries in the future will be examined.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Vaccination against ticks and the control of ticks and tick-borne disease

P. Willadsen

CSIRO Livestock IndustriesQueensland Bioscience Precinct, St Lucia QLD 4067, Australia

E-mail: peter.willadsen@csiro.au

Ticks and tick-borne disease are a major constraint to livestock production in developing countries [1]. The control of ticks is of particular importance given that a major component of the control of tick-borne disease is, in fact, via the control of the vector. Problems with the control of ticks through pesticide application continue to increase, explaining the continued interest in vaccine and biological control strategies as alternatives.

The feasibility of vaccination was demonstrated conclusively with the release, in 1994, of a recombinant commercial vaccine against *Boophilus microplus*. Nevertheless, since then the field has languished commercially, despite ongoing scientific progress. This paper will address the hurdles to further development and some ways in which they might be overcome.

Efficacy and the development of prototype vaccines

Recombinant vaccines have a number of potential advantages over chemical control. These include safety, specificity and freedom from environmental contamination. They are potentially low cost and stable, either minimising or eliminating the need for a cold chain for distribution. Another advantage is less well recognised. Many of the problems with pesticides derive from inaccurate or inappropriate application. In this regard, vaccines are more robust technology, being less dependent on volume or timing of application.

The problem with vaccines is their perceived and real lack of efficacy. The current commercial vaccine against *B. microplus* is a single antigen vaccine that, at best, gives 90% protection. Used in a sustained way, this is adequate in many production situations. In other situations, efficacy may be less and hence inadequate. Efficacy can be increased through the addition of other antigens to a vaccine. Over the last decade a number of possibilities have been identified, though none have been thoroughly evaluated. A reliable evaluation of the existing portfolio of antigens in a field situation would be invaluable. In a commercial and practical sense, the ability of vaccines to protect against multiple species is important, but has been little investigated, though preliminary results are encouraging. In particular, evaluation of putative antigens identified through the conservation of sequence across parasite species has not been made.

The antigens so far identified result from laborious, gene-by-gene or protein-by-protein, investigation. Looking forward, the impact of genomics on the efficient selection of target vaccine antigens is certain to be revolutionary.

An important consideration of efficacy is the accurate definition of the level of performance necessary for adequate livestock protection. This is a complex, but vitally important question that is not adequately addressed.

Commercial Issues

Vaccines are a good example of market failure. The greatest economic benefit from a vaccine usually accrues to the farmer or the consumer, not the vaccine manufacturer. In a developing country, with a multiplicity of tick species and a diversity of farming situations, there may be little commercial incentive to manufacture a vaccine, particularly if the manufacturer is to be an international animal health company.

Importantly, a recombinant vaccine may be relatively simple to manufacture. Potentially very large amounts of recombinant protein can be produced at low cost in multi-purpose and commonly available facilities and then formulated on a contract basis. In principle the manufacture of such a vaccine for the farmers of a developing country does not have to engage the interest of a large company, in many circumstances, it could be done on a small and local scale.

Regulatory Issues

Many of the most striking successes of veterinary vaccines have been with locally manufactured vaccines that would not now pass regulatory hurdles. Recombinant vaccines are sterile and contain small amounts of a rapidly degraded protein of bacterial or yeast origin. As such, they are likely to be safe and non-contaminating. The requirements for registration of such a vaccine however, vary from country to country or are poorly defined. In many countries registration requirements for an anti-parasite vaccine are likely to have been designed around the typical performance characteristics of chemical anti-parasitics. Thus, the guidelines for the novel technologies for producing a vaccine may be either non-existent or inappropriate. In the worst circumstance, the costs of registration, often to satisfy criteria that appear to have little practical relevance, may be a major obstacle to a vaccine's commercialisation.

Summary

Before vaccines against ticks (and similarly other parasitic diseases of importance to the developing world) become available, three conditions must be met: efficacy must be established; the vaccines must be manufactured in a way that is commercially feasible and the regulatory environment must facilitate the development of such desirable technology. This is achievable, though the will and the resources to do so are critical.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Development of marker vaccines for rinderpest virus using reverse genetics technology

S. Parida, S. Kumar, P. Walsh, M. Baron, T. Barrett

Pirbright Laboratories, Institute for Animal Health, Ash Road, Pirbright, Woking, Surrey, GU24 ONF, UK

E-mail: tom.barrett@bbsrc.ac.uk

Rinderpest or "cattle plague" is an economically devastating disease which is still endemic in areas of Eastern Africa, its aetiological agent being a morbillivirus (RPV) closely related to human *Measles* virus [1]. A global rinderpest eradication programme (GREP) is in place and its goal is the eradication of the disease by the year 2010. In the final stages of the eradication campaign, when mass vaccination must be discontinued, it would be desirable to use a vaccine that enables vaccinated animals to be distinguished from animals which have recovered from natural infection, so called "marker vaccines". These are now being produced using reverse genetics, the process whereby the genomes of RNA viruses can be genetically manipulated through a DNA copy and live virus rescued from the altered DNA. This very powerful new technology, in addition to its practical usefulness in allowing the development of marker vaccines, also enables us to investigate, on a rational scientific basis, the molecular determinants of virulence and attenuation in this virus group. This will be an important consideration when assessing the safety of such vaccines for general use. Reverse genetics for RPV was established in the Pirbright laboratory some six years ago [2] and several candidate marker vaccines have been produced which are now ready for field testing.

The first approach we have taken was to place genes encoding "positive" marker proteins into the RPV genome [3]. The markers chosen were the green fluorescent protein (GFP) and a modified form of the influenza haemagglutinin (fluHA) protein. A strong antibody response was generated to the fluHA marker protein in all animals vaccinated with the RPV-fluHA recombinant. The GFP gene was modified so that the protein, in addition to cytoplasmic expression, would be either secreted from the infected cells or expressed as a membrane-anchored protein of the cell surface. The antibody response to the GFP protein elicited in the vaccinated animals depended on its mode of expression. The form of GFP that was only expressed in the cytoplasm failed to elicit an immune response in any of the vaccinated animals, despite very high expression levels of the protein during *in vitro* experiments. Animals vaccinated with a virus expressing a secreted form of GFP varied in their responses to the marker protein with only 50% showing strong anti-GFP antibody levels. Only the membrane-anchored form of the protein gave a strong antibody response in all vaccinated animals and thus was a suitable marker protein.

In addition to positive markers, a "negatively" marked vaccine, i.e. one lacking a RPV-specific antigenic component, was also produced. To achieve this a chimeric virus in which the nucleocapsid protein (N) gene of RPV was replaced by that from the related morbillivirus *Peste des petits ruminants virus* (PPRV), was produced (rRPV-PPRN) and tested in cattle. Use of this recombinant virus vaccine enables vaccinated animals to be distinguished from those that have been naturally infected. This distinction can be made using two currently available ELISAs that detect antibodies specific to either the N or haemagglutinin (H) proteins of these two viruses. Vaccinated animals become positive in the PPRV N-specific

ELISA and negative in the RPV N-specific assay. The opposite is true for the H protein-specific ELISAs. Vaccinated animals which subsequently become infected with wild type virus will become double positive for the N protein antibodies of both viruses. In a further development we have introduced a positive marker gene into this virus genome, namely that encoding the membrane-anchored GFP protein. This vaccine (rRPV-PPRN-ancGFP), in addition to being distinguishable using the above mentioned tests, produces antibodies against the marker protein in vaccinated animals which are absent in naturally infected and recovered animals. Unfortunately this "positive-negative" marker vaccine, unlike the other marker vaccines produced thus far, does not grow to high titre in tissue culture and we are currently trying to improve its growth by introducing a chimeric RPV-PPRV N protein gene to replace the complete PPRV N gene in this virus.

One major consideration when developing genetically modified virus vaccines is safety. The possibility that the marker proteins could be incorporated into the virus envelope and thus alter the tropism and possibly the pathogenicity, of the vaccine produced, must be considered. Generally viruses have mechanisms to exclude the incorporation of foreign proteins into their envelopes and immunoprecipitation studies using antibodies to the marker proteins failed to precipitate the recombinant vaccine viruses, indicating that the marker is excluded from the virion envelopes. As an additional safety precaution, the gene for the HA protein was modified to remove its receptor-binding capacity to ensure that a novel tissue tropism cannot be induced in the recombinant virus. Immunoelectron microscopy also showed that virion envelopes were free of the marker fluHA protein.

These findings have implications for the design of new vaccines based on the rescue of negative strand viruses where either marker proteins or additional immunogens from other pathogens are incorporated into their genomes to produce dual vaccines.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Vaccines against East Coast fever: Where do we stand?

E.L.N. Taracha[a], V. Nene[b], M. Gardner[b], P.Van Der Bruggen[c], A.J. Musoke[a], S.P. Morzaria[d]

[a] International Livestock Research Institute (ILRI), Nairobi, Kenya

[b] The Institute for Genomic Research (TIGR), Rockville, Maryland, USA

[c] Ludwig Institute for Cancer Research (LICR), Brussels, Belgium

[d] Food and Agriculture, Regional Office, Bankok, Thailand

E-mail: E.Taracha@cgiar.org

A live vaccine is currently in use to control East Coast fever (ECF), caused by a tick-borne haemoprotozoan parasite *Theileria parva*, a fatal disease of cattle occurring in eastern, central and southern Africa. The live vaccine, though very efficacious, has serious shortcomings that limit its sustainability. Improved vaccines against *T. parva*, under development taking advantage of recent advances in biotechnology, will be part of an integrated control programme for ECF. A first-generation subunit vaccine based on the p67 sporozoite surface antigen has been evaluated under field conditions. This vaccine, having demonstrated efficacy levels of 50% reduction of severe disease, may be ineffectual on its own, but a useful part of a multicomponent vaccine. Current research to develop a vaccine incorporating antigens from the schizont stage of the parasite has exploited the *T. parva* genome sequence data and a platform of information on the protective immune mechanisms. Applying this rational approach, a number of vaccine candidate antigens have been identified that are being evaluated in cattle to provide proof-of-principle using proprietary technologies.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Evaluation of diagnostic tools for epidemiological purposes: Application to FMD

I.E. Bergmann, V. Malirat, E. Neitzert, E. Correa Melo

Pan American Foot-and-Mouth Disease Center PAHO/WHO, Rio de Janeiro, Brazil

E-mail: ibergman@panaftosa.ops-oms.org

Foot-and-mouth disease (FMD) is economically the most important disease of domestic livestock. Due to its highly infectious nature, ability to cause persistent infection and long-term effects on the animal species it affects, countries that have the disease have many trade restrictions placed upon them. The importance of laboratory information is critical for FMD control and eradication programs. In this regard and in support of the Continental Program for Eradication of FMD, implemented in 1988 in the Americas, a major challenge for the Pan American Foot-and-Mouth Disease Center (PAFMDC) as a regional OIE reference laboratory, was related to the adaptation of approaches to respond to the new demands of diagnostic precision placed to support the epidemiological transition expected in the Continent. Regions with advanced eradication campaigns needed to incorporate approaches for rapid and precise identification of the agent during an emergency, and equally important, for enabling a deeper epidemiological analysis, capable of characterizing the potential sources of infection and dissemination of the virus, either during clinical or subclinical viral circulation.

Since the implementation of the eradication program there have been significant advances in the understanding of FMD epidemiology in South America. These have partly been due to the application of nucleotide sequencing methods for viral characterization and of newly developed tools for seroepidemiological monitoring of persistent viral activity, based on the use of recombinant noncapsideal antigens as serological probes to detect infection specific antibodies, regardless of vaccination condition.

In the PAFMDC, a genetic sequence database of representative South American strains has been built up, which constituted the basis for the phylogenetic analysis of the viruses type O and A, responsible for the major outbreaks recorded, in regions already declared free by the OIE in the Southern Cone of South America (Argentina, Southern Brazil, Paraguay and Uruguay) in 2000/2002, and in endemic regions of the Andean area. This database is a valuable aid in tracing virus movement between geographical regions.

Genetic-based surveillance, although quite relevant, can only be applied on isolation of the agent. A wider epidemiological scenario can be reached when overall viral activity, including asymptomatic circulation, is assessed through serosurveillance. Moreover, the latter constitutes an important pathway to find the agent and thus, to support the genetic database. Effectiveness of active surveillance, irrespective of vaccination, has been attained through its development and validation of an immunoenzymatic system, composed of a screening test that detects antibodies against noncapsideal polyprotein 3ABC through an indirect ELISA and an enzyme linked immunoelectrotransfer blot (EITB) assay that detects antibodies against five noncapsideal proteins to confirm I-ELISA 3ABC suspect or positive samples. As a result of the validation process, we were able to identify the criteria for analysis when applying the system for assessing risk of viral activity and for confirmation of its absence in a population.

Thus, the system has been successfully applied to support epidemiological investigations after an FMD episode, including serological sampling to evaluate regions with epidemiological links with the outbreak area, to follow infection as a function of time and space after an episode, and to ratify the disease free status of the area. In addition, it is the tool used in South America during routine active surveillance, to trace viral niches, to support decision-making prior to interruption of vaccination, to support the OIE requirements for declaration of free regions and as an input for risk analysis during trade.

The application of the methods described has become increasingly important in understanding the epidemiology of FMD, including the role of carrier animals and will play a significant role in the development of future disease control strategies.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Virus evolution in the face of the host response

E. Domingo

Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos 28130, Madrid and Centro de Biología Molecular "Severo Ochoa", (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco 28049 Madrid, Spain

E-mail: edsolans@inia.es

Microbial infections are highly dynamic processes in which the invading pathogen must counteract host responses to complete an infectious cycle. Viruses offer a broad repertoire of strategies to cope with host defences. Complex DNA viruses encode a number of immunomodulatory proteins aimed at interacting with the host to modulate host responses. In contrast with this "interacting" strategy, the highly variable RNA viruses employ an "evasion" strategy, with the frequent selection of mutants capable of evading host immune responses. Mutation rates for RNA viruses are in the range of 10^{-3} to 10^{-5} misincorporations per nucleotide copied. This continuous mutational input has as a result that viral genome populations do not consist of a defined nucleotide sequence but of multitudes of closely related sequences. The entire mutant distribution is termed a viral quasispecies. The spectrum of mutants serves as a reservoir of genetic and phenotypic variants. The continuous replenishment of this dynamic reservoir constitutes an adaptative strategy for RNA viruses since selective pressures can result in dominance of viral subpopulations that were a minority in the parental distribution of mutants. Quasispecies adaptability is reflected not only in evasion of immune responses but also in changes in cell tropism and host range of viruses, with implications for viral disease emergence and re-emergence. Several examples of human and animal viruses in which quasispecies dynamics plays a role in viral persistence and disease progression will be discussed.

Quasispecies dynamics has additional implications for virus evolution, the diagnosis of viral disease and the design of preventive and therapeutic strategies. Viral populations can contain a memory of those genomes that were dominant at an earlier stage of their evolutionary history. This is a genetic memory in the form of minority components of the mutant spectrum, first described with two independent lineages of foot-and-mouth disease virus (FMDV) in cell culture. Memory has been recently described for human inmunodeficiency virus type 1 (HIV-1) *in vivo*. The identification of such memory genomes may be relevant for viral diagnosis during chronic infectious, to avoid rapid selection of mutants present at memory levels (for example in the face of a modification of treatment during HIV infections). Regarding prevention, quasispecies dynamics requires administration of multivalent vaccines (consisting of multiple B-and T-cell epitopes and when possible, several different relevant sequences of those same epitopes) to avoid or delay the selection of escape mutants. Regarding therapy, quasispecies dynamics demands combination therapy with multiple antiviral agents directed to independent functional targets of the virus, to avoid or delay selection of inhibitor-escape mutants. An example is highly active antiretroviral therapy (HAART) to control HIV infections. Quasispecies dynamics has opened also the possibility of a new antiviral therapy based on virus entry into error catastrophe, termed lethal mutagenesis. Recent results on these several implications of quasispecies dynamics will be reviewed.

These recent developments underline the need of trans-disciplinary approaches (involving evolutionary biology, physics and experimental virology) to the understanding of microbial infections and to the design of appropriate preventive and therapeutic treatments.

SESSION III: GENE-BASED TECHNOLOGIES APPLIED TO PLANTS, RUMEN MICROBES, AND SYSTEMS BIOLOGY

Chairperson: C.S. McSweeney, Australia

TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Rumen microbial genomics

M. Morrison[a], K.E. Nelson[b]

[a] The Ohio State University, Columbus, Ohio, USA

[b] The Institute for Genomic Research, Rockville, Maryland, USA

E-mail: morrison.234@osu.edu

Improving plant cell wall (fiber) degradation remains one of the highest priority goals for all livestock enterprises, whether it is the intensively managed dairy herds in the United States, or the nomadic cattle herds in sub-Saharan Africa. The North American Consortium for Genomics of Fibrolytic Ruminal Bacteria was created in 2000 to promote the sequencing and comparative analysis of rumen microbial genomes. High throughput genome sequencing offers the potential to obtain a complete blueprint for the lifestyle of a specific microbe, and to assess its genetic potential in a functional and comparative fashion. So far, a combination of funds from U.S. Department of Agriculture's (USDA's) Initiative for Future Agriculture and Food Systems and the National Science Foundation (USDA-NSF) Microbe Sequencing Program has supported the sequencing of three rumen bacterial genomes to closure. Genome closure and annotation is complete for *Fibrobacter succinogenes,* and the *Ruminococcus albus* and *Prevotella ruminicola* genomes should be completed during 2004. In addition to these sequencing projects, Consortium members have used subtractive hybridization methods to characterize the genomic differences among the sequenced genomes and the genomes of additional strains and species of ruminal bacteria. A database has also been developed by bioinformaticians at The Institute for Genomic Research (www.tigr.org), which will contain the sequence information arising from this project, as well as *in silico* tools for genome examination. Accordingly, our Consortium will have provided a comprehensive suite of resources and tools useful to microbiologists and animal scientists throughout the world, especially those interested in the conversion of cellulose-rich materials into useful commodities, such as meat, milk and draught animal power.

The inherent value associated with whole genome sequencing is already apparent for the *F. succinogenes* project. The genome sequence has revealed that there are many more genes encoding endoglucanases and cellodextrinases than what had previously been isolated from clone libraries propagated in *Escherichia coli.* Additionally, we have yet to identify either exo-acting cellulases or processive endoglucanases in the *F. succinogenes* genome. The cellulase system of *F. succinogenes* S85 therefore differs from all other studied cellulolytic microorganisms. Subtractive hybridization studies between strain S85 and closely related bacteria (*F. succinogenes* strain A3C and *F. intestinalis* DR7) have revealed that the DR7 genome encodes several transporters and a xylanase, which are either absent, or possess limited sequence identity, to genes found in the S85 genome.

Genome sequence data has also empowered studies underway with *R. albus*, by combining the sequence data with findings made from RNA and protein-based studies. For instance, several new processive endoglucanases have been identified by proteomic analysis of *R. albus* wild-type and mutant strains, and *R. albus* is now known to employ a variety of strategies for its adhesion to fiber. The subtractive hybridization studies also include the examination of a non-cellulolytic strain of *R. albus* (B199) with the sequenced genome, and have revealed that

strain B199 is missing several endoglucanases. As such, the genome sequencing projects are revealing, for the first time, those gene products rate-limiting to the process of cellulose degradation.

Even at these early stages, the ramifications of acquiring genome sequence data for rumen microorganisms are profound. The potential to elucidate which enzyme(s) or other ecological or physiological process(es) are rate-limiting to fiber degradation by these bacteria, and how this might change relative to dietary composition, are now greatly enhanced. With specific reference to cellulases, the Consortium projects have revealed that only a small percentage (~25%) of these genes had been previously identified, and that novel cellulase systems are employed by at least some ruminal bacteria. But is that enough? “Metagenomics” is a term coined with reference to the genetic potential resident within an entire microbial community, and is dependent upon high throughput DNA sequencing, advances in recombinant DNA technologies, and computational biology. It is anticipated that metagenomics will significantly augment the rumen genome studies that are already underway, and allow for the genetic characterization of microbes that cannot currently be cultured in the laboratory. The genetic potential of these species, which undoubtedly make a significant contribution to the ecology of the rumen environment have, until now, escaped attention. The "-omics" technologies also offer exciting new opportunities to investigate microbial diversity and physiology in ruminants, other herbivorous animals, and humans. Hopefully, the current model that has been established by the North American Consortium will be just the beginning, but we are aware that many challenges lay ahead in terms of funding, data acquisition, data mining, and data interpretation. The benefits from these studies will however have global implications for animal productivity.

TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Transgenesis and genomics in molecular breeding of pasture grasses and legumes for forage quality and other traits

G. Spangenberg

Plant Biotechnology Centre, Agriculture Victoria, Department of Primary Industries, La Trobe University, Bundoora, Victoria 3083, Australia and CRC for Molecular Plant Breeding

E-mail*: German.spangenberg@dpi.vic.gov.au*

Significant advances have been made in the establishment of the methodologies required for the molecular breeding of temperate forage grasses (*Lolium* and *Festuca* species) and legumes (*Trifolium* and *Medicago* species). Examples of current products and approaches for the application of these methodologies to forage grass and legume improvement are outlined. The plethora of new technologies and tools now available for high-throughput gene discovery and genome-wide expression analysis have opened up opportunities for innovative applications in the identification, functional characterisation and use of genes of value in forage production systems and beyond. Selected examples of our current work in pasture plant genomics, xenogenomics, symbiogenomics and microarray-based molecular phenotyping are discussed.

1. Pasture Plant Transgenesis

Gene technology and the production of transgenic plants offers the opportunity to generate unique genetic variation, when the required variation is either absent or has very low heritability. In recent years, the first transgenic pasture plants with simple 'engineered' traits have reached the stage of field-evaluation. While gaps in our understanding of the underlying genetics, physiology and biochemistry of many complex plant processes are likely to delay progress in many applications of transgenesis in forage plant improvement, gene technology is a powerful tool for the generation of the required molecular genetic knowledge. Consequently, applications of transgenesis to temperate pasture plant improvement are focussed on the development of transformation events with unique genetic variation and in studies on the molecular genetic dissection of plant biosynthetic pathways and developmental processes of high relevance for forage production [1].

Primary target traits for the application of transgenesis to temperate pasture plant improvement are forage quality, disease and pest resistance, tolerance to abiotic stresses, and the manipulation of growth and development. Some representative approaches and selected examples in temperate forage grasses and legumes are discussed [1].

Molecular breeding based on transgenesis to overcome limitations in forage quality may be targeted to the individual subcharacters involved: dry matter digestibility, water-soluble carbohydrate content, protein content, secondary metabolites, alkaloids, etc. These molecular breeding approaches may include modification of the lignin profile to enhance dry matter digestibility, genetic manipulation of fructan metabolism to increase non-structural carbohydrate content, genetic manipulation of condensed tannin synthesis to develop ‘bloat-safe’ forages, and the expression of 'rumen by-pass' proteins to improve the supply of proteins and essential amino acids. Most quality or anti-quality parameters are associated with specific metabolic pathways or the production of specific proteins. This allows target enzymes or

suitable foreign proteins to be identified, corresponding genes isolated, and their expression manipulated in transgenic forage plants.

Pathogen and pest infection can considerably lower herbage yield, persistency, nutritive value, and palatability of forage plants. An armory of genes and strategies for engineering disease and pest resistance in transgenic plants has been developed and tested over the last decade, including chitinases, glucanases, plant defensins, phytoalexins, ribosome-inactivating proteins, viral coat proteins, viral replicase, viral movement proteins, *Bt* toxins, proteinase inhibitors, and α-amylase inhibitors. Some of them have been applied to the development of pasture plants, mainly forage legumes, for enhanced disease and pest resistance [1].

Plants can be used to express recombinant heterologous proteins. Transgenic plants may be an attractive alternative to microbial systems for the production of certain biomolecules. The perennial growth habit, the biomass production potential, the capacity for biological nitrogen fixation, and the ability to grow in marginal areas exhibited by forage plants, particularly pasture legumes, make them potential suitable candidates for molecular farming. Advances in genetic manipulation technologies that allow high levels of transgene expression and transgene containment may, in the not too distant future, make it possible to exploit some forage plants as bioreactors for the production, among others, of industrial enzymes, pharmaceuticals, vaccines, antibodies and biodegradable plastics. Multidisciplinary efforts will, however, be needed to identify the most feasible targets, to generate transgenic plants with suitable expression levels, and to develop efficient downstream processing technology that could adapt transgenic forage plants for non-forage uses and make them a cost-effective alternative for molecular farming. Significant progress has been achieved in recent years in the production of value-added proteins in transgenic lucerne [1].

Small scale planned releases of transgenic plants are required to assess the stability of transgene expression and the novel phenotypes under field conditions and to identify transformation events suitable for transgenic germplasm and cultivar development. Only after the transformation events have been thoroughly evaluated for the stability of the novel phenotype outside of the controlled environment in a glasshouse would it be advisable to continue to integrate these in molecular breeding programs for the development of transgenic cultivars. An illustrative example of design features of such a small scale field trial can be found in a recent field trial of alfalfa mosaic virus (AMV) immune transgenic white clover plants [2].

A range of transformation events in forage legumes and grasses with proof of concept for the technology under containment conditions are being developed. The challenge now is how to best deploy these molecular technologies and tools to evaluate their full potential based on the transgenic transfer of single and multiple valuable genes, to generate novel genetic variability and novel elite transgenic germplasm, and to efficiently incorporate these factors into breeding programs for the development of improved cultivars.

Efficient strategies for the introgression of transgenes into elite parents for the subsequent production of synthetic cultivars have been developed ensuring stable and uniform transgene expression in all plants in the population. One such strategy has been applied to the production of AMV immune transgenic elite white clover plants homozygous for the transgenes. It involves initial top crosses of transformation events chosen after their field evaluation with elite non-transgenic white clover parental lines; selecting for progeny from the harvested seed carrying the transgene and its linked selectable *npt2* marker gene by antibiotic selection or PCR screening followed by diallel crosses between the T_1 progeny. The

T_2 offspring plants homozygous for transgenes can be directly identified by high-throughput quantitative PCR transgene detection. The elite white clover plants homozygous for the transgenes are then planted in a selection nursery together with elite non-transgenic parental lines for identification of the new parents of transgenic experimental synthetic cultivars and their subsequent multisite evaluation [1].

2. Pasture Plant Genomics

Forage plant breeding has entered the genome era. The plethora of new technologies and tools now available for high-throughput gene discovery and genome-wide expression analysis have opened up opportunities for innovative applications in the identification, functional characterisation and use of genes of value in forage production systems and beyond. Examples of these opportunities, include 'molecular phenotyping', 'symbio-genomics' and 'xeno-genomics' [1].

We have undertaken the discovery of 100,000 ESTs from the key forage crops of temperate grassland agriculture, perennial ryegrass (*L. perenne*) and white clover (*T. repens*) using high-throughput sequencing of randomly selected clones from cDNA libraries representing a range of plant organs, developmental stages, and experimental treatments. The DNA sequences were analysed by BLAST searches, categorised functionally, and subjected to cluster analysis leading to the identification of unigene sets in perennial ryegrass and white clover corresponding to 14,767 and 14,635 genes, respectively [1].

We have further developed high density spotted cDNA microarrays with approximately 15,000 unigene sets as a main screening tool for novel ryegrass and clover sequences of unknown function. These EST-based plant microarrays will allow the global analysis of gene expression patterns as a main approach for functional genomics and other applications. Novel applications of EST-based forage plant arrays including 'molecular phenotyping', i.e. the analysis of global or targeted gene expression patterns using complex hybridisation probes from contrasting genotypes or populations and contrasting environments, are now being tested to integrate microarray data with current conventional phenotypic selection approaches used in temperate pasture plant improvement [1].

Comparative sequence and microarray data analyses from ryegrass and clover with data from complete genome sequencing projects in *Arabidopsis* and rice as well as from extensive EST discovery programs in the model forage legume *M. truncatula* have been undertaken to provide insight into conserved and divergent aspects of grass and legume genome organization and function.

3. Pasture Plant Symbiogenomics

Pasture legumes and grasses offer unique and exciting opportunities in genome research to study plant-pathogen interactions, legume/nitrogen-fixing bacteria symbiosis, legume/mycrorrhiza associations, and grass/endophyte endosymbiosis, as well as to the application of the knowledge gained from these studies to develop resistance to pathogens and improved beneficial associations in forages.

We have undertaken a gene discovery program in the fungal endophytes of tall fescue and perennial ryegrass, *Neotyphodium coenophialum* and *N. lolii*, respectively. Approximately 8,500 *Neotyphodium* DNA sequences were generated, analysed by BLAST searches, categorised functionally, and subjected to cluster analyses leading to the identification of a 3,806 unigene set in *Neotyphodium*. The program is focused on the discovery of genes

involved in host colonization, nutrient supply to the endophytic fungus, and the biosynthesis of active pyrrolopyrazine and pyrrolizidine secondary metabolites (e.g. the insect deterrents peramine and N-formylloline, respectively) and their regulation. It will provide insight into the molecular genetics of the grass endophyte/host interaction as well as into the physiological mechanisms leading to the increased plant vigour and enhanced stress tolerance. These genomic tools and knowledge will underpin the development of technologies to manipulate grass/endophyte associations for enhanced plant performance, improved grass tolerance to biotic and abiotic stresses, and altered grass endophyte host specificity, to the benefit of the grazing and turf industries [1].

4. Xenogenomics

Genome research with exotic plant species, i.e. 'xeno-genomics', includes gene discovery by high-throughput EST sequencing and large-scale simultaneous gene expression analysis with EST-based microarrays. Xeno-genomics has opened up opportunities for a 'genomic bio-prospecting' of key genes and gene variants from exotic plants. This approach is particularly suited for the discovery of novel genes and the determination of their expression patterns in response to specific abiotic stresses.

We have undertaken a xenogenomic EST discovery focussed on selected Australian native and exotic grasses and legumes that show unique adaptation to extreme environmental stresses. Genes which allow certain plant species to tolerate extreme abiotic stresses including drought, salinity and low fertility soils are being isolated and characterised. The targeted species in the xenogenomic EST discovery program include Australian native grasses, such as the halotolerant blown-grasses (*Agrostis adamsonii* and *A. robusta*) and the aluminium-tolerant weeping grass (*Microlaena stipoides*); as well as exotic species such as antarctic hair-grass (*Deschampsia antarctica*), one of only two vascular plant species native to Antarctica [1].

The discovery of novel genes and their functional genomic analysis will facilitate the development of effective molecular breeding approaches to enhance abiotic stress tolerance in forages and other crops.

5. Molecular Breeding for Forage Quality

Molecular breeding based on transgenesis to overcome limitations in forage quality may be targeted to the individual subcharacters involved: dry matter digestibility, water-soluble carbohydrate content, protein content, secondary metabolites, alkaloids, etc. These molecular breeding approaches may include modification of the lignin profile to enhance dry matter digestibility, genetic manipulation of fructan metabolism to increase non-structural carbohydrate content, genetic manipulation of condensed tannin synthesis to develop 'bloat-safe' forages, and the expression of 'rumen by-pass' proteins to improve the supply of proteins and essential amino acids. Most quality or anti-quality parameters are associated with specific metabolic pathways or the production of specific proteins. This allows target enzymes or suitable foreign proteins to be identified, corresponding genes isolated, and their expression manipulated in transgenic forage plants.

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TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Investigation of the rumen microbial community responsible for degradation of a putative toxin in *Acacia angustissima*

E. Collins[a,b], C.S. McSweeney[b], D.O. Krause[b], L.L. Blackall[a]

[a] School of Molecular and Microbial Sciences, University of Queensland, St Lucia, Australia

[b] CSIRO Livestock Industries, Queensland Biosciences Precinct, Carmody Rd, St Lucia, Brisbane, Australia

E-mail: chris.mcsweeney@csiro.au

The tropical legume *Acacia angustissima,* has been proposed as an alternative feed source for ruminants in countries where limited choice and restricted availability of feedstock is an issue for farmers, due to extreme, harsh environments. The widespread use of *A. angustissima* as fodder is currently impeded by its potential toxicity to the animal, as observed in feed trials. Consequently, much research has been directed towards identifying the possible causes of its toxicity. Non-protein amino acids present in *A. angustissima* have been hypothesised to be responsible for the toxic effect of this plant. The non-protein amino acid 4-N-acetyl-2, 4-diaminobutanoic acid (ADAB) has been implicated in the toxicity of *A. angustissima* due to its high abundance and similarity to well known neurotoxins [1]. Thus, investigation into members of the rumen microbial community responsible for the degradation of ADAB has resulted.

Selective enrichments were carried out for ADAB degrading microorganisms from the rumen. Defined rumen-simulating media with an amino acid extract from *A. angustissima,* as the source of ADAB, were used. Additionally, ADAB degrading rumen enrichments were decimally diluted in an effort to determine at which dilution ADAB degrading activity was still present, and to further define the microbial community present. Investigations into the members of these mixed ADAB degrading rumen enrichments was achieved through culture-independent techniques.

Fluorescence *in situ* hybridisation (FISH) with oligonucleotide probes specific for the bacterial and archaeal domains and for major taxonomic groupings were used [2], highlighting that the microorganisms in the ADAB degrading mixed cultures were *Bacteria*. Consequently, bacterial 16S rDNA clone libraries were constructed from dilutions of an ADAB-degrading mixed culture and of a non-ADAB-degrading mixed culture [3]. The two bacterial 16S rDNA clone libraries were then subjected to restriction fragment length polymorphism (RFLP) analysis, partial and complete insert sequencing followed by sequence analysis. The ADAB degrading clone library yielded 57 full-length 16S rDNA clones with eight distinct RFLP patterns, while the non-degrading clone library consisted of 62 full-length clones and only four RFLP patterns. Approximately 60% of the clones from four of the eight RFLP patterns from the ADAB degrading clone library belonged to the *Firmicutes,* yet only two clones from the non-ADAB degrading clone library belonged to this group, and were represented by a single RFLP pattern. It was thus hypothesised that some members of the *Firmicutes* were likely to be of importance in ADAB degradation. Phylogenetic analysis of these *Firmicutes* sequences demonstrated they formed a monophyletic group supported by high bootstrap values. An oligonucleotide probe, ADAB1267 was designed to specifically

target this group for use in FISH. The ADAB1267 oligonucleotide probe hybridised a high proportion of long thin rods in the ADAB degrading mixed culture enrichments.

Since ADAB1267-targeted cells were abundant and distinctive in the ADAB degrading enrichment cultures, further research is currently underway investigating their significance in ADAB degradation. Proof that ADAB1267-targeted cells can take up ADAB could be accomplished by combining FISH with *in situ* microautoradiography (ISMAR), using radioactively-labelled ADAB. The identification of microorganisms responsible for ADAB degradation in the rumen, will lead the way for further research into the mechanisms of degradation, the ultimate goal being to confer toxin tolerance to ruminants in the field, thus allowing ruminant subsistence on *A. angustissima*.

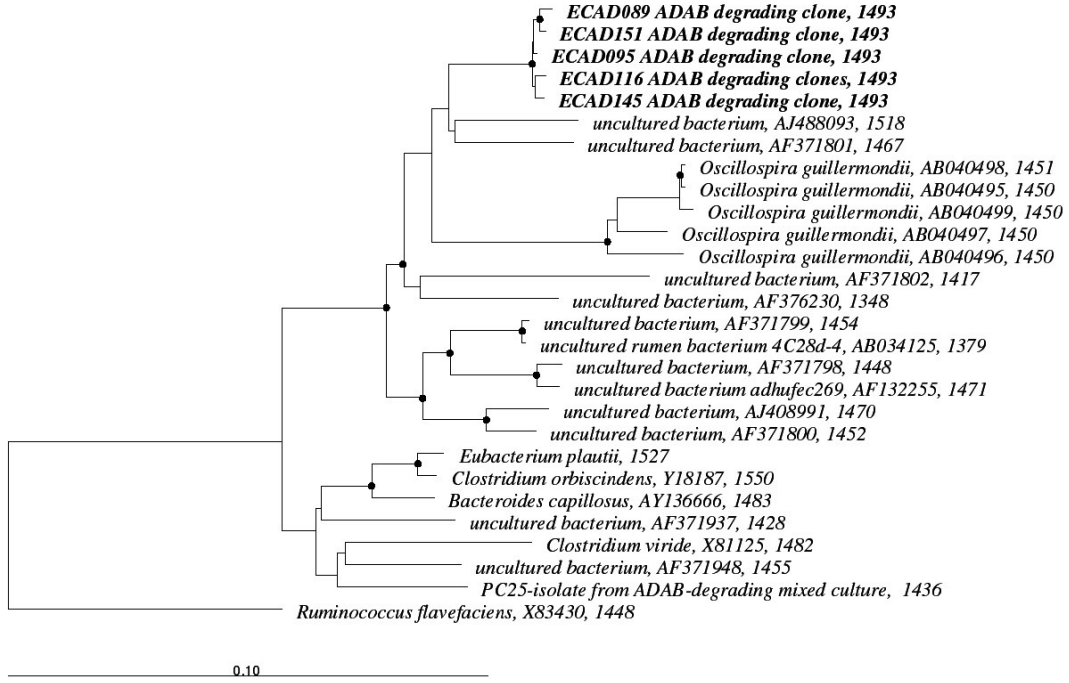

FIG.1. Evolutionary distance tree of 16S rDNA sequence data of clones from the ADAB degrading mixed culture belonging to the Firmicutes. Solid dots represent bootstrap values of 75% or greater for all four methods used in the bootstrap analysis.

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TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Ecology of tannin-tolerant streptococci in the rumen

D.O. Krause[a], W.J.M. Smith[a], J.D. Brooker[b], C.S. McSweeney[a]

[a] CSIRO Livestock Industries, Long Pocket laboratory, Meiers Rd, Indooroopilly, Brisbane 4068, Australia

[b] University of Adelaide, Roseworthy Campus, Roseworthy SA 5371, Australia

E-mail: john.brooker@adelaide.edu.au

Tannins are polyphenolic compounds that can be found in significant quantities in plant tissue, although the chemical structures are highly variable. In extensive ruminant grazing systems, particularly in developing nations, protein rich forage trees and shrubs are often integrated into grazing systems as protein supplements but these plants often contain tannins [1,2] which have a negative impact of tannins on ruminal microbiota and nutrient digestibility. Condensed tannins from the pasture legume *Lespedeza cuneata* [3] inhibit pectinases and cellulases that are often associated with the bacterial cell wall. Ruminal bacteria that are inhibited include *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Ruminobacter amylophilis* and are correlated with ultra-structural changes of the cell wall, while these cell wall changes do not occur with the tolerant *Prevotella ruminicola* [4, 5] . Other organisms which appear to tolerate tannins, are *Streptococcus gallolyticus* [6], *Clostridium* spp., and some proteobacteria [7].

Tannin tolerant *S. gallolyticus* 2.2 produces an extracellular polysaccharide (EPS) coat in response to tannic acid and condensed tannins from *Acacia angustissima* and is probably the electro dense layer typically visible at the cell surface in the presence of tannins [8]. We have examined the molecular basis of tolerance to condensed tannins in this organism, and to do this we measured expression levels of EPS genes in the presence of tannins. We found that there was a significant increase in cellular polysaccharides and EPS gene expression in response to tannic acid, acacia tannin, and calliandra tannin in *S. gallolyticus* 2.2 but not *S. bovis* JB1. We also found that tannin tolerant streptococci could be distinguished from tannin sensitive streptococci based on 16S-rDNA oligonucleotide probes. These probes were applied to ruminally extracted rRNA from animals consuming diets containing acacia or calliandra. To examine the response *in vivo* we fed sheep either *A. angustissima*, or *C. callothyrsus* and measured the abundance of *S. bovis* and *S. gallolyticus* populations using 16S-rRNA based oligonucleotide probes that differentiated these two populations. The *S. bovis* population declined significantly when acacia was fed at 37% of the diet but recovered when polyethylene glycol (PEG) was added to the ration; PEG has a neutralising effect on tannins. The *S. gallolyticus* population was more resistant to the presence of tannins in the diet but this effect was diminished when PEG was added to the diet. Similar population dynamics were observed with sheep fed calliandra at 30% of the ration. In conclusion, it appears that EPS production is correlated with tannin tolerance *in vitro*. Tannin resistant *S. gallolyticus* is more abundant in the rumen in the presence of tannins than is *S. bovis* indicating the presence of tannin resistant organisms may have significant nutritional consequences for the animal.

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TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Application of molecular microbial ecology and functional genomics tools to elucidate mechanisms of tannin resistance in intestinal bacteria

A.H. Smith, E.G. Zoetendal, M.A. Sundset, R.I. Mackie

Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801, USA

E-mail: r-mackie@ux1.cso.uiuc.edu

Tannins are polyphenolic compounds produced by plants to protect themselves against herbivory, tissue decay and infections by pathogens. Tannins can be divided into two structural groups namely hydrolysable and condensed tannins. Condensed tannins or proanthocyanidins consist of polymers of flavonol units. Tannin-resistant bacteria in the rumen are thought to prevent detrimental effects on animals due to tannins in the diet and may be able to confer protection to animals not adapted to a tannin-containing diet. Tannin-resistant bacteria have been isolated in recent years from the intestinal ecosystem although the mechanisms mediating this resistance remain enigmatic.

In order to elucidate these mechanisms, the effect of dietary condensed tannins extracted from *Acacia angustissima* on rat fecal bacterial populations was determined. Comparative studies were carried out to determine the proportion of the bacterial populations resistant to tannins on tannin-free and tannin-containing diets. The proportion of tannin-resistant bacteria increased significantly when tannins were present and returned to pre-exposure levels in the absence of dietary tannins. A shift in bacterial populations was confirmed by molecular fingerprinting of fecal bacterial populations by Denaturing Gradient Gel Electrophoresis (DGGE). Post-treatment samples were generally still distinguishable from controls after 3.5 weeks indicating a long-term population shift. Sequence analysis of DGGE amplicons and characterization of tannin-resistant isolates indicated that tannins selected for Enterobacteriaceae and *Bacteroides* species. Dot blot quantification confirmed that these Gram-negative bacterial groups predominated in the presence of dietary tannins with a corresponding decrease in the Gram-positive *Clostridium leptum* group and others. A long-term shift was confirmed, as the *Bacteroides fragilis* group was significantly higher than controls 3.5 weeks post-treatment. Metabolic fingerprint patterns revealed that functional activities of culturable fecal bacteria were affected by the presence of tannins and again a long-term shift in bacterial populations was indicated. Thus condensed tannins were shown to alter fecal bacterial populations resulting in a shift in predominant bacteria towards tannin-resistant Gram-negative Enterobacteriaceae and *Bacteroides* species. However, the exact mechanisms behind this resistance are still unknown.

The aim of the next study was to unravel the main mechanisms of tannin resistance of *Escherichia coli* BW13711 using a DNA microarray approach. The strain was cultured in a chemostat under anoxic conditions in medium containing either 0.4% (wt/vol) glucose (control) or 0.4% (wt/vol) glucose with 1% (vol/vol) of an aqueous extract from wattle bark (*Acacia mearnsii,* 66% total phenolics) (adaptation response). In addition, a sample was taken two hours after *E. coli* was transferred from the control steady state culture into fresh medium containing glucose and 2% tannin extract (shock response). From all samples total RNA was isolated, quantified, and used for hybridization on the GeneChip® *E. coli* Antisense Genome Array (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's instructions.

Microarray data were analyzed using MicroArray Suite 5.0 (Affymetrix Inc.) and GeneSpring 5.0 (Silicon Genetics, Redwood City, CA, USA) software. The duplicate tannin adaptation array data were used to determine the 95% confidence interval for gene expression levels. The Colibri database (http://genolist.pasteur.fr/Colibri/index.html) was used to determine the major responses of *E. coli* by finding the corresponding open reading frames (orfs) and functional pathways of the 40 most up-regulated orfs during the shock and adaptation responses. The 95% confidence interval of the tannin adaptation array duplicates demonstrated that genes >1.9 times up- or down-regulated could be considered as significantly regulated. This resulted in a total of 1372 and 1648 genes that were up- or down-regulated during the tannin shock and tannin adaptation response of *E. coli*, respectively. Under both conditions, the majority of these genes (882 and 1202 respectively) were up-regulated.

The highest (52 fold) up-regulated gene of the shock response was *spy*, encoding the SPY protein (spheroblast protein y) which is involved in the outer membrane protein folding and cell envelope synthesis. Analysis of the 40 most up-regulated genes revealed that 12 of these genes belong to or were related to genes induced by heat shock, phage shock and other shock conditions. All genes belonging to the multiple antibiotic resistance *mar*RAB operon were also found in the 40 most up-regulated genes. Up-regulation of this operon resulted in the down-regulation of *omp*F (12.5 fold), a gene encoding for a porin for organic molecules. With the exception of *yeg*O (6 fold up-regulated) genes of the *yeg*MNOB operon, a newly discovered multidrug transport system were also present in the 40 most up-regulated genes. The 40 most up-regulated genes of the shock response were also up-regulated in the tannin adaptation response, although the level of up-regulation was in general much lower. Overall, DNA microarray analysis of gene expression of *E. coli* in response to the presence of tannins indicated that the major strategies utilized by *E. coli* to develop resistance against the adverse effects of tannins are cell envelope-related.

TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

The application of molecular microbial ecology tools to facilitate the development of more efficient feeding systems and reduce adverse environmental effects of rumiant livestock in the developing world

G.J. McCrabb[a], C.S. McSweeney[b], S. Denman[b], M. Mitsumori[c], H.P.S. Makkar[d]

[a] International Livestock Research Institute (ILRI), P.O. Box 5689, Addis Ababa, Ethiopia

[b] CSIRO Livestock Industries, Queensland Biosciences Precinct, Carmody Rd, St Lucia, Brisbane, Australia

[c] National Institute of Livestock and Grassland Science, Ikenodai 2, Kukizaki, Ibaraki, 305-0901 Japan

[d] Animal Production and Health Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Wagramerstr. 5, 1400 Vienna, Austria

E-mail: g.mccrabb@cgiar.org

Ruminant livestock in the developing world are a major global anthropogenic, human induced, source of methane gas emissions. Methane gas is produced in the digestive tract of ruminant livestock during feed digestion and released into the environment. As a result of international concern over climate change induced by rising greenhouse gas emissions, international policy makers and national governments are considering cost effective methods for reducing global greenhouse gas emissions.

Global inventories of greenhouse gas emissions indicate that this source of methane is the largest individual source of methane, and in 1990 accounted for 23% of global methane emissions. Cattle are responsible for 73% of methane emissions from all livestock species, and more than half of the global cattle population is located in developing countries. The largest cattle populations in the developing world in the South Asia, Sub Saharan Africa, Latin America and China. Projections of ruminant meat and milk consumption in the developing world indicate a doubling of the livestock population by the year 2020, which is an increase of about 3% per annum in developing countries. These increases in livestock populations are projected to be associated with an almost doubling of methane emissions from livestock between 1990 and 2020. If significant abatement of global enteric methane emissions is to be achieved in the short and long term, policy makers will need to look towards the developing countries for significant abatement.

Feeding systems of ruminants in developing countries are typically based on lower quality tropical forages, and associated with higher emissions of methane per unit of meat and milk production than livestock systems in developed countries. Reducing methane emissions from ruminants is a mean to increase feed conversion efficiency (i.e. more meat or milk production per unit of feed intake).

A large proportion of the global ruminant population are located in tropical environments, where animals feed predominantly on low quality highly fibrous forages. Methane emissions

from ruminants fed on fibrous diets are higher than outputs from better quality temperate forages. The excretion of methane from the rumen can represent a loss of 8–10% of the digestible energy depending on the type of diet. Therefore, reducing methane production could benefit the ruminant energetically provided the efficiency of ruminal metabolism is not compromised. Studies indicate that a reduction in methanogenesis in the rumen can be associated with improvements in feed conversion efficiency without affecting intake. The relationship between methane production, feed digestion and animal production efficiency will be discussed and the results of recent experimental studies will be presented.

Any attempt to reduce methane emissions from livestock is unlikely to be adopted unless production efficiency is at least maintained if not enhanced. The challenge therefore is to devise strategies, which reduce methane emissions from ruminants and improve production efficiency. Approaches such as inhibitors of methanogens (e.g. synthetic or plant secondary metabolites), dietary approaches (e.g. use of polyunsaturated fatty acids or ingredients containing these acids), vaccination against methanogens, supplementation strategies, etc. have the potential to achieve this objective. The use of rumen molecular techniques in conjunction with conventional approaches such as measurement of methane by GC in *in vitro* and by sulphur hexafluoride (SF6) tracer or gas mask technique in *in vivo*, apparent and true dry matter digestibility, and efficiency of microbial protein synthesis using purine as a marker or ^{15}N incorporation in *in vitro* and urinary purine derivative methodology in *in vivo* studies could result in innovative technologies to reduce methane emission and increase microbial protein and energy supply to animals from fibrous feed resources. Current approaches to the evaluation of digestibility and nutritive value of feed resources using conventional *in vitro* feed evaluation and animal studies have resulted in a large body of information about nutrient composition, digestion kinetics and digestibility. However, these techniques are unable to describe the microbial mechanisms involved in ruminal digestion, and are unlikely to result in the development of rational feeding strategies. Conventional culture-based methods of enumerating rumen bacteria are being rapidly replaced by the development of nucleic acid based techniques which can be used to characterise complex microbial communities. The foundation of these techniques is SSU rDNA (eg. 16S rRNA sequences) sequence analysis which has provided a phylogenetically based classification scheme for enumeration and identification of microbial community members. The 16S rRNA sequences in DNA extracted from a mixed digesta sample can be amplified by PCR using primers and the diversity and identity of the amplified 16S rDNA can be further analysed by several molecular techniques including: 1) restriction enzyme analysis of amplified polymorphic DNA (RFLP); 2) 16S rDNA based cloning, sequencing and probing; and 3) denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and single strand conformation polymorphism (SSCP). Also the more variable sequence regions of the 16S rDNA are hybridisation sites for genus, species and sometimes even strain specific hybridization probes. Therefore oligonucleotide probes targeting the respective 16S rRNA of the methanogenic archaea, and the major fibrolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens*) in the rumen would be a robust approach to quantifying the effect of reduced methanogenesis on important functional microbial groups. Similarly PCR primers that are species-specific can be designed to enumerate these populations by real time PCR. The molecular based ecology techniques are also likely to provide better insight into the interactions between methanogens and the other rumen microorganisms. All this information should assist in the development of strategies for improving production by reducing methanogenesis. A logical strategy would be the *in vitro* examination of various potential approaches for reduction of methanogenesis, followed by in depth *in vivo* evaluation of the promising approaches. Another interesting aspect is the establishment of correlation between methane production and methanogen numbers using molecular probes. This information

could lead to the development of a simple tool based on the methanogen number, for investigations on strategies being developed and tested for methane reduction, without the need to measure methane emission, which is complex, time consuming and requires substantial resources. A Coordinated Research Project to address these aspects will be initiated under the auspicious of the Joint FAO/IAEA Division in 2005.

TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

The effect of secondary compounds on the rumen microbial population structure measured by 16S rRNA and 18S rRNA

E. Wina[a,b], S. Muetzel[a], H.P.S. Makkar[c], K. Becker[a]

[a] Institute for Animal Production for Tropics and Subtropics (480B), University of Hohenheim, Stuttgart 50793, Germany

[b] Research Institute for Animal Production, Jl.Veteran III, Banjarwaru-Ciawi, Bogor, Indonesia

[c] Animal Production and Health Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Wagramerstr. 5, 1400 Vienna, Austria

E-mail: wina@uni-hohenheim.de; balitnak@indo.net.id

Plant secondary compounds in the forages have an important role in determining forage quality. A method for evaluating their effects on microbial population structure was carried out using the *in vitro* gas syringe system [1] followed by extraction of RNA and gel separation of 16S rRNA and 18S rRNA. Quantification of 16S rRNA and 18S rRNA bands indicated the prokaryote and eukaryote populations, respectively [2].

Five types of plant materials, i.e. *Nothopanax scutellarium* (Mangkokan) leaves, *Morinda citrifolia* (Mengkudu) fruit, *Sapindus rarak* (lerak) fruit and two types of *Sesbania sesban* leaves (hgh saponin and low saponin) were tested and *Pennisetum purpureum* (rumput gajah, Indonesian name) was used as a control roughage. Presence of saponin in these plant materials was determined qualitatively by thin layer chromatography.

Eukaryote population was found to be significantly affected by the above plant materials. Both types of *S. sesban* leaves caused total elimination of eukaryotes. *S. rarak* reduced both eukaryote and prokaryote populations. The observed inhibition of eukaryote population might be due to the presence of saponin in these plant materials.

In another experiment, a methanol extract of *S. rarak* which contained saponin was included and its effect on *in vitro* fermentation of *P. purpureum* was evaluated. The results showed that at higher levels of inclusion of *S. rarak* methanol extract, eukaroytes were totally eliminated. Comparison was made between microbial mass calculated based on difference between apparent undigested residue and true undigested residue and microbial mass calculations based on 16S rRNA and 18S rRNA. Microbial mass calculated by difference method was much higher than the microbial mass calculated on the basis of 16S rRNA and 18S rRNA.

The quantification of RNA can be a useful and rapid technique for an accurate assessment of the effect of new forage materials on the microbial population structure. Other parameters from *in vitro* fermentation like short chain fatty acid, efficiency of microbial protein synthesis are additionally required for determining the nutritional quality of a new forage material.

The measurement of microbial mass in *in vitro* using 16S rRNA and 18S rRNA must be validated using other methodlogies, both in *in vitro* and *in vivo*.

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TOPIC: Gene based technologies applied to plants, rumen microbes and systems biology

Nutrition-gene interaction (post-genomics): Changes in gene expression through nutritional manipulations

G.S. Harper[a], S.A. Lehnert[a] , P.L. Greenwood[b]

[a] CSIRO Livestock Industries, Queensland Bioscience Precinct, St. Lucia, Qld, 4068, Australia

[b] NSW Agriculture Beef Industry Centre, University of New England, NSW, 2351, Australia

E-mail: gregory.harper@csiro.au

Consumers in the developed world have demanded quality and consistency from animal products marketed to them, and as a result implementation of quality assurance systems has been a major industry proirity. These systems are based on the principle that quality, as defined by the consumer, can be influenced at critical control points in the production chain.

Consumer demands for sustainable production practices have also led to adoption of strategies sometimes counter to eating quality objectives. For example, *Bos indicus* animals are used in extensive grazing systems of northern Australia because of their drought and parasite tolerance, but have inferior eating quality. Given the environmental rigours of this northern production system, cattle producers are attempting to optimise decisions regarding all variables that influence their enterprises. These include breed and genotype, slaughter age, lifetime nutrition, health management strategy, growth promotion strategy, weather predictions, stocking rate, and eating quality criteria.

In this paper, we will review what is known about development of the intrinsic factors (those extant at the time of slaugher) of bovine skeletal muscle as they relate to the subsequent eating quality attributes of meat. These attributes include toughness, colour, fat content, and nutritive value, but exclude food safety. We will focus on current understanding of nutritional regulation of the candidate genes known to underlie each of these attributes, and where appropriate draw on understanding developed in other mammalian species.

We will discuss two experimental studies that are likely to contribute to our understanding of functional gene networks that are responsive to nutritional restriction. The first investigated the effects of severe nutritional restriction and compensatory growth in *Bos indicus* cross cattle during the post-weaning period. The study was motivated by our previous finding that nutritional effects on meat toughness were long-lived and of sufficient magnitude to be of commercial and scientific interest [1,2]. The second study involved pre-weaning nutritional restriction of cattle of specific genotpye: heterozygotes for the double muscling (*mh*) mutation; and Wagyu cross cattle. The aim of the study was to determine whether the expression of double muscling or marbling can be affected by restricted prenatal and preweaning nutrition.

The cattle used in these studies were selected on the basis of sire breeding values for the marbling, or double muscling traits. Where appropriate, individual animals were genotyped for key meat quality genetic polymorphisms (e.g. the TG5 marker for marbling). Post-weaning nutritional treatments were designed to at least halt weight gain in the animals, and at

most lead to a loss of 15% of body weight over 100 days. Pre-weaning nutritional treatments involved restricting nutrition of the pregnant cows from ~100 days of pregnancy, and continued restriction of both cow and calf until the calves were weaned. Tissue samples were collected by repeat biopsy and at slaughter. Muscle and fat phenotyping is currently underway and involves: muscle fibre typing; muscle fascicular structural characterisation; carcass yield; and eating quality objective measures. Gene expression profiling is being performed on a subset of the animals using a bovine muscle and fat cDNA microarray. Bioinformatic tools have been developed to not only identify the genes that are either up- or down-regulated in the respective nutritional treatments, but also to identify co-regulated genes.

In the longer term, animal productionists need accurate models for breed and nutritional effects on the intrinsic properties of muscle, so that they can predict the effects of nutritional regimens on eating quality of meat. Polymorphic responses of individual genes are important contributors to the whole response, but need to be viewed in perspective with the larger scale homeostatic and homeorhetic responses of the animal. Given the wealth of information that has come from the Cattle Industry Cooperative Research Centre [3], we now have a framework on which to build a mechanistic understanding of at least the genetic effects on meat eating quality. We acknowledge with thanks, the work of a large team of researchers within the Cooperative Research Centre who have contributed to the experimental studies we will review.

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Options for development of transgenic pigs with enhanced performance traits

C.W. Forsberg, S.P. Golovan, J.P. Phillips, A. Ajakaiye, M.Z. Fan, R.G. Meidinger, R.R. Hacker

University of Guelph, Guelph, Ontario N1G 2W1, Canada

E-mail: cforsber@uoguelph.ca

Traditional breeding practices have yielded a slow but steady genetic improvement of domestic animals [1]. Unfortunately, these practices often do not enable the separation of desirable production traits from undesirable traits, and furthermore, do not enable the transfer of advantageous genetic traits from one species to another. Transgenic methodologies surmount these barriers, and transgenic pigs have been developed that have a variety of novel enhanced performance traits, the capability to serve as factories for the production of pharmaceuticals, and soon may provide a reliable supply of organs for xenotransplantation. This presentation will focus primarily on the expression of novel performance traits, since they have the potential to provide the greatest benefit to farmers in countries with a less well-developed agricultural infrastructure.

The first hurdle in the development of animals with novel production characteristics is the availability of reliable methods for the production of transgenic animals. This requires the combination of a suitable transgenic technique and an appropriate genetic construct. Classic pronuclear microinjection, the original method for producing transgenic animals may soon be surpassed by the more convenient sperm-mediated transgenesis [2], use of a retroviral vector system, or by techniques involving nuclear transfer [3].

Despite the complexity involved in generating transgenic animals, a variety of interesting performance enhancing genes have been introduced into pigs. These include porcine growth hormone and IGF1 to enhance growth characteristics and carcass quality [4], α-lactalbumin to enhance the growth of nursing piglets [5], plant oleate desaturase (http://www.newscientist.com/hottopics/gm/gm.jsp?id=ns99991841) to increase the proportion of unsaturated fatty acids in tissues, and phytase to enhance plant phosphorus utilization [6]. No information is available on the performance or meat quality characteristics of pigs expressing the desaturase gene, however, expression of growth hormone, α-lactalbumin and phytase genes in pigs has been shown to be efficacious. As an example, phytase in transgenic pigs enable practically complete utilization of the phosphorus in cereal grain as compared to less than half by non-transgenic pigs. This new trait would be particularly beneficial in a production environment without extensive infrastructure characteristic of modern intensive Western-style agriculture. In addition to bypassing the need for expensive supplemental phosphorus, it also could help avoid environmental problems associated with intensive agriculture.

In addition to genes already tested that enhance performance characteristics of pigs, other genes of interest include those coding for plant cell wall hydrolases, genes coding for disease resistance, and yet others that could improve protein utilization.

Expression of a novel gene in a pig is only the first step in bringing a new line of transgenic pigs into the pork production system. Once a transgenic pig has been documented to perform according to expectation, and several generations have passed to ensure that the novel gene is stably inherited and expression maintained, governmental regulatory requirements must be satisfied to ensure that the pig has no deleterious effect on the environment, and that the meat is safe for human consumption [7]. The food safety standards may vary among countries, but those established by the FAO (http://www.fao.org/es/ESN/food/risk_biotech_papers_en.stm) are usually taken as the primary requirement and country specific requirement overlaid. Meeting these standards dictates that, at least initially, only traits with great utility will be funded at a sufficiently high level to afford the essential testing to meet national and international safety requirements.

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SESSION IV: GENE-BASED TECHNOLOGIES IN ENVIRONMENT, FOOD SAFETY AND ANIMAL INDUSTRY AND RELATED ETHICAL AND INTELLECTUAL PROPERTY RIGHT ISSUES

Chairperson: J. Hodges, Austria

TOPIC: Gene-based technologies in relation to the environment, food safety and livestock industry, and related ethical and intellectual property rights issues

Ethical, social, environmental and economic issues in animal agriculture

P.C. Kesavan, M.S. Swaminathan

M.S. Swaminathan Research Foundation, Third Cross Street, Taramani Institutional Area, Chennai - 600 113, India

E-mail: pckesavan@mssrf.res.in; mssrfed@vsnl.net

Livestock are vital to sustainable agriculture in most developing countries. In Asia, in general, the integration of livestock, fish and crops has proved to be a sustainable system through centuries of experience. Traditional use of dung for manuring the fields and bullocks for ploughing is the "biodynamic farming", which has special significance in dry land agriculture comprising about 65 percent of India's cropped area. Gene-based technologies will be useful for developing special draught breeds of cattle so that their valuable source of energy remains available especially to the small and marginal farmers. Further, the livestock in the developing countries form an essential part of an integrated agricultural system and, therefore, development strategies for gene-based applications should consider the total production system (i.e., breeding fodder and forage crops, animal and crop disease and pest management etc).

The gene-technologies of relevance to the developing countries are (i) rumen molecular techniques for reducing methane production and for increasing protein and energy supply; ruminant food stuffs currently used in India and a few other developing countries are fibrous, low in nitrogen, and contain anti-nutritive factors, (ii) improving animal productivity in developing countries by manipulation of nutrition *in utero* to alter gene expression of key metabolic hormones and enzymes for a long period after birth in cattle, (iii) genetic resistance to *Helminthes* in sheep, and (iv) molecular methods of diagnosis, molecular epidemiology and treatment of swine fever. Although not gene-based, artificial insemination (AI) for genetic improvement of dairy cattle and buffalo, and embryo-transfer (ET) for rapid multiplication of elite cattle are also relevant. Cloning (of the 'Dolly' – the sheep kind) will be useful to revive the rare and endangered animal species such as one-horned rhinoceros, swamp deer, wild buffalo and dugongs in India, for restoring environmental balance and social harmony with the forest, coastal and hill communities of various regions of India.

The ethical, environmental and economic issues of animals in agriculture are best discussed in the context of mixed farming for the sustainable livelihood security of millions of resource-poor rural communities with small or no land holdings at all. India has about 16% of the cattle, 57% of the buffalo, 17% of the goat and 5% of the sheep population of the world. India's population of cattle and buffaloes estimated at about 32 million in 2001, forms nearly 31% of the global cattle population of about 1.1 billion. Further, 70% of the livestock is in the hands of small and marginal farmers and landless labourers, who own less than 30% of the land area! About 19 million people (about 11 million in principal status and 8 million in subsidiary status) secure their livelihood in the livestock sector. Women constitute 71% of the labour force in livestock farming. In the dairy sector, 75 million women are engaged as against 15 million men. Production systems are based on traditional low-cost input systems and many of the cattle and livestock owners subsist at or below poverty line.

The ethical issues from a technological point of view centre around both gene-based and non-gene-based technologies to improve the nutrition, health and productivity of the farm animals. In particular, a reference needs to be made to bovine somatotropin (bST), a natural growth hormone secreted by the anterior pituitary in all animals, with a major effect on the regulation of growth and also milk production. Since the quantities of bST obtained from slaughtered animals are quite small, recombinant DNA technology-based r-bST is produced, and widely used in the USA to increase the milk production by 10% to 20%. Exhaustive evaluation tests conducted in the USA have shown that r-bST has no harmful effects in milk, but a high production of milk makes higher demands on animal physiology, and if an adequate food supply is lacking, negative effects are observed on fertility besides other health problems, especially mastitis and ketosis. Presently, neither r-bST, nor adequate nutritious feed is available for millions of dairy cattle in the developing countries. In the Indian scenario, the non-availability of r-bST to enhance the milk productivity of dairy cattle has an economic dimension to the "production of milk by masses" as compared to "mass production" in a developed country, say USA. During 2002-03, India produced about 88.0 million tons of milk, but this involved pooling the milk by as many as 75 million women and 15 million men from their dairy cattle. In contrast, the milk production for the corresponding period in the USA has been about 69.0 million tons, involving just about 0.25 million farmers. The economic benefits of "mass production" over "production by masses" are obvious; further, the access to the r-bST in the "mass production" system, but not in the system of "production by masses" could accentuate the economic disparity. The above-said scenario has further ramifications in view of the implications arising from the WTO-related Agreement on Agriculture. With an array of domestic supports, products of "mass production" could be dumped into developing countries causing a substantial rise in the already high levels of livelihood and food insecurity.

Since the r-DNA based technologies for "pharming" or for human food are not yet applied to farm animals in the developing countries there are no serious concerns of bio-safety, and violation of ethical norms. In the countries, where animals for human food are genetically altered, the issues of "animal sentience" and "telos" become quite pertinent. For example, the "growth genes" introduced into pigs (i.e. Beltsville Pigs) to accelerate growth and produce leaner meat also result in a number of serious physical disabilities to the pigs. Of course, in the case of "broiler" chickens too, it is true that their skeleton and cardio-vascular systems are more poorly developed than their muscles and gut. The broiler chickens never live their natural life and thus their rights are ignored. With an ever increasing concern for "animal welfare", the need is to recognize the "animal right" (the right not to be tortured and left in pain) and to introduce humane and kindly treatment so that the unpleasantness of pain is greatly reduced. The objection is about treating animals as "raw materials" upon which our ends and purposes can be imposed regardless of the ends and purposes natural to them. Legislation introduced in Sweden stipulates that farm animals be allowed to live their lives in accordance with their telos - e.g. cattle to have the right to graze and chicken and pigs have the right to freedom of motion. This is abundantly relevant to the developing countries where mixed farming with goals of "evergreen revolution" and organic approach can still allow a greater degree of telos and freedom of movement to the animals maintained for milk, meat or draught purposes. The challenge before us is to foster the advancement of science, particularly the new genetics, while addressing the concerns of society.

TOPIC: Gene-based technologies in environment, food safety and animal industry and related ethical and intellectual property right issues

Risks of gene transfer from GMOs to livestock and its consequences for health and nutrition

R.H. Phipps[a], D.E. Beever[a], R. Einspanier[b]

[a] School of Agriculture, Policy and Development, The University of Reading, Reading RG6 6AR, UK

[b] Institute of Veterinary Biochemistry, Oertzenweg 19b, 14163 Berlin, Germany

E-Mail: r.h.phipps@reading.ac.uk

Background

The global area of GM crops has increased from 2 million ha in 1996 to 59 million ha in 2002. The principal crops modified for agronomic input traits such as herbicide tolerance and/or insect resistance are soyabean, maize, cotton, and canola. These crops are all used extensively in livestock production rations as either energy and/or protein feed resources. They are included either in the form of whole crop, (eg. maize silage); a specific crop component (eg.maize grain); or as co-products (eg. oilseed meals, or maize stover). One of the main concerns expressed over the use of GM feed ingredients arose from the fact that some GM crops contained genes coding for antibiotic resistance which were used as markers in the initial plant selection process. The other main concern was linked to the possible accumulation of transgenic DNA and protein in milk, meat and eggs derived from animals receiving diets containing GM feed ingredients and over possible human and animal health risks. These concerns resulted in renewed interest in the fate of DNA and protein in the gastrointestinal tract. Key issues in this debate will be addressed in the paper.

Fate of DNA and protein in the digestive tract of livestock

The paper will discuss the principles of digestion of DNA and protein and then consider the factors that effect the fate of transgenic (tDNA) and protein in the digestive tract of livestock.

1. Effect of conservation and processing of feeds used for animal production. The ensiling process with chopping of plant tissue and the subsequent lowering of pH by lactic acid fermentation produces a harsh environment for DNA and will accelerate its degradation. It has been noted that while the origin of silage could be confirmed it showed that the ensiling process resulted in major fragmentation of the tDNA and that the presence of intact, functional genes after an extended time of ensiling was highly unlikely. While grinding and milling has little effect on DNA fragment size, mechanical expulsion or chemical extraction of oil from seeds can cause extensive fragmentation. Dry heat applied at 90^{o}C appeared to have no effect, while 95^{o}C for 5 minutes caused considerable fragmentation of the plant DNA. Equally steam at low to moderate pressures caused substantial fragmentation. Workers have shown that processed feed samples may contain DNA fragments large enough to contain functional genes and thus farm livestock will consume both tDNA and protein.

2. Presence of transgenic tDNA and gene products in milk, meat and eggs. Numerous studies will be presented to show that to-date, neither tDNA nor recombinant gene products have

been detected in milk, meat or eggs derived from animals receiving GM feed ingredients as part of their diet. Although tDNA has not been detected, small fragments (199 bp) of plant chloroplast DNA have been detected in lymphocytes and milk of cattle, and in muscle, liver, spleen and kidneys of chickens fed either conventional or Bt maize. However, all published studies have shown that tDNA has not been found in animal tissue. The obvious explanation may be that the chloroplast genome is present in each plant cell in far greater numbers than the nuclear genome in which current transgenes are integrated. This means that there is far more chloroplast DNA than tDNA in feed material. Consequently its uptake would be a much rarer event than that of chloroplast DNA and therefore more difficult to detect. Thus it is possible that the detection of DNA is a function of its abundance and analytical sensitivity. However, if there is no concern over the presence of fragments of plant and animal DNA in milk, meat and eggs, why should there be if tDNA were to be detected in animal products at some point in the future given that it would not differ from that ingested from foods already deemed safe.

3. Uptake of DNA by intestinal microflora. The presence of antibiotic resistance marker genes (eg. kanamycin, hygromycin, ampicillin) in first generation GM crops raised concerns that these genes may be transferred to bacteria which may cause diseases in animals and humans, making them resistant to antibiotics often used for their control. This is an understandable concern but should be put into perspective with the over prescription for humans and widespread use on farms. To our knowledge some antibiotic resistance genes e.g. ampicillin resistance (AmpR), are found wide-spread naturally in intestinal bacteria. Therefore a specific gene transfer of particularly that AmpR DNA fragment from Bt maize towards rumen bacteria will be hard to analyse. Even though many authorities consider that the potential for any transfer and thus risk to public health is virtually zero, it has been agreed to phase out the use of antibiotic resistance markers in the development of new biotech products.

Effects on animal and human health

If the use of GM feed ingredients had detrimental effects on animal health it would not be unreasonable to expect those to be manifested in not only an increased occurrence of health related incidents but also impaired animal performance. Neither of these effects have been noted in the literature and evidence on the nutritional equivalence of GM feed ingredients has noted where their inclusion has improved actually improved performance. Amongst others the World Health Organization has stated that the consumption of DNA from all sources – including plants improved through biotechnology– is safe and does not produce a risk to human health, given the long history of safe consumption of DNA. While not proofed positive in the classical experimental form it is worth noting that for each of the last 5 years 300 million US-Americans have consumed authorised GM food (70% of all items on supermarket shelves contains GM ingredients directly or indirectly) in the form of milk, meat and eggs from animals fed GM feed, yet there has not been a single authenticated negative health related incident attributed to GM crops and that includes allergic reactions.

Conclusions

The rate of adoption of first generation GM crops such as soybean, maize, cotton and canola has been dramatic and these crops are used extensively in livestock rations. To-date tDNA and protein has not been detected in milk, meat and eggs derived from animal fed diets containing GM ingredients and there have been no authenticated cases of health related incidents recorded in animals and humans associated with the medium term consumption of GM feed or food.

TOPIC: Gene-based technologies in environment, food safety and animal industry and related ethical and intellectual property right issues

Regulatory and bio-safety issues in relation to transgenic animals in food and agriculture, feeds containing GMO and veterinary biologics

H.P.S. Kochhar, G.A. Gifford, S. Kahn

Animal Health and Production Division, Animal Products Directorate, Canadian Food Inspection Agency, Ottawa, Canada

E-mail: hkochhar@inspectin.gc.ca

The development of an effective regulatory system for genetically engineered animals and their products has been a subject of increasing discussions among researchers, industry and policy developers, as well as the public. Transgenic technology alters an animal's genome to achieve desired production or health effects of commercial or societal value. Since transgenesis itself is a relatively new scientific approach, transgenic animals are new organisms for which there is no existing information relevant to their performance under domestication or to their behavior in the wild, nor is there any firm basis for predicting their potential. The issues associated with the regulation and biosafety of transgenic animals pertain to environmental impact, human food safety, animal health and welfare, trade, and ethics. Many concerns related to genetic modifications in animals or plants focus on safety of the human food chain. Given the present public perception of animal biotechnology in general, transgenic animals are viewed with varying degrees of optimism and skepticism. In light of these divergent views the question that remains to be answered is how to develop regulations that safeguard the public concerns and at the same time allow this technology to benefit agriculture, in a manner that neither 'restricts' nor 'facilitates'.

Transgenic animals are produced for four basic reasons: to improve animal health, to increase productivity and improve product quality, to mitigate the environmental impact of food-animal production, and to produce therapeutics. To date, scientists have been able to add, delete, silence or partially activate genes of interest. To regulate such a powerful technology predicated on limited background information is a challenge not only to the regulators, but also to the developers who strive to prove that these animals are safe by demonstrating bio-equivalency to their conventional counterparts. The regulations are based on the principle of substantial equivalence from the point of view of human food safety, and data is required to elucidate molecular characterization, nutritional similarities, and toxicological studies to substantiate that the animal product is safe. To address the concerns connected with the environmental release of transgenic animals, the regulatory framework should take into account the ability of genetically modified animals to survive and compete with the conventional populations. They should consider biosafety issues to prevent adverse effects of genetic modification on bio-diversity and to prove that the animals have no negative environmental impact. The challenge to regulate the animal health component of transgenic animals is addressed by animal welfare considerations and risk assessments, to ensure that such animals are not susceptible to diseases or acting as vectors for disease promoting organisms by virtue of their transgenic origin.

The Canadian regulatory system relies on the "precautionary principle" in its approach to regulate the "product" instead of the "process". The regulatory framework captures transgenic animals under a 'safety net' created by the Canadian Environmental Protection Act (CEPA),

administered by Environment Canada. The New Substances Notification Regulations (NSNR) (http://www2.ec.gc.ca/substances/nsb/enq/qui_e.htm) of this Act define the standards and guidelines for assessment of transgenic animals before their introduction in to the environment. Assessment must be based on the intended use, whether it is food, bio-pharmaceutical or organ transplantation. Food from transgenic animals is assessed for safety by Health Canada under its Novel Foods Regulations of the Food and Drugs Act. Transgenic animals developed for food purposes are considered novel foods if (i) they have no previous history of safe use; (ii) the manufacturing process has never been previously used; and (iii) food derived from plants/animals/ organisms that have been genetically modified falls outside the expected standard range. To date, no transgenic animal has been approved for food use or for release in Canada.

The link between animal feed and the human food chain necessitates establishing strict criteria for the evaluation of feeds containing genetically modified materials. The regulatory issues related to feed assessments tend to overlap the assessment standards for novel foods and the environmental assessment of genetically modified organisms. Feed containing any genetically modified organism is considered *Novel Feed*, defined as a feed comprising an organism or organisms (i.e. from plant, microbial or animal source) or parts or products thereof that is not set out in schedules or has a novel trait. Novel traits include characteristics which have been intentionally selected, created, or introduced through a genetic change or based on valid scientific rationale that are not substantially equivalent in terms of their use and safety from environmental, human, and animal health points of view. The Canadian Food Inspection Agency (CFIA) administers the national livestock feed program under the authority of the *Feeds Act* and *Regulations*, to verify that livestock feed manufactured and sold in Canada is safe, effective, and labelled properly.

The regulation of veterinary biologics, in an effort to prevent and diagnose infectious diseases in animals, relies on effective science-based regulatory controls that take into account safety, efficacy and availability. To meet the licensing requirements, veterinary biologics must be shown to be pure, potent, safe, and effective when used according to label recommendations. The detailed description of the process followed in preparing a veterinary biologic, tests used to establish its efficacy, purity and potency, and the methods employed in handling, storing, and labelling a veterinary biologic are critical to effective regulation. Veterinary biologics are regulated by the CFIA under *Health of Animals Act* and *Regulations*. The aspects of permit issuance and the bio-safety protocols followed in production and testing of biologics are critically assessed before products are approved for use.

The Canadian system of regulation for feeds, veterinary biologics and transgenic animals may be applicable to other countries in the process of establishing a regulatory framework for biotechnology derived products. An effective regulatory filter can permit safe products while forming a secure barrier for those that pose an unacceptable risk. However, even though extrapolation of regulatory principles from the Canadian system may be adaptable in developing countries, there remains a wide scope for improvements, just as the Canadian system is undergoing further development. The fast pace at which methodological advancements are currently being introduced indicates that the regulatory system will have to be constantly reviewed, altered and improved in a manner that keeps up with the technological leaps in order to address the public concern over transgenesis in agriculture.

TOPIC: Gene-based technologies in environment, food safety and animal industry and related ethical and intellectual property right issues

IPR issues with relevance to the application of gene-based technologies to animal production and health in developing countries

G.M. Dutfield

Queen Mary Intellectual Property Research Institute , Queen Mary, University of London, John Vane Science Building, Charterhouse Square, London EC1M 6BQ, UK

E-mail: g.m.dutfield@qmul.ac.uk

Patents protect inventions that are considered by the national or regional granting authorities to be new, to involve an inventive step or be non-obvious to a skilled person, and to be susceptible to industrial application. Before the 1980s non-biological and microbiological processes were patentable in Europe and North America, but the situation for life forms as well as their structural and functional components was uncertain to say the least.

There is now much more clarity in these parts of the world, and the extent to which discoveries arising from gene-based technological processes can be patented, including living things, is now quite substantial. Nonetheless, these and other countries diverge widely with respect to how their patent systems deal with the new biotechnologies, and the current international rules contribute to harmonisation in this area only to a moderate degree.

For example, the United States makes no explicit exceptions in biotechnology and the related fields of animal and plant breeding. In Europe, animal and plants are patentable but not animal or plant varieties. Few if any developing countries follow the United States, and some of them are extremely restrictive in terms of how far they permit the patenting of biotechnological inventions or even how far they are willing to accept that any kind of natural product or living thing can be classed as an invention at all. The international rules accommodate these differences except that micro-organisms and non-biological and microbiological processes must be patentable. They do not refer directly to DNA sequences, and so the situation is open to interpretation.

The World Intellectual Property Organization, a United Nations specialised agency dealing with the promotion and further development of intellectual property rights, is holding negotiations on the harmonisation of patent standards so that national and regional patent regulations would be virtually identical. It is far from clear that this is a good thing, especially for the developing countries.

With respect to the interests of developing countries and patenting in this area, several questions arise that will be dealt with in the paper; first, how do Europe and North America treat gene-based technologies and the products derived from them in their patent regulations? Second, what are the consequences in terms of attracting research investment and enhancing welfare-enhancing innovation? Third, is the harmonisation of patentability standards in respect of gene-based technologies relating to animal production and health a good or bad thing for developing countries if this means using either Europe or the United States as their model? Fourth, given the widely divergent capacities possessed by different developing countries to generate, absorb and adapt such technologies, should the international rules

actually be relaxed so as to allow as much differentiation as possible to fully accommodate each countries' capacities, needs and priorities?

TOPIC: Gene-based technologies in environment, food safety and animal industry and related ethical and intellectual property right issues

Antibiotic resistance and plasmids carriage among *Escherichia coli* isolates from chicken meat in Malaysia

Tin Tin Myaing[a], A.A. Saleha[b], A.K. Arifah[b], A.R. Raha[c]

[a] Pharmacology and Parasitology Department, University of Veterinary Science, Yezin, Myanmar

[b] Faculty of Veterinary Medicine

[c] Department of Biotechnology, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia, 43400UPM, Serdang, Selangor, Malaysia

E-mail: tmyaing@hotmail.com

One hundred and thirty-one *Escherichia coli* isolates from raw chicken meat were tested for antibiotic susceptibility to twelve antibiotics, namely ampicillin 10 μg, cefoparazone 30 μg, cephradine 30 μg, ciprofloxacin 5 μg, chloramphenicol 30 μg, enrofloxacin 5 μg, erythromycin 15 μg, kanamycin 30 μg, nalidixic acid 30 μg, tetracycline 30 μg, trimethoprim 5 μg, and vancomycin 30 μg. The plasmid isolation was carried out according to the method described by Maniatis et al [1], with modifications as in the protocol provided by *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (ABI P/ N 401150). The newly modified method is a mini alkaline-lysis / PGE precipitation procedure and easy to perform on large numbers of samples. The graphical method of relating the logarithm of the molecular weight of a DNA molecule (log C) to its electrophoretic mobility (m) in gels was used to determine the molecular weight of plasmid. Plasmids of known molecular weight from *E. coli* V517 were used as standards for calibrating the size of plasmid DNA molecules. In this study DNA fragments are referred to as plasmids.

In all *Escherichia coli* isolates resistance to ampicillin (96.2%), cefoperazone (83.3%), cephradine (93.9%), ciprofloxacin (78.0%), chloramphenical (75.6%), enrofloxacin (72.0%), erythromycin (84.0%), kanamycin (50.8%), nalidixic acid (94.7%), tetracycline (90.2%), trimethoprim (94.7%) and vancomycin (100%) was observed (Table I). Plasmid occurrence rates of 81.7% were observed among *E. coli* isolates from the chicken meat. The number of plasmids ranged from 0 to 8 and the sizes of plasmids ranged from 1.2 MDa to 118.6 MDa. Plasmids were detected in 93.8% of *E. coli* isolates that were resistant to all 12 antibiotics and in 90.5% of *E. coli* isolates resistant to 11 antibodies (Table II). Three (2.8%) *E. coli* isolates harboured 8 plasmids and showed resistant to 12 antibiotics (Table III). The antibiotic resistance among the *E. coli* isolates in this study was compared and it was found a higher percentage of *E. coli* isolates resistant to ampicillin (96.9%), cephradine (94.7%) and trimethoprim (96.3%) had plasmids. It is known that antibiotic resistant genes in bacteria are usually carried in extrachromosomal DNA and therefore it may be concluded that when *E. coli* contained a high number of plasmids, it possessed a higher resistance to antibiotics.

TABLE I. PERCENTAGE OF *ESCHERICHIA COLI* ISOLATES RESISTANT TO INDIVIDUAL ANTIBIOTIC

Antibiotic tested	No (%) of *E. coli* isolates resistant to
Ampicillin	127 (96.2%)
Chloramphenicol	100 (75.6%)
Cephradine	124 (93.9%)
Cefoperazone	110 (83.3%)
Ciprofloxacin	103 (78.0%)
Erythromycin	111 (84.0%)
Enrofloxacin	95 (72.0%)
Kanamycin	67 (50.8%)
Nalidixic acid	125 (94.7%)
Tetracycline	119 (90.2%)
Trimethoprim	125 (94.7%)
Vancomycin	131 (100%)

TABLE II. NUMBER OF DNA FRAGMENTS HARBOURED BY *ESCHERICHIA COLI* ISOLATES IN RELATION TO NUMBER OF ANTIBIOTICS THEY ARE RESISTANT TO

E. coli isolates resistant to number of antibiotics	Number of *E. coli* isolates	*E. coli* isolates	
		With plasmids (n=107)	Without plasmids (n=24)
12	48	45 (93.8%)	3 (6.3%)
11	21	19 (90.5%)	2 (9.5%)
10	27	18 (66.6%)	9 (33.3%)
9	9	8 (88.9%)	1 (11.1 %)
8	17	10 (58.8%)	7 (41.2%)
7	3	3 (100%)	0
6	1	1 (100%)	0
5	1	1 (100%)	0
4	3	1 (33.3%)	2 (66.7%)
2	1	1 (100%)	0

TABLE III. *ESCHERICHIA COLI* ISOLATES RESISTANT TO NUMBER OF ANTIBIOTICS AND HARBOURED PLASMIDS

No. of plasmids	Resistant to number of antibiotics												
	12	11	10	9	8	7	6	5	4	3	2	1	0
8	+												
7	+	+				+							
6		+	+	+									
5	+	+	+		+	+	+						
4					+				+		+		
3								+	+				

+ = resistant to antibiotics and possessed plasmids

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TOPIC: Gene-based technologies in relation to the environment, food safety and livestock industry, and related ethical and intellectual property rights issues

Comparison of DNA probe, PCR amplification, ELISA and culture methods for the rapid detection of Salmonella in poultry

J.A. Qasem, S. Al-Mouqati, G.P. Rajkumar

Kuwait Institute for Scientific research, Food Resources Division, Biotechnology Department, P.O. Box 24885, SAFAT Kuwait 13109

E-mail: qjafar@safat.kisr.edu.kw

The detection of foodborne microorganisms has traditionally been done using microbiologically based methods. Such methods are generally reliable but have the disadvantage of being labor intensive, subjective, and time consuming. This study was conducted to compare molecular techniques and immunoassay to the culture methods for detection of *Salmonella* in food.

The identification of *Salmonella* sp. from poultry meat was studied by comparing bacterial detection using the Gene-Trak colorimetric hybridization method, PCR amplification kit and ELISA (Enzyme Linked Immuno Sorbent Assay), compared to the conventional methodology proposed by US FDA for detection of *Salmonella* in food samples. A total of forty samples which included positive and negative controls, were studied. The detection limits of the PCR assay were 10^2 and 10^1 CFU/ml after enrichment at 37^{o}C for 6 and 9 hr, respectively. When the assay was validated, *S. enteric* in artificially inoculated meat, 10^1 CFU/g was detected. The hybridization assay was able to detect as little as 100 pg of purified chromosomal DNA of *S. typhimurium* and 10 CFU g-l of artificially contaminated food sample. The sensitivity and specificity value of ELISA compared to the DNA amplification were 91.3% and 100%, respectively. Good enrichment procedures are very important to achieve a detection limit of approx. 10^5 cell/ml.

All the three methods investigated demonstrated high sensitivity and specificity for *Salmonella* in comparison to conventional standard bacteriological methods. Economic reasons may determine whether these alternative methods can be used routinely in food inspection.

TOPIC: Gene-based technologies in relation to the environment, food safety and livestock industry, and related ethical and intellectual property rights issues

Control of bovine spongiform encephalopathy by genetic engineering: Possible approaches and regulatory considerations

J.S. Gavora[a], H.P.S. Kochhar[b], G.A. Gifford[b]

[a] Consultant, 51 Forest Hill Avenue, Ottawa, ON K2C 1P7 Canada

[b] Animal Biotechnology Unit, Veterinary Biologics Section, Canadian Food Inspection Agency, Ottawa, Canada

E-mail: gavora@sympatico.ca

Transmissible spongiform encephalopathies (TSE), including bovine spongiform encephalopathy (BSE) and scrapie in sheep, are diseases in which affected animals exhibit abnormal neurological behavior associated with accumulation of prion protein in nervous system tissues. TSEs also occur in humans and include sporadic Creutzfeldt-Jakob disease (CJD), as well as its new variant (nvCJD) believed to be related to consumption of meat from BSE cattle. Since the emergence of BSE, also known as 'mad cow disease' in 1986, significant numbers of BSE cattle have been diagnosed in several countries and there have been more than 100 deaths from definite or probable nvCJD in humans. This paper examines a possible genetic modification that may render cattle resistant to BSE.

Prion proteins (PrP), present in both animals and humans, consist of approximately 250 amino acids and play a role in copper transport and regulation in tissues. The conversion of a normal, wild-type PrP^{C} into the pathogenic prion PrP^{Sc}, involves a change in protein folding. Presence of aberrantly folded PrP^{Sc} stimulates the conversion of PrP^{C} to PrP^{Sc} and the accumulation of PrP^{Sc} leads to a dysfunction of the central neural system. The aberrant PrP^{Sc} is virtually indestructible as it is not digested by proteases, unaffected by sterilization techniques, high doses of gamma irradiation, or incineration.

Naturally occurring scrapie resistant sheep that do not convert PrP^{C} to PrP^{Sc}, have the 'ARR' genotype, i.e. amino acids alanine, arginine and arginine in PrP positions 136, 154 and 171 [1]. Sheep selection for scrapie resistance based on DNA analyses is now in progress. In humans, the presence of lysine instead of glutamine at position 219 of the PrP amino acid chain results in resistance to sporadic CJD [2]. Any publication on existence of a similar resistance to BSE has not been found in available literature.

Research with laboratory mice [3] indicated a possible genetic engineering approach to the introduction of BSE resistance. At mouse PrP amino acid position 167 (corresponding to sheep position 171) the authors substituted arginine for glutamine, or at mouse PrP amino acid position 218 (corresponding to human position 219) they substituted lysine for glutamine. For each mouse amino acid positions 167 or 218, there were two groups of mice: (1) those producing only the engineered PrP, and (2) those producing the engineered PrP in combination with normal wild type PrP. When the mice were inoculated with a pathogenic prion protein, group (2) showed limited neural tissue damage, but group (1) was unaffected. The modifications did not appear to influence the normal function of PrP. The inability of mice carrying the modified PrP genes to support prion replication raises the possibility of producing prion-resistant livestock expressing genetically engineered PrP with a single

amino-acid substitution. The impact of such genetically engineered BSE resistant cattle could be rather rapid and widespread if the PrP gene in somatic cells from elite bulls were modified in tissue culture, as suggested above, to produce elite bull clones. Such clones could introduce BSE resistance to cattle populations through artificial insemination.

A possible problem with introducing resistance to BSE by genetic engineering is that prion-resistant animals might still harbor acquired prion infectivity [4]. Regulatory assessment of such engineered animals would need to ascertain, besides their general acceptability, also that possible existence of such 'carriers' does not result in a threat to animal and human health.

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TOPIC: Gene-based technologies in relation to the environment, food safety and livestock industry, and related ethical and intellectual property rights issues

Genetically modified organisms in New Zealand and cultural issues

R. McFarlane

Lincoln University, Christchurch, New Zealand

E-mail: *mcfarlan@lincoln.ac.nz*

Since 1996, the development or importation of genetically modified organisms (GMOs) in New Zealand has been covered under regulations contained within the Hazardous Substances and New Organisms (HSNO) Act. Previous to this time, and especially since, there has been spirited opposition to policies - promoted by commercial and scientific interests - relating to GMOs based on environmental, economic or ethical concerns. The latter reason has particular relevance to the Maori people of New Zealand, although Pakeha (Europeans) may have similar views. Indeed, Section 8 of the HSNO Act requires that the national committee regulating GMO activities (Environmental Risk Management Authority-ERMA) takes account of the Treaty of Waitangi which is a founding document signed between Maori and the British Crown in 1840. Within this treaty Article 3 stipulates that Maori shall retain guardianship of their natural resources and not loose possession of their treasures (taonga).

A review of recent applications (July 1998-May 2003) for the development or importation of GMOs is indicated in the accompanying table.

	Applications	Approved	Withdrawn	Declined
Import or develop GMO	144	129	12	0
Field test GMO in containment	15	13	**2**	0
Field release GMO	0	0	0	0
Import new organism (non GMO)	33	28	4	0
Release of new organism (non GMO)	7	5	1	1

The main reason for the initial withdrawl of the 2 field test applications (bold and underlined above) was that they would "impinge on the relationship of Maori culture and traditions with other taonga (treasures)". These 2 case studies will be described to illustrate the dilemma that has arison between a Western science and secular-based paradigm which emphasises quantitative risk assessment, versus a traditional belief system that is fundamental to an indigenous (Maori) culture.

Both examples involve the production of transgenic livestock incorporating human genes. In the first case study, transgenic sheep were to be produced in containment (grazed pasture within a securely fenced enclosure), for the purpose of producing human alpha-1-antitrypsin (hAAT) in milk. Following purification, this substance would be used for clinical trials, as a potential treatment for cystic fibrosis or congenital deficiency of hAAT, in humans. This application was eventually approved with a substantial list of controls. The second application

was for the production of transgenic cattle in containment, expressing myelin basic protein (MBP) in milk and again following initial postponement of a decision, approval followed.

The majority of the opposition to both applications stemmed from the strongly held spiritual beliefs of Maori living adjacent to the proposed research area (Manuwhenua), although there was a variety of opinions within Maori.

Inheritance or geneology (Whakapapa) is the foundation on which all tribal Maori values, including spiritual and ethical, are based. Whakapapa establishes the family origins of individual Maori within the family (whanau), subtribe (hapu) and tribe (iwi). Whakapapa is the way in which the people of the land (tangatawhenua) relate to mountains, rivers and the physical features within an area where they reside and allows them to claim governorship (rangitiratanga) over these taonga. To compromise the integrity of the whakapapa by altering gene structures of species through artificial manipulation, which would not occur naturally, is inherently against natural rules (tikanga). In addition, some Maori are particularly of the view that the crossing of human genes with other species is abhorrent.

In both instances the applications were eventually approved. ERMA decided that the advantages of the generation of scientific knowledge and possible pharmaceutical developments overrode the recognized affront to the spiritual beliefs and values of local Maori. The decisions highlight the difficulty in ascribing a value to spiritual issues and indeed in finding a compromise position that encompasses both spiritual and scientific interests.

PANEL DISCUSSION 2: ROLE OF INTERNATIONAL ORGANIZATIONS AND FUNDING AGENCIES IN PROMOTING GENE-BASED TECHNOLOGIES IN DEVELOPING COUNTRIES

Chairperson: G. Apostolatos, European Commission

TOPIC: Role of international organizations and funding agencies in promoting gene-based technologies in developing countries

Opportunities and constraints for using gene-based technologies in animal agriculture in developing countries and possible role of international donor agencies in promoting R&D in this field

M.L. Madan

Livestock Systems Group, INDRI, Karnal, India

E-mail: *mlmadan@hotmail.com*

Gene-based technologies for livestock are in varying stages of development and implementation mainly focussed on analysis of individual componants of complex animal system. Most of these technologies, evolved through International initiatives in the developed countries, have found limited application for the livestock of developing world. Several genes and traits of excellence have been associated with livestock of developing countries of Asia and Africa. High milk fat in buffaloes, protein quality for mozzarella cheese in buffalo milk, the fiber of 'Pashmina' and 'Toos' in the hair coat of goats in cold dry regions, the high prolificacy gene in Black Bengal goats, twinning genes in Garole sheep, high salt tolerance of Andaman goats, high altitude mountain adaptability of Yak and Mithun, high lignin content feed utilization in tropical arid ecology by camel, sheep and goat and disease as well as stress resistance by different species are examples where livestock of developing world has a global advantage. Efficient epidemiology, disease surveillance and monitoring, value addition in meat, leather and diary industry, economic biopharmaceutical production from transgenic livestock and developing of comprehensive data base, are other areas in which gene-based technologies are directly involved and hold great prospect in developing economies.

Animal owners in the developing world are resource poor, small scale operators, mostly landless or with marginal lands, having limited animal holdings, dependent on agro-ecology of temperate climate, with limited purchasing power and having little opportunity to access resources or make resource allocation decisions for animal production. This situation, for the poorest of the poor who live on livestock, is fast deteriorating mainly due to fragmentation of limited holdings, exhaustion of land resource, fatiguing of green revolution and increasing human and animal population pressure.

Biotechnologies in the developing world have to be applied in the shadow of paradox of plenty of food grains in the middest of poverty, mal-nutrition, disease, poor hygiene and unemployment. Poverty can be addressed only if there is convergence and synergy between various agriculture and animal production programmes. Past decades of green revolution have brought in comparative prosperity among the farmers with land. Such farmers had all the infrastructure and funding support to increase crop production in a commodity centered approach. An unintended consequence of this policy was neglect of a majority of farmers, who are landless or marginal farmers and subsist only on livestock, thus remaining poor. To address poverty there exists, therefore, no other way but to address animal improvement directly.

The major constraints for the utilization of the above technologies would include, (a) absence of an accurate and complete batabase on livestock and animal owners with a programme compatibility, (b) extensive animal biodiversity with respect to species and breed in agro-

ecological sub systems, (c) models of biotechnological intervention distinctly different than those in the developed economics, (d) animal species and breeds unique to the developing world as each breed/species has distinct developmental, production, disease resistance and nutriant utilisation niche (e) absence of a critical mass of trained scientists, technicians and field workers for developing and application of technologies both in the government and private sector, (f) absence of an industry-university interface for translating technologies to products, (g) non existant means to access technologies from the developed world at an affordable price to make a rightful, positive and sustainable contribution to livestock and economic welfare of people, (h) intensive and expensive technological inputs in terms of material, biological, equipment cost, (i) lack of policy issues on biosafety and risk analysis of new biologicals, gene products, transgenics and modified food items, and above all (j) negligible investment in animal biotechnology.

Globally it has been realized that biotechnology will play a strategic role for the economic development of the animal owners. But, it has to be recognized that world wide R&D and production trends do not conform to the concerns, needs and opportunities of developing world. Developing world has the largest concentration of diverse livestock population. There is a limited private and public sector investment in animal agriculture, particularly when modern biotechnologies are 'resource hungry'. Inspite of several laboratory advances in the developing world, in most cases, they have not been converted into a transferable technology or product. The key players and the potential users, the resource poor farmers with little knowledge base or literacy, do not regard these technologies as worthwhile in relation to the effort, resource and risk involved. This is mainly because there is no agency or industry to scale up and package the technology. Also, as in the developed world, the remarkable economic interest in marketting the services and products of biotechnology does not exist due to limited purchasing power by resource poor stake-holders.

A pragmatic approach is needed in providing adequate multi-institutional (National and International) support or an International Donor Consortium setup for supporting cost effective, cheap, easily adaptable biotechnological interventions. The total financial spending by International Agencies for animal biotechnolgy, targeted to developing countries, is currently very meager and constitutes a small percentage of its total spending on agriculture. World Bank, FAO, CGIAR, UNDP, USAID, SIDA, IDRC, ADB, etc., and other Donor and Funding Agencies have to proportion a higher percentage of funds for livestock, as the economy of the developing country in tropical agro-ecology is sustained by the livestock. Biotech strategy should include, a Foundation Fund to support cutting edge R&D, increase access to investment capital and create humen resource capacity. Financial audit in terms of investment on livestock improvement and Human Development Index relationship is convincing enough, with a dramatic and far reaching impact, to commit massive financial support to animal production and health biotechnologies for economic prosperity, nutritional security, rural development and health of poor populations in the developing world.

TOPIC: Role of international organizations and funding agencies in promoting gene-based technologies in developing countries

Objectives, capabilities and dangers in the role of international organizations and funding agencies in promoting gene-based technologies in developing countries

J. Hodges

Lofererfeld 16, A-5730 Mittersill, Austria

Email: *hodgesjohn@compuserve.com*

The paper examines the objectives, capabilities and dangers facing international bodies and funding agencies concerned with promoting the new and still emerging gene-based technologies in developing countries. First, major issues are considered, followed by an examination of the context within which public activities and funding must take place.

MAJOR ISSUES:

Who will be the decision-makers in transferring gene-based-technologies?
Donor or recipient?
Governments, institutions, individual scientists or private sector?
Scientists, development specialists, public or private investors?

Identification of need, benefit and opportunity
Complexity of using gene-based technologies
Who are the beneficiaries: local, regional, national or global?
Evaluation of benefits of funding biotechnology
Understanding the local situation

The nature of gene-based technologies
How do gene-based technologies differ from previous development approaches?
Biotechnology: information, technique, or product?
Mechanisms for delivery of public funding and support

Private versus public funding
Motivations: conflicting or complementary?
Speculative or mission oriented research and technology?
Transfer of existing technology or encouragement of new research?

Potential conflicting issues in role of international bodies
Patents and intellectual property rights
Scientific certainty and probability
Public consequences: assessment of risk in short and long terms
Ethics and cultural issues.

CONTEXT FOR PUBLIC ACTION AND FUNDING:

In considering the above issues the paper recognizes some key infrastructures which place important constraints upon the availability of the technologies and their possible use in developing countries.

Public versus private involvement in gene-based technologies
The paper interprets the term "international organizations and funding agencies" to mean the public sector as contrasted with private organizations and investments. The contrast is important and the paper examines critical differences in motivations, procedures, expectations, benefits and likely output between public and private activities. An understanding of this contrast is vital to any serious consideration of the role of the public sector.

Global or local technologies
The paper considers the issue of technologies that are capable of bringing benefit wherever they are applied against those technologies which need to be designed in the research stage for specific environments and cultures.

Poor versus rich
The growth of gene-based technologies in the developed west has been possible because of the wealth of the west in finance, intellectual resources and the existence of a highly structured economic system offering a route for application. This has been true of research in both the public and the private sectors. Most research to date has been within the context of wealthy states which are likely to be made richer economically or in quality of life by prudent use of the new technologies. By contrast, many people in developing countries are poor. It is not so clear that gene-based technologies will automatically improve the wealth or the quality of life in developing countries. Further, these technologies combined with current WTO trade policies for agricultural products have great potential to increase the gap between rich and poor.

Locations of food production in the future
Clearly the potential impact of the WTO on food production in developing countries is great. The long-term impact of gene-based technologies must be evaluated with this context. The use of these technologies in the west will continue to exceed their application in developing countries. There will be competition for urban markets in developing countries from imported western foods. The question is how gene-based technologies in developing countries funded by public organizations can play a role and what type of role should this be?

Conclusion
The paper concludes with recognition that the most important issues in the role of international organizations and public funding will not be the techniques issues but rather the impacts upon the social, economic, cultural and ethical aspects of life. Public organizations and funding agencies must equip themselves in creative ways to be effective in facing these new situations?

TOPIC: Role of international organizations and funding agencies in promoting gene-based technologies in developing countries

Role of international organisations and funding agencies

M. Ståhl

International Foundation for Science (IFS), Sweden

E-mail: Michael.Stahl@ifs.se

Sustainable development in the 21st century must be science-based and technology-driven. This also holds true for Sub-Saharan Africa where current trends paint a gravely worrying picture. Poverty and malnutrition are on their way up while life expectancy is on its way down. Africa was given special attention at the UN Sustainable Summit in Johannesburg (2002) where the priority sectors for development interventions were identified as Water, Energy, Health, Agriculture and Biodiversity (WEHAB). In order to reverse the trends toward degradation in these sectors, both conventional development efforts and sophisticated research are being called for.

The Millennium Goals set by the UN includes *inter alia* halving the number of food insecure and malnourished by 2015. In the African context, this implies achieving a level of 6 % annual growth of agricultural production by 2015. More than half of this growth must be based on productivity increases, which in turn would require considerable inputs of agricultural research.

The international development community is prepared to invest in the agricultural and environment sectors in Africa. However, there is much less preparedness, both by donors and by national governments, to invest in home grown scientific capacity. Instead, it is argued that the scientific underpinnings and technology solutions can be brought to Africa as imported packages, to be unwrapped and put in operation by expatriate experts, if possible with input from the "African Diaspora".

Such an approach would not work as it rests on flawed assumptions. Instead, a home grown African scientific community must take on the scientific and technological challenges. There are strong arguments for this. Science based technologies for disease control, for crop and animal management etc. emanating from the North cannot be readily transferred to the humid, sub-humid and semiarid agro-ecosystems of Africa. The crops and their pests, the animals and their diseases must be studied in their own local environments as a basis for sustainable production and management.

In the same vein, almost all gene-based technologies are taking place in the developed world. Once the dust of the controversies which surround the issues of gene technology has settled, there are few scientists who would doubt that the technology will lead to important new developments and significant increases in production of food both in animal and crop production. In the poor countries where food needs are most urgent, the generation of new knowledge in biotechnology and the patenting of genes and germplasm require a new paradigm for using biotechnology to improve germplasm It is often said that biotechnology research is too expensive for African institutions. If so, it may be unavoidable to focus attention on upgrading those facilities and increasing capacity in those institutes and universities which are attempting to embark upon this path, even at the risk – where this is

possible only on a regional basis - of siphoning off talented scientists from research institutes which are not in a position to undertake research in this area.

Global climate change also impacts on Africa. It is no longer possible to rely on traditional farming systems and husbandry methods. More frequent and erratic episodes of heat and water stress, jeopardising agricultural production, have been recorded. Climate change in Africa is even less understood than elsewhere. How the global changes will impact in various regions in Africa is little understood and must be studied in the regions themselves.

The global research community gives only scant attention to agricultural research focussing on African issues. However, the efforts by the Consultative Group for International Agricultural Research (CGIAR) are commendable and represent a concerted effort to provide science-based solutions to African agriculture. CGIAR nowadays works increasingly closely with national and regional research institutions.

Nevertheless, the CGIAR scientific establishment contains only a minority proportion of African researchers. If research is to be institutionalised as a component of agricultural development in local African communities, then it is imperative that African scientists, based in national and local institutions, take the lead. This is not to say that international experts and members of the African scientific Diaspora cannot play a role. They may provide crucial inputs, but they are not in a position to lead the process.

An analysis of the African human resources in the field of science shows that the research community is ageing. In the 1980's and 1990's most African countries suffered from general economic decline while at the same time pressure from vastly growing cohorts of secondary school-leavers put pressure on the universities. Undergraduate training was prioritised while research dwindled. Research institutes saw their budgets severely cut. The number of research positions and scholarships was restricted. Many scientists left their institutions and moved out of Africa while others moved into consultancy and business.

As a result, the recruitment of research students, notably in the natural sciences, went down and became wholly dependent on project funding from external donors. As Africa enters the 21st century there is a glaring deficit of researchers in the age 25 - 35. In many institutions most staff members are above the age of 40 and quite a number are above 50. The present population of professors and senior researchers are a "greying" lot. The average age is high and many are close to retirement. Therefore, a new generation of scientists must be stimulated to take over the task of pursuing science for sustainability.

Only a few aid agencies support scientific capacity building on a regular basis. Many more give *ad hoc* support to individual research projects. Following approaches are common:

- Overseas scholarships for bright young researchers. While thousands of students from the South have received their Ph.D. abroad, a great number never return. Thus they end up strengthening research capacity in the North.

- Sandwich programmes allow Southern students to do field work at home while theoretical and laboratory work is provided at a Northern institution, where the degree is granted. This system can also lend itself to the brain drain.

- Support to the establishment of post graduate training programmes at Southern universities. This approach has a greater chance of becoming sustainable at it tackles the

root causes of the brain drain: unattractive and low quality training at national institutions in the South.

- Support to regional centres of excellence whereby a number of neighbouring countries pool resources to establish a sophisticated research institute. The argument behind this approach is that poor countries cannot be expected to set up sophisticated institutions all by themselves. However, as mentioned before a regional centre may drain national institutions of their scarce capacity.

Scientific capacity building must be considered at different levels:

- Individual (enhancing scientific competence).
- Research programme (strengthening equipment, management).
  University/Research institute (providing scientific infrastructure, Information and Communication Technology (ITC), post graduate training programmes).
- National (budgetary resources for research, science policy, enabling conditions for the scientific community, interface between research and development)
- Regional (joining forces to provide the scientific underpinnings to solve joint regional development problems).

Scientific capacity building in poor countries can only succeed if donor support is matched by coherent science and research policies on the part of governments in recipient countries. The problem is that research funds are among the first to be cut in a situation of budgetary constraints. This holds both for national budgets in poor countries and for aid agencies. The reason is that governments and donors see few immediate results of support to science. The 'new contract' between science and society may present a solution: the scientific community gives priority to developmentally relevant research issues while the government resolves to provide infrastructure and an enabling research environment.

Today the prospects for young scientists in Sub Saharan Africa are extremely disheartening. A great number of M.Sc. students are graduated annually. Many want to continue on a scientific career only to find barriers everywhere – no research funds, no mentorship, grim career opportunities, outdated and out of order laboratories, isolation from the international scientific developments.

The major challenge for science in Sub Saharan Africa today is to mobilise the scientific potential; a great number of talented and enthusiastic young M.Sc. holders want to pursue the scientific profession. International organisations and donor agencies should join hands with national governments to initiate a major post graduate research training enterprise and provide the students with meaningful research tasks: to tackle food insecurity, ill health, environmental degradation and inadequate technologies which keep the African population trapped in poverty.

We must not let them down!

POSTER PRESENTATIONS

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Suitability of blood protein polymorphisms in assessing genetic diversity in indigenous sheep populations in Kenya

J.M. Mwacharo[a], C.J. Otieno[b], A.M. Okeyo[a]

[a] University of Nairobi, Department of Animal Production, P.O. Box 29053 Nairobi, Kenya

[b] Iowa State University, Department of Animal Science, 2255 Kildee Hall Ames, Iowa 50011 USA

E-mail: mwaharojoram@hotmail.com

Several techniques for assessing genetic diversity exist. However information on their suitability for use in indigenous sheep populations in Kenya is sparse in literature. Using seven blood protein-coding loci analysed in six indigenous sheep populations found in Kenya, the polymorphism in blood proteins as a rapid method for assessing genetic diversity was evaluated. Blood samples were obtained from 457 unrelated animals in different populations found in Kwale, Makueni, Siaya, Kakamega, Kajiado and Isiolo Districts. Forty animals of the fine wooled Merino were used as the reference breed. Blood samples were typed for biochemical polymorphisms using discontinuous polyacrylamide gels for Transferrin and starch gel electrophoresis for Haemoglobin, Albumin, Carbonic anhydrase, X-protein, Esterase-A and Esterase-C. A more detailed description of the analytical technique is published elsewhere [1]. Of the seven loci studied, Carbonic anhydrase and X-protein did not give interpretable results. This is attributed to the long period of 2 years between sample collection and analysis. This time lag may have affected the quality of the samples. Most proteins, even the most stable such as lactate dehydrogenase, begin to degrade seriously in activity and resolvability after 2 years under even the lowest temperatures [2]. The remaining five loci however, showed low levels of polymorphism, low variability in allele numbers and frequencies and low heterozygosity values. These levels of variability are lower than those observed in microsatellite DNA markers [3]. Each marker showed the same predominant allele in each population. Transferrin was the only locus that deviated significantly from the Hardy-Weinberg equilibrium. The multilocus F_{ST} values indicated that 8.3% of the total genetic variation was explained by population differences; the remaining 91.7% corresponding to differences among individuals (Table I). These significant between-population F_{ST} estimate indicates a relatively low degree of gene flow between the populations studied due to reproductive isolation. In this study it was observed that 8.3% of the total genetic variation is attributed to population differentiation. This value is close to that found in other domestic species studied using microsatellite DNA markers, i.e. 10% in European cattle breeds [4]. On average each population had a 31.8% deficit of heterozygotes whereas the combined populations had a 37.4% deficit in heterozygotes. In spite of their low variability, blood proteins are still ideal for the rapid assessment of genetic diversity between populations that have not been characterized. However microsatellite DNA markers are superseding their usefulness.

TABLE I. SUMMARY OF THE F-STATISTICS AT ALL LOCI

	F-statistics		
Locus	**F_{IS}**	**F_{IT}**	**F_{ST}**
Albumin	- 0.059	- 0.010	0.046
Haemoglobin	- 0.025	0.250	0.268
Transferrin	- 0.170	- 0.131	0.034
Esterase – A	1.000	1.000	0.122
Esterase - C	1.000	1.000	0.082
Mean value	**0.318**	**0.374**	**0.083**

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Preliminary investigation on genetic characterization of native and endemic fowl types in Sri Lanka

L.P. Silva[a], W.R.K.J.S. Rajapakshe[b]

[a] Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka

[b] Veterinary Research Institute, Gannoruwa, India

E-mail: pradeepas@pdn.ac.lk

Sri Lanka is a tropical island, which shelters a large number and variety of wild as well as domesticated animals. As an oceanic island Sri Lanka has a high percentage of endemic species that have evolved because of the isolation, but they are particularly vulnerable. Its location, astride the sea routes between the east and west throughout the history, has exposed the country to be a recipient of variety of animal species transported throughout the world. This history had made the gene pool of native animals very unique and diverse. In this context native poultry species of Sri Lanka demonstrate an incomparable scenario in evolution of domestic poultry species. According to one of the hypotheses regarding the evolution of poultry, the Red Jungle Fowl (*Gallus gallus*) is considered as the main ancestor of the domestic fowl *(Gallus domesticus)*. However, it is also believed that the domestic fowl descent from different ancestral groups, one of which is Ceylon Jungle Fowl [1]. Ceylon Jungle Fowl *(Gallus laffeyatti)* is endemic to Sri Lanka. Nevertheless, different native fowl types found in Sri Lanka resemble varying characteristics of Asiatic fowl. However, except for the few studies on *G. laffeyatti* [2,3] there is hardly any information available on the origin of Sri Lankan native fowl. Also there is only one investigation [4] done so far on the relationship of the Ceylon Jungle Fowl and native fowl population in Sri Lanka. Therefore, the present study was conducted, in order to investigate the origin of native fowl in Sri Lanka and to find out the genetic relationship among them.

Observations of morphological characters of endemic, indigenous and exotic fowl types were carried out using Ceylon Jungle fowl, eleven types of native chicken and two exotic chicken breeds (Cornish and Rhode Island Red). Blood samples for DNA extraction were collected from the above three categories of chicken. Randomly Amplified Polymorphic DNA (RAPD) analysis were carried out using sixteen non-specific primers.

The results of morphological characterization revealed many variations in plumage color pattern. Single and pea comb types were found in both native and exotic types of chicken. A prominent yellow color marking on red color comb was a unique feature in Ceylon Jungle fowl. In the sample tested only one indigenous chicken type showed feathered shank character. Another distinguishing feature observed was the presence of white spot in red color earlobes of all native chicken types except naked neck type, which is believed to be a cross of exotic and indigenous.

Sixteen non-specific primers used in the study produced 22 polymorphic bands ranging from 500 base pair (bp) to 1957.6 bp. There were two monomorphic bands common to all chicken types tested. Genetic similarity coefficient detected according to Noeingen Index ranged from 0.5 to 1.1 indicating a wide genetic base of tested samples of chicken. According to the results of cluster analysis there was a clear separation of Ceylon Jungle fowl from the other chicken

types used in the study. This indicates that there was an early separation and divergent evolution of Ceylon Jungle fowl from all the other domestic chicken types tested. It appears that the contribution of Ceylon Jungle Fowl in development of Sri Lankan native chicken is minute or very marginal. However, the present study was carried out with limited sample size and from the present results it can be confirmed that RAPD is an effective method, though the repeatability is low, in genetic characterization of animal populations with wide genetic basis.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Transgenic rabbits as a model organism for production of human factor VIII

D. Vasicek, P. Chrenek, A. Makarevich, M. Bauer, R. Jurcik, K. Suvegova, J. Rafay, J. Bulla, L. Hetenyi

Research Institute of Animal Production, Nitra, Slovakia

E-mail: vasicek@vuzv.sk

Here we report the generation of transgenic rabbits as a model species for testing the expression of human clotting factor VIII (hFVIII) in the mammary gland. Human factor VIII is a very complex and large protein whose expression is difficult as the hFVIII reqiures extensive posttranslational modifications to be biologically active. First transgenic pigs, where the expression of the hFVIII cDNA was targeted to the mammary gland, produced 0.62U/ml of rhFVIII in their milk [1]. Expression of recombinant hFVIII in the transgenic sheep was also achieved albeit at low levels [2]. In the present study we used for microinjection a fusion gene construct consisting of 2.5kb murine whey acidic protein (mWAP) promoter, 7.2 kb cDNA of the hFVIII, and 4.6 kb of 3‘ flanking sequences of mWAP gene [1].

Totally 130 microinjected zygotes were transferred into recipients and 30 offsprings were delivered. The pups were screened for the transgene by PCR of DNA isolated from ear, and results were confirmed by Southern blot analysis. The transgene was identified in one female founder animal, and it was transmitted to the offspring in a Mendelian fashion. These data demonstrate a stable integration of the gene construct into the germline of transgenic rabbits.

Mammary gland biopsies were taken from lactating founder and her female offspring rabbits on the day 20 of lactation. The presence of hFVIII mRNA is currently under detection by RT-PCR. Further studies to analyze level of FVIII expression in further lactations are being carryied out.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Hepatic and duodenal expression of β, β-carotene 15, 15' oxygenase in beef cattle

A. Morales[a], A. González[a], A. Shimada[a], M. Cobos[b], A. Varela[c], O. Mora[a]

[a] Facultad de Estudios Superiores – Cuautitlán, Universidad Nacional Autónoma de México (UNAM), México

[b] Colegio de Postgraduados, México

[c] Instituto de Neurobiología – UNAM, México

E-mail: amorales24@yahoo.com

In Mexico most bovines are finished on pasture: the adipose tissue of those animals shows a yellow pigmentation which results in an important economic loss for the producer, because pg downgrading or even rejection of resulting carcasses [1]. Yellowness of fat is caused by excessive carotenoids in the diet, of which β-carotene is predominant [2]. β, β-carotene 15, 15'oxygenase (β-ox) is the enzyme that cleaves it into two molecules of vitamin A [3]. In cattle not all the absorbed β-carotene is transformed into vitamin A; the excess is transported to the liver where it might be cleaved by the enzyme and stored or packaged with other lipid compounds and carried to different tissues, including the adipose [4]. The aim of this study was to determine the expression pattern of β-ox in duodenal mucosae and hepatic tissue of grass-fed compared with grain-fed cattle.

Biological samples were taken at Querétaro's Municipal abattoir from bovines at slaughter. For tRNA assays, intestinal mucosae was taken in Trizol reagent (Gibco BRL cat. 15595-026), homogenized and transported on ice to the laboratory. For *in situ* hybridization duodenal and hepatic tissue samples were taken and fixed on 3.5% paraformaldehyde.

A partial cDNA for β-ox of 744 nt was obtained by RT-PCR amplification, this product was inserted into TOPO vector 4.0 (Invitrogen cat. K457501) and cloned into *E. coli* bacteria. Then two sense and antisense digoxygenin labeled probes were synthesized and used for the hybridization of duodenal and hepatic cryosections of 15 μm from four pigmented and four non-pigmented carcasses. The hybridization conditions were a four-hour pre-hybridization at 65°C and a overnight hybridization at the same temperature. The slides were washed and incubated overnight at 4°C with digoxygenin/alkaline phosphatase antibody. The slides were washed and incubated with alkaline phosphatase-nitro blue tetrazolim/5-bromo-4-cloro-3-indoly-phosphate in darkness overnight at room temperature [5].

Results from *in situ* hybridization shows the location within a given tissue sample. The hybridization signal on duodenum was observed in the epithelial cells of crypts and villi; similar results have been reported in the chicken [6]; in this site, the β-carotene is absorbed, cleaved and/or packed to be carried to the liver [7]. Apparently there were no differences in duodenum *in situ* hybridizations between pigmented and non-pigmented animals. This could mean that the expression levels at this site were not affected by the diet β-carotene.

However large differences were found in liver hybridizations between pigmented and non-pigmented animals. The pigmented ones showed a higher level of expression of β-ox near the portal space and less at the central vein in the liver lobule, but in non-pigmented animals the expression level was either very low or not detected. This could mean that as grass-fed animals (yellow adipose tissue) consume high quantities of β-carotene and its cleavage is insufficient at the intestinal level, thus the compound is carried to the liver where its cleavage is continued, stimulating a higher expression of β-ox. At this point, there are contradictory opinions on the relationship between the level of dietary β-carotene and the activity of β-ox [8,9].

Future studies are required to confirm that liver ß-ox might play a central role in the metabolism of ß-carotene in bovines.

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Parentage determination in three breeds of Indian goat using heterologous microsatellite markers

N.A. Ganai, B.R. Yadav

Livestock Genome Analysis Laboratory, National Dairy Research Institute, Karnal – 132 001, India

Email: bry@ndri.hry.nic.in

The identification of proven sires has been of utmost importance in animal improvement programmes. Failure to record correct parentage can cause bias in sire evaluation, by inducing errors in estimates of heritabilities and breeding values. Error in recording the sire of a progeny can occur at many stages such as handling of semen during freezing, transportation and artificial insemination or in open range grazing of the animals, particularly in sheep and goat. Different types of markers have been used for correct parentage determination. Parentage can be solved adequately by conventional testing (blood groups, serum proteins, red cell enzymes, lymphocyte antigen systems), however, these tests lack conclusion and need further confirmations.

There are different approaches based on DNA polymorphism for paternity testing including RFLPs, DNA fingerprinting using multilocus minisatellite and oligosynthetic probes and PCR based amplification of minisatellites and microsatellites. However, RFLPs generally suffer from low heterozygosities and low PIC, while the DNA fingerprints are difficult to interpret or reproduce owing to complex nature of banding pattern revealed. Microsatellite markers not only overcome many of the difficulties, but also have added features and are markers of choice for paternity verification and individual identification owing to their high heterozygosity, Mendelian co-dominant inheritance, ubiquity throughout the genome and ease of scoring.

The heterologous microsatellite markers are often used in genetic studies due to the fact that there is significant genome conservation across different related species. This circumvents the need for obtaining a suitably large panel of polymorphic markers for each species, especially those that are less common and less studied. Cross-species utilisation of microsatellite loci not only saves time and effort in the laboratory, but also enables the construction of comparative maps between related species and their use for genetic distance studies and parentage determination. The present study was carried out to study the suitability of the bovine microsatellite markers for parentage verification in Indian goat breeds for dubious parentage of three types, viz. type-1: exclusion of a putative parent, when the genotype of one parent and offspring are known; type-2: exclusion of a putative parent, when the genotype of the other parent is not available; and type-3: exclusion of both the parents of an offspring if falsely recorded.

The investigation was carried out on 116 unrelated goats and six pedigreed families of three breeds of goat viz. Jamnapari, Barbari and Sirohi. The former were chosen to generate the allele frequency data, PIC and heterozygosity for different markers in each breed, and estimation of the exclusion probabilities of false parents; while the latter were analysed to verify the usefulness of the microsatellite markers for parentage determination.

A set of 12 bovine microsatellite markers was analysed for parentage determination in goats for different types of misidentifications. Allele frequencies were estimated and probabilities of exclusion of the wrong parentage were calculated from allele frequency data of microsatellite DNA analysis. The marker-wise exclusion probabilities of false parent (s) in three breeds were calculated for dubious parentage types 1, 2 and 3, respectively. For Type-1 dubious parentage, the exclusion probability for each marker varied widely from as low as 13.4% (locus BM-5004 in Jamnapari) to as high as 67% (locus BMS-1237 in Sirohi). The markers with exclusion probabilities of > 30% in 3 breeds were BMS-357, BM-7160, BMS-1237, BMS-585, BMS-332, BMS-820, BR-6027 and BM-7228. For Type-2, the values of probability of exclusion ranged from 5% (locus BMS-1237 in Barbari) to 50.1% (locus BMS-1237 in Sirohi). For Type-3 these values ranged from 21.6% to 84%.

The exclusion probabilities of falsely recorded parents were estimated for different combinations of marker sets (1 to 5) and number of markers in these sets was 12, 8, 6, 5 and 4 respectively. Among three breeds, these values were lower in Barbari for all sets of markers in all the three types of misidentifications. In type-1 misidentification, the exclusion probability of a false parent was 99.9%, when all 12 markers were considered together (Set-1). Using only 4 markers (Set-5) the exclusion probability was 94.5%. The probability of exclusion of the false parent decreased for all sets of markers when the information on one of the parents was missing (type-2 misidentification, 98.9% for Set-1 to 80% for Set-5). In Barbari goats the Set-5 of 4 markers would exclude only 65% false misidentifications. For type-3, the probability estimates of excluding both the falsely recorded parents were highest of all the three types of misidentifications for any set of marker (99.9 % for Set-1, and 99.4% for Set-5 of four markers). In conclusion, there is a need to identify the marker systems for parentage verification for each livestock species for which microsatellites serve a useful tool. They are highly informative, quite simple to use and interpret.

TOPIC: Gene-based technologies applied to livestock genetics and breeding

DNA polymorphism of Arabian, thoroughbred and Anglo-Arabian Horses in Morocco: Application to identification of individual horses and parentage verification

L. Ouragh

Laboratoire d'Analyses Génétiques Vétérinaires, Institut Agronomique et Vétérinaire Hassan II, Rabat, Morocco

E-mail: *l.ouragh@iav.ac.ma*

New techniques of molecular biology used in analyzing DNA polymorphism give access to the whole genetic variability of a given individual while the traditional bloodtyping (red cell typing and biochemical polymorphisms) gives access only to the transcribed fraction which is then translated to protein. In addition, this fraction represents only a very small part (5–10%) of the genome's incoding fraction.

One of the newer testing methods in identifying horses is a DNA-based test using microsatellite marker analysis.

The objective of this work was to evaluate the efficacy of this new technology in the identification and parentage verification of Arabian, Thoroughbred and Anglo-Arabian horses in Morocco. Anglo-Arabian horse is a crossbreed between Arabian and Thoroughbred.

Three samples from the three breeds were analyzed for 12 microsatellites (HMS2, HMS3, HMS6, HMS7, HTG4, HTG6, HTG7, AHT4, AHT5, VHL20, HTG10 and ASB2). A total of 1541 horses were used: 804 Arabians, 559 Thoroughbreds and 178 Anglo-Arabians.

Allelic frequencies of the 12 studied systems were calculated in the three groups. The results allowed the determination of intra-population genetic parameters: heterozygosity ratio (H), probability of identification (PI) and probability of exclusion (PE).

Based on average heterozygosity ratio, variability was relatively lower in Thoroughbred horse (.7036), while it was almost the same in Arabian and Anglo-Arabian horses (respectively .7217 and .7232).

Probabilities of exclusion obtained with the 12 systems were higher than 99.9% for the three studied populations and probabilities of identification of individual horses were15 x 10^{-12}, 4 x 10^{-12} and 20 x 10^{-12} in Thoroughbred, Arabian and Anglo-Arabian horses respectively.

These results indicate that the test using microsatellite marker analysis constitute a highly efficient and reliable alternative for the identification of individual horses and parentage verification and is a useful tool for horse breeders and horse registries.

TOPIC: Gene based technologies applied to livestock genetics and breeding

New polymorpic microsatellite loci for analysis of genetic diversity in camel species

S. Preuss, H. Bartenschlager, H. Geldermann

Department of Animal Breeding and Biotechnology, Institute for Animal Husbandry and Animal Breeding, University of Hohenheim, D-70593 Stuttgart, Germany

E-mail: preuss@uni-hohenheim.de

In this study, new microsatellite loci were screened and sequenced on the basis of the genomic DNA of a male *Camelus bactrianus* (bactrian). The DNA was restricted with Sau3AI, and the restriction fragments were enriched using magnetic beads (Dynabeads, DYNAL) coated with the biotinylated oligonucleotides $(CA)_{10}/(GT)_{10}$ and $(ATCC)_7$. The enriched fragments were cloned and then sequenced. Primers were defined for PCR and applied to genomic DNA from 20 bactrians from the Russian Altai region, 220 dromedaries from four Kenyan breeds, and 7 bactrians, 6 llamas and 4 alpacas from German zoos. Locus specific allelic fragment sizes were analysed on an automated DNA sequencer. Basic statistical tests were conducted using the software package BIOSYS-1.7 [1]. The between-breed diversity was measured using the bootstrapping procedure of PHYLIP [2] and the standard Nei's distance [3].

Among 32 sequenced microsatellite loci, specific PCR products were generated for the 23 loci in bactrian as well as in dromedary. Amongst them, 19 loci could be amplified in llama and 20 in alpaca. Different repeat motifs were found, which were not always complementary to the oligonucleotides used for screening. Fragment lengths per locus were more similar between llama and alpaca and between dromedary and bactrian than between New and Old World *Camelides* [4].

The loci were largely different with regard to their polymorphism. No significant ($p<0.05$) deviation from Hardy-Weinberg equilibrium was observed for all animal groups. Five of the investigated loci were monomorphic and had identical fragment sizes in all investigated species. One locus was monomorphic in bactrian and dromedary and could not be amplified in the other species. Three loci were monomorphic in each of the species, but had different fragment sizes in individual species. More than one allele within a species were observed in 13 loci for bactrian, 12 loci for dromedary, 11 loci for llama and 12 loci for alpaca. Seven (50%) of the polymorphic loci showed more than ten alleles, and in most loci the allelic fragment lengths were similar in different species. Since microsatellite alleles of the same length can be different from one species to another, sequence analysis of allelic microsatellite regions in the different species is recommended.

The new informative microsatellite loci can be efficiently used in several species, e. g. for parentage control, gene mapping or phylogenetic analysis. As a first application of the new polymorphic loci, four of them were genotyped in unrelated dromedaries belonging to four breeds from Kenya. The allele frequencies were similar and genetic distances point to a close relationship between the breeds.

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TOPIC: Gene based technologies applied to livestock genetics and breeding

Molecular characterization of zoogenetics resources

L.A. Alvarez Franco[a], J.E. Muñoz F.[a], J. Torres[a], A. Piedrahita[a], A. Oslinger[a], A.G. Rodas[a], J.D. Palacios[b], A.M. Posso[a], M.V. Gomez[c]

a Universidad Nacional De Colombia Facultad De Ciencias Agrpecuarias Sede Palmira, Colombia

b Instituto Alexander Von Humboldt, Bogotá D.C., Colombia

c Inmunogen

E-mail: lalvarez@palmira.unal.edu.co

A resume of research papers based on gene technology that has been developed during the year 2002 at National University of Colombia campus Palmira on native bovines (Hartón del Valle), Native Swine and poultry, Tilapia (Oreochromis spp) and equines, is given below.

(1) Bovine Hartón del Valle: Colombia possesses the major variety of criolle Bovine of America; one of them, Hartón del Valle cattle originated from the cattle brought by the Spanish in the XV century and has adapted to conditions and general environment of Valle del Cauca (Colombia) with high rates of reproduction and survival and low susceptibility to diseases. The "Hartón" inventory has been estimated to be 5000 animals of a total of 24 millions cattle in the country.

A resources program was established between the National University of Colombia and Agricultural Secretary (Ministerio de Agricultura) and breeders association (ASOHARTON) and as of 1996 the following research has been conducted: Inventory and characterization of production systems; genetic improvement, crossbreeding and quality caracterization of milk, blood and physiological constants and identification of endocrine values. The project has a germoplasm bank *in vivo* and in vitro.

In order to identify the genetic diversity to establish conservation criteria and for breeding criteria, at present genetic variation is being evaluated by RAPD`s markers. Allelic variants of k-cassein are being determined and microsatellites characterization has been initiated.

The aim in the short terms is to evatuate adaptability characteristics, resistance to deseases and parasites, and in the medium term the selection assisted by markers.

(2) Criolle Swine: The actual size of the population is unknown, however it is believed there is close to 1 million which are established in small farm production systems. At present genetic diversity has been characterized by RAPD`s markers with the purpose to evaluate genetic distances between three groups of criolle swine: Zungo, Casco de Mula and San Pedreño. In the short term, the AFLP´s markers will be used.

(3) Criolle Chicken: Colombia imports all of its genetic stock for the systems of intensive poultry production. The criolle chicken are found only in small farmer systems because they require few inputs and because of their capacity to replace themselves. An *in vivo* germoplasm bank was established with 200 birds and five chicken criolle types.

Morphological descriptors, hatchability curves, growth and egg production are been evaluated. The genetic diversity between and within the groups is being studied by RAPD`s markers and later by AFLP`s markers.

(4) Equine: Using breeders association data bank that test their genotypification in the EEUU was used to evaluate Colombian equine diversity. A high association was found between markers and a characteristic gait ("pasofino"). It follows that tests conducted in Colombia using capillary electrophoresis and automatic sequencing with 15 microsatellites; the results matched those conducted in the EEUU in 99.9%.

(5) Oreochromis sp: Since the introduction of Tilapia in Colombia in 1982, the consumption of these fish has increased dramatically. There are very few alevines production that provide good quality. The researches conducted at present have been focused on mass selection by evaluating growth curves and red color (consumer preferences). In order to determine the genetic composition of five population and to cull out inbreeding problems five alevine producers populations were evaluated and were comparee to parental line of O. niloticus, O. aureus and O. mossambicus, using RAPD`s markers. High variation was found in and between populations. At present there is a project to make diallelic croos to evaluate growth heterosis.

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Proliferation index of camel skin fibroblast cells as nuclear donor

S.C. Gupta, N. Gupta, S.P.S. Ahlawat, A. Kumar, R. Taneja, S.S. Bulandi, R. Sharma, M.S. Tantia

National Bureau of Animal Genetic Resources, Karnal 132 001, India

Email: *scgupta@nbagr.nic.in*

Jaiselmeri is an excellent breed of riding camel, found in Jaiselmer and other adjoining districts of Western Rajasthan in India. Jaiselmeri camel like other pack animals are declining in India over the years due to increased mechanization and control of desert agriculture to some extent. The deep freezing technology on camel semen is poorly developed in India. The somatic cell technology has been developed at this Institute as an alternative tool of long-term conservation on endangered livestock breeds. For this study, samples of (0.25 cm^2) skin tissue were collected from ear biopsy from elite male germplasm from National Research Centre on Camel, Bikaner. Skin tissues were cultured at 37ºC in Medium (DMEM+ Ham's F-12 nutritive mixture) supplemented with 10% fetal bovine serum, L-Glutamine and antibiotics in an incubator under 98% humidified and 5% Co_2 atmosphere. The cell explants were visible from 12–16 days of culture. The cells were allowed to confluent in the TC flasks for additional 3–5 days till nearly 80% surface area is covered by the cells. The primary cells were harvested by usual trypsin-EDTA protocol [1]. The cells were counted using Neubar's haemocytometer and cells were passaged subsequently. Since no reference values were available for camel skin fibroblasts, the present experiments were conducted to study the cell proliferation index, population doubling time, standard growth curve and cell viability using standard growth and MTT assays.

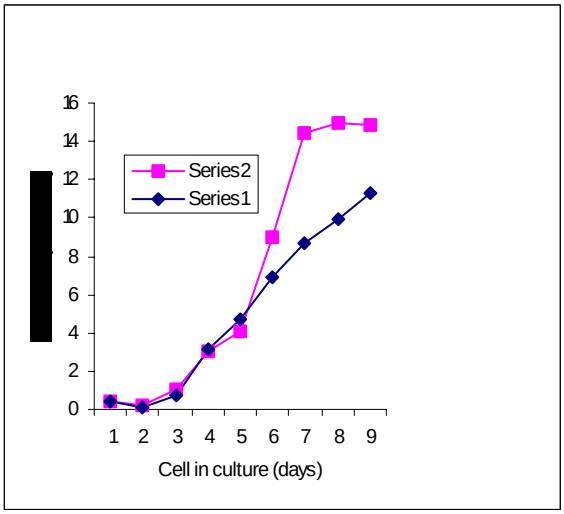

FIG.1. Growth curve in skin fibroblast

Growth Curve: Fibroblast cell lines (JC3 and JC5) from log phase of 4th Passage (15 population doublings) were harvested. Cells (40,000) were re-seeded in each of 20 flasks of 25 cm^2 size (Nunc) and were incubated at 37ºC for 8 days. Two flasks were harvested after every 24h and cell counts were made. Data was fitted on log_{10} scale in MS Excel, 2000.

Figure 1 shows that growth curves showed true sigmoid shape but a marked variation between the cell lines was observed. Moreover, cells, which grew faster attained plateau on day 6 while in slow growing cultures, the curve showed elevation even on day 8. This is probably due to non-availability of growing space for cells having faster growth rate. It was concluded that all animals do not produce karyoplast donors at equal rate or efficiency. Therefore, the growing cultures need to be compared with standard growth curve each time the cells are used as nuclear donor cells for cloning.

Cell Proliferation Index: Cell multiplication rates vary considerably under different culture condition and slight change in environment or composition of medium may affect the proliferation of cells significantly. For camel skin fibroblast cells, the standard multiplication rate and the population doubling time was not known earlier. In order to study the proliferative indices of the growing cells using objective parameters, MTT assay was conducted. In this assay, the dividing and viable cells take up MTT [3– (4,5- dimethylthiozol-2yl) 2,5 diphenyltetrazolium bromide] and a colour is developed. The intensity of colour is measured by ELISA reader at 540-570 nm. For this, 4000 cells per well were seeded in 96 well ELISA plate (flat bottom, Nunc) and cultured at 37ºC. First two rows of eight wells each were kept as negative and positive controls respectively. Rest of the 10 rows were kept as treatments. One row was harvested at an interval of 24 hours and adjoining row was treated with MTT solution for 4 hours. The MTT treated cells were fixed in 10% DMSO.

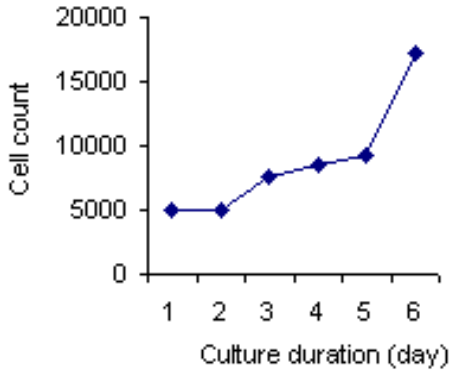

FIG.2. Cell proliferation index

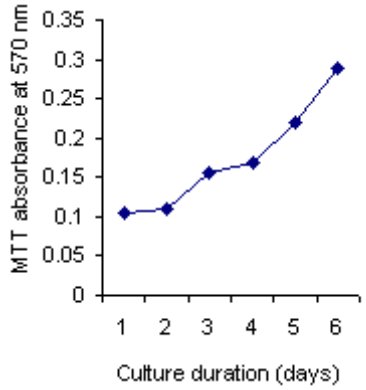

FIG 3. MTT absorbance at 570 nm

Figures 2 and 3 show that the cell proliferation index both in terms of cell count and absorbance values in ELISA reader at appropriate wavelength was similar. From this study it is clear that MTT assay can give fairly accurate figures of cell proliferation rate of skin fibroblasts.

Ploidy level: During long-term culture, the cells are likely to develop one or other type of chromosomal abnormalities. It must be ensured that the cells in different passages are checked for normal ploidy so that the viable clones can be developed from them. In order to see the utility of cells from Jaiselmeri camel as nuclear donor, the chromosomal profile was studied following the protocol described by [1]. The 2N chromosomes up to passage No 4 (15^{th} population doubling) was found to be normal (74XY) in 97% of the cells. From these preliminary studies it appears that camel skin fibroblast cells behave normally in culture and can serve as nuclear donors. These results will further help in camel cloning.

Acknowledgements: Financial grant by World Bank under NATP project Animal Genetic Resource Biodiversity (Mission Mode) are duly acknowledged.

Reference:

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TOPIC: Gene-based technologies applied to livestock genetics and breeding

Study of genetic diversity in Algerian sheep breeds using microsatellite markers

S. Gaouar[a], N. Tabet Aoul[a], A. Derrar[a], K. Goudarzi-Mouazami[b], N. Saïdi-Mehtar[a]

[a] Laboratoire de Biologie Moléculaire et Génétique USTO, Oran, CRSTRA, Algeria

[b] Laboratoire de Génétique Biochimique et de Cytogénétique, Département de génétique Animale, INRA, 78352 Jouy-en-Josas, France

E-mail: nmehtar2002@yahoo.fr

In order to realize the preservation and exploitation of domestic animal biodiversity and to define a global strategy for the management of Algerian sheep genetic resources, the goals of our research are the knowledge of the degree of genetic variability, the partition of this variability and the genetic distance among ovine breeds. These informations will be obtained by analyzing directly the genome at molecular level.

In this study DNA of 85 animals from two breeds, Ouled-Djellal and Hamra, was PCR amplified at the following microsatellite loci : McM42, TGLA53, MAF65, McM527, OarFCB20, INRA49, OarFCB11, OarCP49, CSSM66, MAF36, Oar HH56 and ILST05. Number of alleles and allele frequencies were calculated.

The results showed that these breeds presented a polymorphism with these microsatellite markers reflecting a genetic variability among them. However, highest differences in allele frequencies were found at loci : McM527, ILSTS05, OarFCB20, INRA49, MAF36, McM42, MAF65, OarFCB11, OarCP49, CSSM66, while at the other loci the alleles at the highest frequency are the same.

This study is being extended to five other Algerian breeds: Taadmit, Barbarine, D'men, Sidaou and Berbere to estimate their variability and genetic distance between them. Now, we are collecting blood from these different breeds to constitute a DNA bank. The results will allow establishment of the strategy to promote the use and development of locally adapted sheep resources.

TOPIC: Gene-based technologies applied to livestock genetics and breeding

Somatic cell banking — an alternative technology for conservation of endangered sheep breeds

N. Gupta, S.C. Gupta, S.P.S. Ahlawat, R. Sharma, K. Gupta, R. Taneja

National Bureau of Animal Genetic Resources, Karnal 132 001, India

E-mail: neelug@yahoo.com

Each cell of an animal's body contains full genetic code for the whole animal and nuclear transfer provides a way of converting cells to whole animal. Cells from endangered breeds collected by biopsy or from scrapings of soft skin or ear tissue or from hair follicle can be grown and multiplied in a laboratory and this would then be stored frozen indefinitely at 196^{o}C in liquid nitrogen. Mammary gland cells from sheep, mouse cumulus granulosa cells [1], bovine mural granulosa cells [2] and fibroblast cells have all generated viable clones. The currently available methods of conservation, deep freezing of sperms (haploid genome) and storage of a large number of embryos are too expensive. In comparison, adult skin fibroblast cells are easy to obtain, hardy in culture and freezing, a good source of donor DNA without the limitations of age, sex and physiological state. Progenies were successfully obtained from nuclear transfer of serum-starved fibroblast cells from cattle, sheep and goat. Several other cell types successfully used for cloning are limited to female donors (cumulus and mammary epithelial, mural granulosa and oviductal cells) and are more difficult for long-term culture. Live progenies using skin fibroblasts have been produced in cattle [3].

Sample collection and development of primary cultures: Samples were collected by biopsy of skin from ear pinna and transported in a complete medium (DMEM + HamsF12 with 10% FBS and penicillin and streptomycin) at 4^{o}C. Tissue samples were processed by removing hair form both sides, cut into small pieces and seeded in petridish containing fibroblast culture medium (DMEM + HamsF12, 10% FBS, penicillin and streptomycin and L-glutamine). The primary skin fibroblast cells started emerging out of tissues within 4–6 days and were allowed to grow up to 12–15 days till nearly 80% confluency was attained.

Purification and sub-culturing of skin fibroblast cells: In isolated cases, there were contaminations of epithelial cells but these were removed mechanically as well as enzymatically to get pure fibroblasts. Sub-culturing or 'splitting cells' was done periodically removing growth media, washing the plate, dissociating the cells and diluting cell suspension in fresh media.

Standard growth curve: Whenever, a new batch of culture media supplement was introduced, it was checked for its efficacy for growth of cells in culture and compared with standard growth curve. Goat skin fibroblasts remained in lag phase for initial two days when they settled on the solid surface of culture vessel and then came to log phase when maximum growth took place spanning from the third to the seventh day. As the confluencey level increased and media supplement was depleted, cells stopped dividing and a platue was attained from the eighth day onwards and then showed decline due to contact inhibition.

Cell proliferation index: Under standard culture conditions, skin fibroblast cells divide once in 24 hours but it is rarely achieved in normal culturing. The population doubling time and cell proliferation rate per day were checked at regular interval for quality assessment. For this,

ELISA based MTT assay, incorporation of 5-bromo-de-oxyuridine method, and flow cytometer methods were used.

Evaluation of cells for ploidy level: During long-term culturing the cells are likely to develop one or other type of chromosomal abnormalities. It must be ensured that the cells in different passages be checked for normal ploidy so that viable clones can be developed from them. Cultures showing increased frequency of aneuploidy or polyploidy must be terminated from further passaging. DNA from cultured somatic cells can be isolated using available DNA isolation kits and checked for its quality on 2% agarose.

Cryo-freezing of cells: Cells are best frozen as cell suspension. Healthy culture were always employed to provide the stock to freeze cells. The cells were frozen at controlled freezing rate. The cells were kept at –80^{o}C as well as at –196^{o}C in Liquid nitrogen. Cells were found viable for subsequent growth after thawing at 38^{o}C even after 12 months.

Applications: Skin fibroblast cells are widely used as nuclear donor and have produced viable clones in different livestock species. These cells after a series of passaging behave like totipotent cells and can be used as substitute of ES cells for gene targeting and production of transgenic animals. Cells can have wider application in understanding disease models and for cell therapy, and can be used for *in vitro* secretion of useful therapeutic protein in a culture medium. These can also be used for *in vitro* model studies on aging and cancer.

Acknowledgements:

Authors are thankful to the Director, NBAGR, and Karnal for the facilities provided. Funding by World Bank under NATP is duly acknowledged.

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TOPIC: Gene-based technologies applied to plants, rumen microbes, and systems biology

Addition of tannins to ruminant feed: Investigation of the effects on ruminal microbiota by DGGE

N. Selje, S. Muetzel, E.M. Hoffmann, K. Becker

Institute for Aquaculture Systems and Animal Nutrition, University of Hohenheim (480B), Fruwirthstr. 12, D-70599 Germany

E-mail: nselje@uni-hohenheim.de

Tannins are polymeric phenolic substances that show antimicrobial properties against filamentous fungi, yeasts and bacteria. They form complexes with proteins and polysaccharides. Protein complexation is supposed to reduce the availability of feed protein to ruminal microorganisms, and in turn increase the supply of aminoacids to the ruminant. In *in vitro* trials tannins were shown to bind cell walls of ruminal bacteria preventing growth and protease activity. In pure cultures they inhibited growth of predominant rumen bacteria, such as *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Ruminococcus amylophilus*, *Streptococcus bovis*.

This study presents the effects of tannin addition to ruminant feed on the microbial community in the rumen *in vivo*. Two non-lactating fistulated Holstein cows were adapted for two weeks to a total mixed ration-concentrate diet fed in two equal meals per day. After the adaptation period samples were collected as control one hour before, and one, three, five and seven hours after morning feeding four and one day before tannin addition. The diet was then supplemented with 7.2 g/kg tannins from chestnut (*Aesculus hippocastanum*) and fed for another two weeks. During this period samples were collected at day one, two and four, five hours after morning feeding. On day eight and ten, five consecutive samples were collected as described for the control.

Rumen fluid was collected from the liquid phase by a pump and from the feed mat by hand squeezing. Parameters investigated were ammonia and short chain fatty acid (SCFA) concentration, enzyme activities, and concentration of 16S (bacteria and archaea) and 18S rRNA (protozoa and fungi).

The daily pattern of parameters investigated for two animals were similar and highly reproducible although the absolute concentration of some parameters (SCFA, isovalerate, 16S rRNA) differed between animals. The concentration of ammonia, enzyme activities and rRNA content, different between the liquid and meat phases. The presence of tannins affected neither the concentration of SCFA nor the concentration of isovalerate, a degradation product of protein. In contrast, the mean concentration of NH3 were lower during the entire period of tannin administration. The average cellulolytic enzyme activities, CMCase and xylanase (Figure 1), decreased as well, the maximum decrease being after three days of tanni introduction. Thereafter the enzyme activities increased.

RNA concentration revealed that ruminal microorganisms showed variable responses to tannin administration, both with respect to the donor animal and to the fraction collected, as shown in Figure 2. The immediate response of prokaryotic organisms was a rise in animal 1 and a decline in animal 2. During further exposition to the tannins 16S rRNA concentrations came back to the original equilibrium in the liquid phase, but continued to decrease in the feed

mat. Initially the concentration of eukaryotic rRNA decreased even more than the prokaryotic rRNA.

These findings indicate that the addition of chestnut tannins showed only short-term effects on metabolic parameters such as enzyme activities. This raises the question whether the transient decrease of activity is due to the adaptation of organisms in the existing rumen community or to a change in microbial community composition. For further investigations it will be important to know, whether the variations in prokaryotic rRNA content of the two fractions indicate quantitative and/or qualitative differences of the microbial community. Molecular techniques are the suitable tools to answer these questions.

To investigate changes within the rumen microflora several specific PCR protocols were evaluated. Primer pairs were chosen to selectively amplify 300–600 base pair fragments of the ribosomal RNA gene of bacteria, archaea (Figure 3), bacteroidetes, low-GC gram positives, fungi and protozoa. Subsequently, PCR products were separated by denaturing gel gradient electrophoresis (DGGE), where fragments of the same length are separated within a gradient of denaturing compounds according to differences in GC content and melting domains. This approach increases the resolution of detection over using universal primers, but gives a broader, simultaneous overview of the rumen community than hybridisation with single taxonomic probes

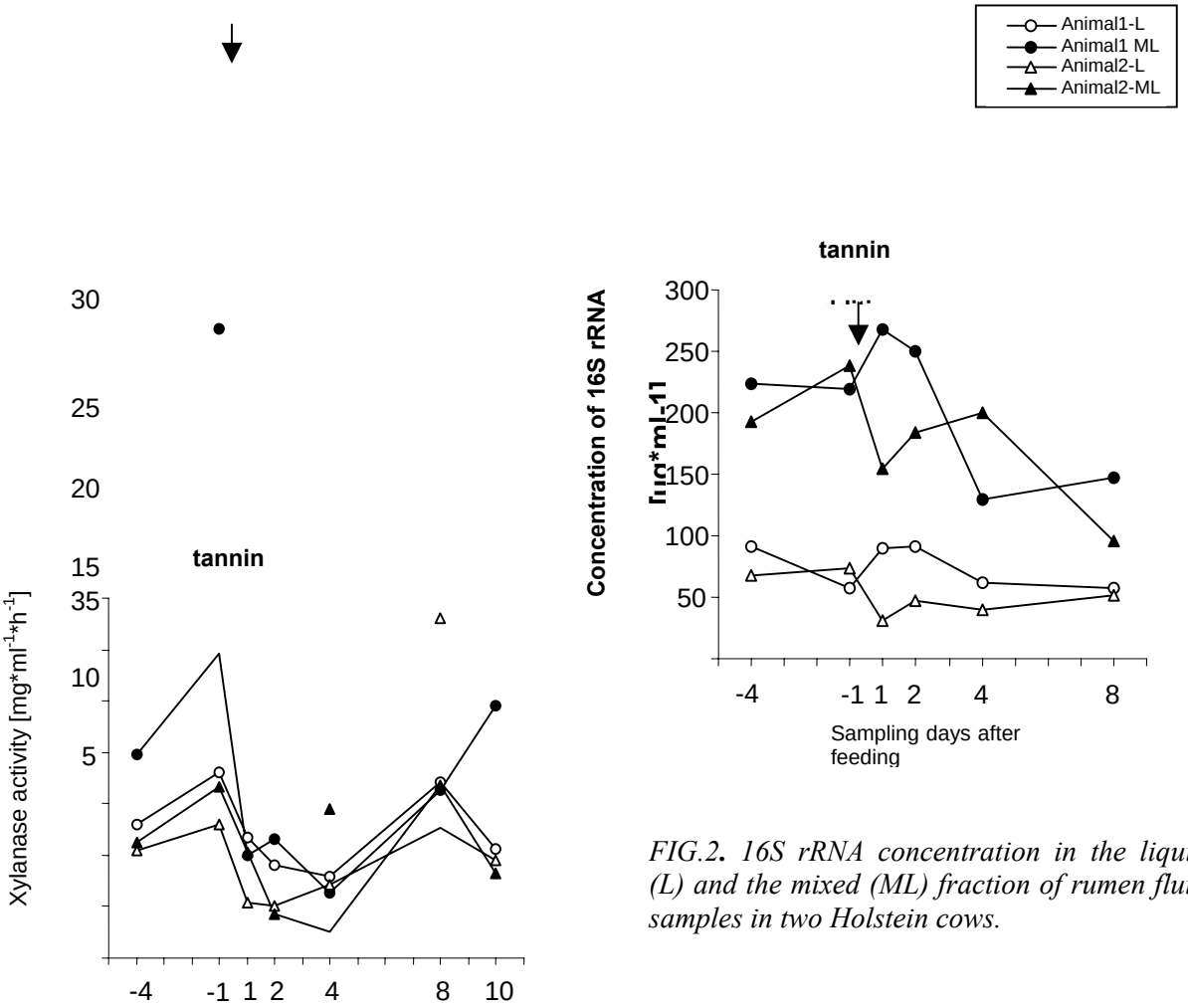

FIG.1. Xylanase activity in the liquid (L) and the mixed (ML) fraction of rumen fluid samples in two Holstein cows.

FIG.2. 16S rRNA concentration in the liquid (L) and the mixed (ML) fraction of rumen fluid samples in two Holstein cows.

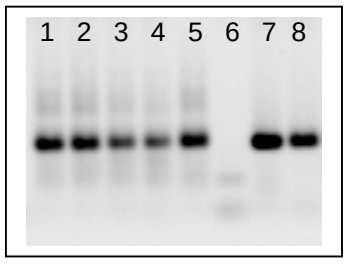

FIG.3. Products of PCR specific for archaea from rumen samples (lane 1-5), negative control (Variovorax paradoxus, bacteria, lane 6) and two positive controls (Methanomicrobium mobile, lane7; Methanobrevibacter ruminantium, lane8).

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

The vaccine properties of a Brazilian BHV-1 strain with an induced deletion of the gE gene

A.C. Franco[a,b], F.R. Spilki[a,b], F.A.M. Rijsewijk[c], P.M. Roehe[a,b]

[a] FEPAGRO-Centro de Pesquisa Veterinária Desidério Finamor, Eldorado do Sul, Brazil

[b] UFRGS-Faculdade de Veterinária, Porto Alegre, Brazil

[c] ID-Lelystad, The Netherlands

E-mail: claufran@terra.com.br

A Brazilian strain of bovine herpesvirus type 1.2a with a deletion of the glycoprotein E (gE) gene was constructed (BHV-1.2a gE$^-$). The deletion was introduced by the co-transfection of a deletion fragment containing the 5´and 3´gE flanking regions and genomic DNA of wild type BHV-1 into bovine cells. Identification of gE deletion mutant was performed by immunoperoxidase staining with an anti-gE monoclonal antibody. This gE deletion mutant was plaque purified and further examined by restriction endonuclesase digestion and Southern blot hybridization. The *in vitro* growth characteristics of this gE negative mutant were studied and compared with the parental strain. The results of these experiments showed that the BHV-1.2a gE$^-$ had a significantly reduced cell-to-cell spread in three different host cells. No statistical differences were observed when single step growth curves or penetration assays were performed using the BHV-1.2a gE$^-$ and the parental strain. *In vivo* studies were performed to access the potential of this virus as a vaccinal strain. In order to examine its attenuation for cattle, four BHV-1 seronegative calves were inoculated intranasally with $2x10^{5,3}$ $TCID_{50}$ of the gE$^-$ virus. Another group of three calves was inoculated with $5x10^7$ $TCID_{50}$ of the wild type virus. Two other calves were kept as uninfected controls. The deletion mutant had a markedly reduced virulence for calves, whereas the wild type virus was highly virulent. The gE$^-$ virus was excreted to lower titres and for a shorter period of time than the wild type virus. Calves immunized with gE$^-$ virus and challenged with $5x10^7$ $TCID_{50}$ of wild type virus developed very mild clinical disease with a significant reduction in virus excretion. These results show that the gE$^-$ recombinant was attenuated and capable of prevent clinical disease upon challenge. The gE$^-$ deletion mutant is a promising candidate virus for a differential vaccine to BHV-1 infections.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Expression of the classical swine fever E_2 recombinant protein and examination of DNA-vaccine based on one subunit

O. Deryabin[a], O. Deryabina[a], P. Verbitskiy[a], V. Kordyum[a]

[a] Institute of Veterinary Medicine UAAS, Kyiv, Ukraine

[b] Institute of Molecular Biology and Genetics NASU, Kyiv, Ukraine

E-mail: *deriabin@i.kiev.ua*

The aim of the work was to study the possibility of using the Classical Swine Fever Virus (CSFV) E_2 (gp51–55) recombinant protein expressed in *E.coli* in an immunologically active form and the creation of a DNA-vaccine model based on the gene.

For minimizing the refolding problems of the recombinant protein one of two subunits of CSFV E_2 protein was chosen for production. This subunit has the domains A and D with the conservative epitopes some of which, in the domain A, induce synthesis of virus neutralizing antibodies.

Viral RNA was isolated from the CSFV virulent strain Shi-Min, which was produced in a continuous swine kidney PK-15 cell culture (passage 37) Oligonucleotide primers were designed based on the Brescia (GeneBank, M31768) strain sequence Sn 2647–2668 and Asn 3492–3471. For additional RT-PCR controls the obtained fragment (846 n. p.) from isolates from CSF outbreak in Ukraine in 1994. It was shown that only the samples from the acute form of disease, wherer the amplicon sizes corresponded to the Shi-Min fragment, readily hybridized under this in stringency conditions. The subsequent sequence analysis of the fragment and the phylogenetic analyses [1] with the use of sequences of various origin strains and isolates, placed the irus in group 1, subgroup 1.1, according to the classification suggested by D. Paton in 1995.

For protein synthesis 3 constructions for plasmids expressing with the insert of CSFV E_2 gene were created: CSFV-pET24a (+) – as the individual form of the protein with the molecular mass (m.m.) 34 kD; CSFV-pGEX-2T – as fused with glutathion-S-tsansphesase, m.m. 59 kD and CSFV-pLY – as fused with one subunit of β-galactisidase, m.m. 107 kD. All 3 variants of the protein were synthesized as inclusion bodies with an expression level of 15–20%. After optimizing the purification and refolding conditions, antigenic properties of the proteins were characterized in an indirect ELISA and immunoperoxidase test with homologous and heterologous sera obtained from laboratory animals and pigs. It was shown that the fused form of the protein of 107 kD reacted specifically with the sera from pigs which were immunized with the proteins of 34 and 59 kD. The individual protein reacted only with 20% of pig sera which were produces against the protein of 107 kD. In some sera against the 34 and 59 kD, virus neutralizing antibodies were examined.

To try and increase the efficiency of swine immunization with the CSFV recombinant proteins various adjuvants were tested. The best results were obtained with mantonide ISA-25 firms "Seppic" (France).

The immunogenic properties of CSFV E_2 recombinant protein variants were tested by direct challenge of vaccinated pigs. Piglets at 3 months and 18–20 kg weight were vaccinated twice intramuscularly, with an interval in 2 weeks. Challenge was made with the CSFV virulent strain "Washington". In the control group all animals died between 9 and 10 days.

It was shown that for the three variants, only the recombinant protein of 34 kD protected pigs from lethal infection. Next effective dose of the variant was determined. Its use led to the absence of disease clinical signs in swine after challenge. Temperature elevation also was not observed. Thus, the possibility of using the CSFV E_2 recombinant protein expressed in E. coli and reproducing one subunit with A and D epitopes to prevent lethal infection was demonstrated.

The fragment of CSFV E_2 gene was used to create a DNA-vaccine model [2]. The genetic construction was developed on the basis of the eucaryotic vector pTR-UF, which except the CSFV E_2 protein gene, contained a CMV promoter and AAV inverted terminal repeats. The resulting plasmid DNA was used for intramuscular immunization of 2 month old female mice, line Balb/C. The DNA administration three times in liposomes or in buffer, induced synthesis of specific antibodies. Additional single immunization with E_2 recombinant protein, expressed in *E.coli*, considerably increased the titer of specific antibodies. This indicated the effectiveness of animals' beyond the DNA-vaccine and the possibility for using the E_2 recombinant protein over vaccination.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Development of thermostable peste des petits ruminants (PPR) virus vaccine and assessment of molecular changes in the F gene

K.S. Palaniswami, A. Thangavelu, R. Velmurugan

Dept. of Veterinary Microbiology, Madras Veterinary College, Chennai – 7, India

E-mail: kspswami@yahoo.com

Two PPRV isolates, MIB 187 (from the first Indian report in sheep in 1987) and MIB 197 (outbreak in1997 in goats), both at vero cell passage thirty were incubated at 38°C, 39°C & 40°C for 15 min, 30 min and 1h in succession. This treatment (three cycles at every stage) was designed to increase the proportion of temperature resistant virions, as described for NDV [1]. to. After each step there was loss in infectivity however, the titre increased on subsequent cell passages at 37°C. The thermostable virus titre was assessed before and after "adaptation" of the PPR viruses to the higher temperatures.

The "thermostable" viruses (70^{th} passage (MIB 187(T)$10^{6.7}$ $TCID_{50}$/100 µl, MIB 197(T) $10^{5.7}$ $TCID_{50}$/100 µl) were tested for immunogenicity by host animal inoculation along with virulent PPRV challenge (a local PPR isolate maintained as tissue suspension) following the standards prescribed in the OIE Manual [2].The viruses were also confirmed as safe for use as per the OIE test protocols and no clinical signs were apparent during the 3 weeks post vaccination observation period. Antibodies produced were assessed using the VNT. Vaccine viruses were not isolated or identified by PCR from ocular and nasal swabs however, virus nucleic acid and antigens have been demonstrated in spleen and lymph nodes by FAT & PCR in earlier studies, indicating replication of vaccine virus. The MIB187(T) vaccine gave 100% protection against challenge' while the MIB197(T) virus gave only 66% protection, although the numbers of animals used was low (2 out of 3 and 3 out or 3 (100%) respectively). The vaccine was freeze dried in vials (50 dose each) at a titre no less than 10^3 $TCID_{50}$ / dose). The OIE stabilizer was used. The minimum protective dose was calculated by having safety with 100 doses and protection with 1/10 dose.

The MIB187(T) virus was chosen as a candidate virus for experimental vaccine production. Possible changes in the virus genome after thermo adaptation were examined using a RT-PCR technique to amplify portion of F gene, followed by sequencing, using the following primers

F_1 5'ATCACAGTGTTAAAGCCTGTAGAGG3'

F_2 5'GAGACTGAGTTTGTGACCTACAAGC3'

F_1 A5'ATGCTCTGTCAGTGATAACC3'

F_2 A5'TTATGGACAGAAGGGACAAG3'

The nucleotides sequences of F gene of thermostable PPR virus both before and after thermo adoption were analyzed, 242 nucleotides of the F gene of two thermostable strains before and after thermo adaptation were compared (Figure 1). Three base changes were observed at positions 1075, 1084 and 1085 in the thermostable PPR virus (MIB 187(T) strain) and one change was noticed at position 1068 with the another strain (MIB 197(T) strain). The nucleotide positions were identified in comparison with the F. gene sequence of the Nigerian

PPRV strain in Gen bank (Z37017.1) The viruses sequences of MIB 187, MIB 197 and the challenge virus have been published in Gene Bank , accession numbers AF 344885.1,AF 344884.1and AF 344886.1 respectively.

The stability of "thermoadapted" PPR virus was compared with the non-thermo adapted PPR vaccine virus, at room temperature, as well as 37°C with incubation for two months. The results were promising up to one month at 37°C. At room temperature the titre and potency of the thermadapted vaccine remained constant up to one month at the $10^{5.5}$ $TCID_{50}$ and after two months the titre was found to be $10^{4.5}$ $TCID_{50}$ / 100µl. The stability of the freeze dried thermostable PPR virus at 37°C incubation for 30 days has also proved the retention of thermostable character.

Field trials with the experimental thermostable PPR vaccine have exceeded 40,000 doses under variable environmental conditions and during disease outbreaks. The serum samples collected from the field trials have a serum neutralization titres exceeding 2^3, and are assumed protective. The reconstituted vaccine could be used for 3 hours with 10^3 sheep infective dose.

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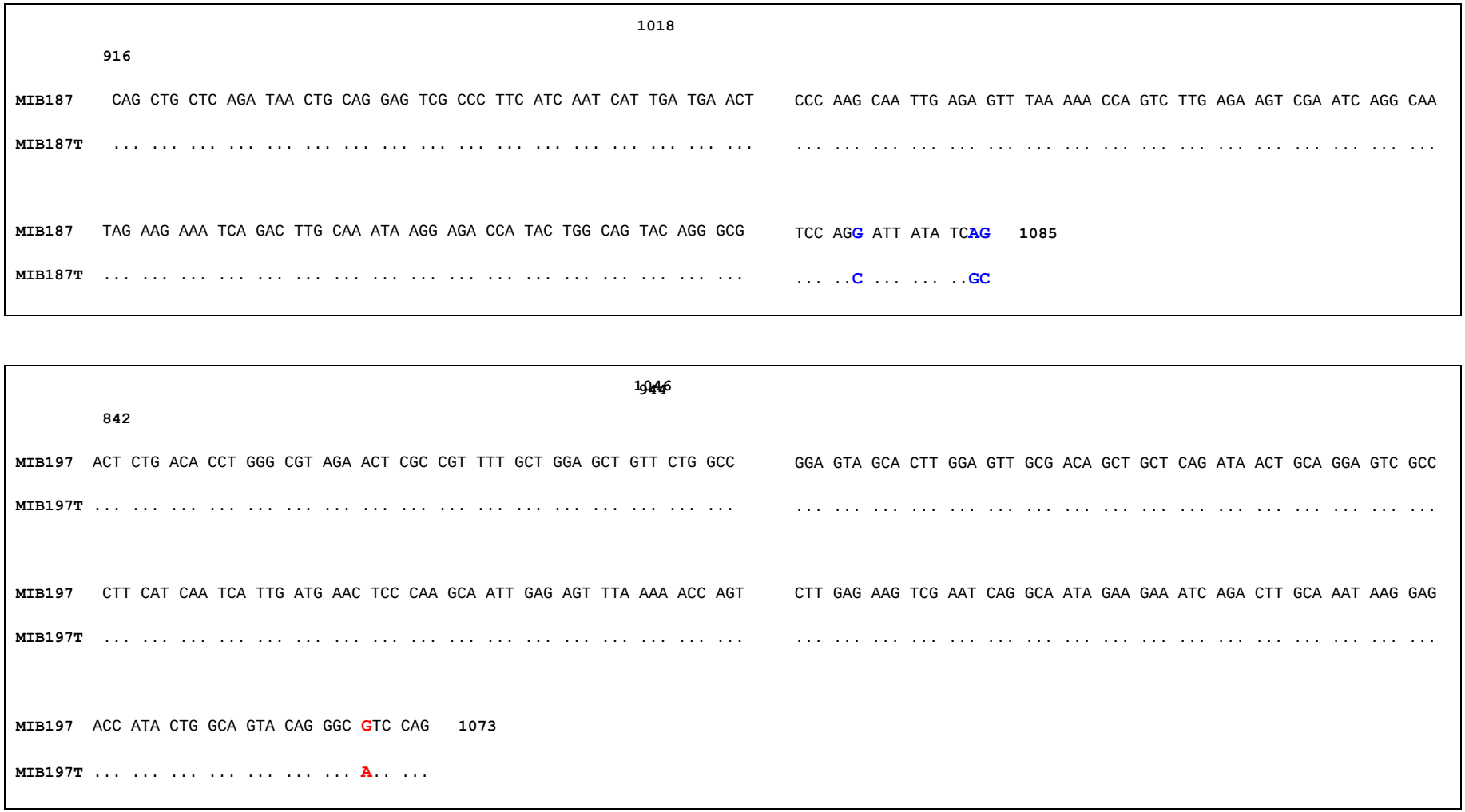

Fig:1 Comparison of Nucleotide sequence of PPR fusion gene (before and after thermoadaption)

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Molecular cloning of a Bangladeshi strain of highly virulent infectious bursal disease virus of chickens and adaptation in tissue culture by site directed mutagenesis

M.R. Islam[a], R. Raue[b], H. Müller[b]

[a] Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh

[b] Institute of Virology, Faculty of Veterinary Medicine, University of Leipzig, D-04103 Leipzig, Germany

E-mail: mrislam@royalten.net

Infectious bursal disease virus (IBDV) causes a highly contagious immunosuppressive as well as fatal disease known as infectious bursal disease (IBD) or Gumboro disease in young chickens [1]. IBDV is a dsRNA virus belonging to the family *Birnaviridae* having a bisegmented genome [2]. Very virulent (vv) IBDV does not replicate in common tissue culture; adaptation to tissue culture following repeated passages in embryonated eggs results in too much attenuation. It has been reported that only a few amino acids in the VP2 are responsible for tissue culture adaptation.

In the present study, full-length cDNA corresponding to the genome segments A and B of a Bangladeshi vvIBDV strain BD-3/99 [3] were synthesised and amplified by reverse transcription — polymerase chain reaction (RT-PCR) in overlapping fragments. The PCR amplicons were used to construct full-length cDNA clones of both segments in plasmid vectors along with the T7 promoter tagged at the 5'-end. Complete nucleotide sequences of both genome segments of BD 3/99 were established (GenBank Accession No. AF352776 and AF36270) and compared with 16 and 17 published sequences of segment A and segment B of IBDV, respectively, using Clustal V method of multiple alignment analysis (MegAlign, Lasergene, DNASTAR Inc., USA). In phylogenetic analysis BD-3/99 clustered with other vvIBDV strains isolated earlier from Europe, Asia and Africa.The reverse genetics technique [4] was optimised for BD-3/99. The plasmids were linearised with appropiate restriction enzymes and cRNA corresponding to both segment A and segment B of BD-3/99 were transcribed *in vitro* under the control of T7 promoter. Freshly prepared chicken embryo fibroblast (CEF) cell monolayer was then transfected with the transcribed cRNA. Transfection of CEF cells with this wild-type cRNA resulted in the formation of infectious virus particles but the progeny virus could not be passaged any further in fresh CEF cells. However, this molecularly cloned virus (BD-3mc) could be rescued by passaging the supernatant of transfected cells in embryonated chicken eggs.

Site-directed mutagenesis of selected nucleotides in the cloned cDNA was performed by megaprimer PCR method [5] to bring about amino acid substitutions at position 253 (Q→H) and 284 (A→T) in the VP2. A tissue culture adapted strain (BD-3tc) was successfully regenerated following transfection of CEF cells with cRNA transcribed *in vitro* from the plasmids having desired mutations.

The pathogenicity of the tissue culture adapted strain BD-3tc was tested in 4-weeks old commercial chicks. The original wild-type strain BD-3wt (BD-3/99) and the molecularly

cloned parental strain BD-3mc were also included in the experiment for comparison. Absence of clinical signs and milder pathological lesions in the bursa of Fabricius followed by quick follicular regeneration in BD-3tc infected birds suggested that the amino acid substitutions, Q253H and A284T in the VP2, made BD-3tc partially attenuated. The suitability of BD-3tc as a possible vaccine candidate is currently being investigated and the preliminary results are encouraging.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Improving resistance to trypanosomosis in mice through marker-assisted introgression

O.D. Koudande[a], J.A.M. van Arendonk[b], F. Iraqi[c]

[a] Laboratoire de Recherches Zootechnique, Vétérinaire et Halieutique / Institut National des Recherches Agricoles du Bénin, Bénin

[b] Department of Animal Science, Wageningen University, Wageningen, Holland

[c] International Livestock Research Institute (ILRI), Nairobi, Kenya

E-mail: dkoud2002@yahoo.fr.

A marker-assisted introgression experiment aiming at introgressing each of the three trypanotolerance QTL alleles from the mouse strain C57BL/6 (donor) into the mouse strain A/J (recipient) was performed at ILRI through a backcross design. A reciprocal cross was performed between founder parents to produce F_1 animals, followed by four subsequent backcross (BC) generations and two intercross (IC) generations [1]. From BC_1 up to BC_3 mice carrying donor QTL allele on chromosome 1 and 5 on one hand and mice carrying donor QTL allele on chromosome 5 and 17 on the other were selected. From BC_4 onward, only mice single carrier of donor QTL allele were selected. Selection troughout the experiment was based on markers information. Genotyping was performed on genomic DNA that was extracted from tail tissue collected on three week-old mice using the conventional method described by Sambrook et al., [2]. The extracted DNA was adjusted to 0.05 mg/ml for PCR and three microsatellite markers were used per QTL region to assess the alleles inherited by each mouse along the experiment. At the end of the introgression experiment, different synthetic mice were challenged with 10^4 parasites (*T. congolense*). The survival time in days i.e. the time from inoculation to death was recorded and analyzed. The experiment was ended at 150 days post-inoculation.

Homozygous mice for the donor QTL allele on chromosomes 1, 5 and 17 were selected at IC_2 and correspond to groups 1, 2 and 3 respectively in Table I. Some mice were homozygous for the recipient QTL allele on the three chromosomes and were selected as internal control group (Group 4). We observed that only animals from one sex were selected at a stage where both sexes were needed. Sometimes no mice of the desired genotype were found after a first round of birth. These problems can substantially delay the introgression program and expand involved costs. Markel et al. [3] experienced similar problems when introgressing the *Apoe null* allele to multiple inbred strain genetic backgrounds. We also observed that few offspring produced from homozygous mice for the donor QTL allele on chromosome 17 did hardly survive after weaning. Observations on these mice when still alive showed that animals grew slowly, became weaker, and finally died. The exact cause is not known but results suggest that the QTL on chr.17 interacts with A/J background to generate negative effect on survival after weaning. This could be the result of the presence or absence of a genetic modifier [4]. It could also explain the low number of mice in Group 3.

The infection rate was 98 per cent over 366 inoculated mice. The survival data of all groups is summarized in Table I. As is shown in Figure 1, all survival curves were generally located between the controls' curves, i.e. the donor and the recipient groups of mice. Groups 1, 2 and

3 showed longer survival time (P = 0.035) than the donor line A/J but did not reach the level of the donor line (Table I). No difference was found between Group 4 and the recipient (Table I) line. This gives evidence for the success of the introgression experiment that has resulted in splitting up the three chromosomal regions related to trypanotolerance into different groups of synthetic animals.

TABLE I. STATUS AND MEAN SURVIVAL OF DIFFERENT GROUPS OF MICE USING A NON-PARAMETRIC ANALYSIS

Group	Genotype			Infected mice (no.)	Censored mice (no.)	Mean survival (days)	S.E. mean survival
	Chr.1	Chr.5	Chr.17				
A/J	AA	AA	AA	78	0	29.7	2.6
1	CC	AA	AA	60	1	57.9	3.3
2	AA	CC	AA	63	2	49.5	3.8
3	AA	AA	CC	21	2	46.8	5.1
4	AA	AA	AA	58	0	34.2	3.4
C57BL/6	CC	CC	CC	77	5	68.8	2.1

(A=A/J recipient allele, C=C57BL/6 donor allele)

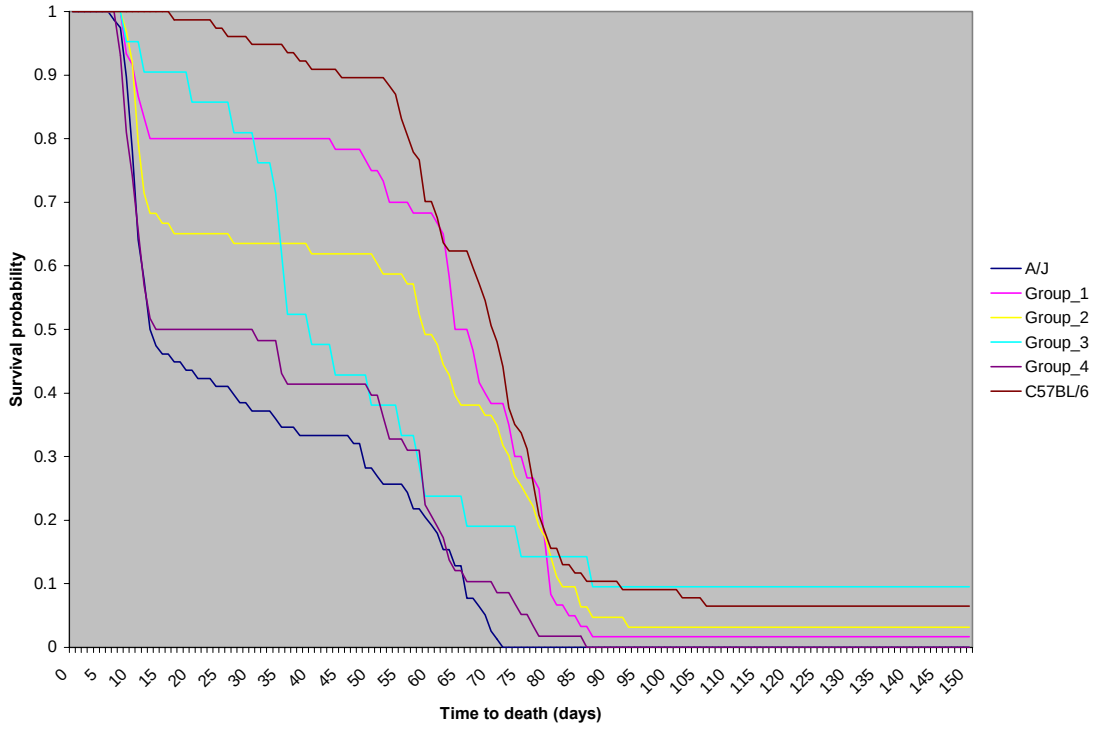

FIG.1. Survival of different genotype groups of mice following Trypanosoma congolense inoculation

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Serial analysis of gene expression (SAGE) in the genetic control of a *Trypanosoma congolense* infection in a trypanotolerant N'Dama cattle

D. Berthier[a], S. Thevenon[b], R. Quéré[c], D. Belemsaga[b] , D. Piquemal[c], L. Manchon[c], J. Marti[d], J.C. Maillard[a]

[a] CIRAD-EMVT Baillarguet, Montpellier, France

[b] CIRDES, Bobo Dioulasso, Burkina Faso

[c] Skuld Tech Cie, Montpellier, France

[d] CNRS/ UMII, Montpellier, France

E-mail: thevenon@fasonet.bf

In central and sub-Saharan Africa, the most important constraint to livestock production is trypanosomosis. This tsetse-transmitted disease represents an important risk for about 60 million cattle spread over 7 million km^2. Several indigenous West African taurine (*Bos Taurus*) breeds, such as longhorn (N'Dama) cattle are well known to control trypanosome infections. This genetic capability named "trypanotolerance" results from numerous biological mechanisms most probably under multigenic control, these include the control of the trypanosome infection by a parasitaemia limitation and the control of a severe anemia due to parasite pathogenic effects, both main criteria of trypanotolerance. Past methodologies were not succeeded in identifying the complete pool of genes involved in trypanotolerance. New post genomic biotechnologies, such as transcriptome analyses, are now efficient enough to characterize the full complex of genes involved in the expression of specific biological functions. One is the Serial Analysis of Gene Expression (SAGE) technique which consists in the construction of transcript libraries for the quantitative analysis of entire genes expressed or inactivated at a particular step of cellular activation. We used the SAGE method to compare the total expressed genes during an experimental *Trypanosoma congolense* infection in four cattle, one N'Dama individual (*Bos taurus*), one Baoule (*Bos taurus*) and two zebu (*Bos indicus*). These animals were infected by the *Trypanosoma congolense* strain SER/71/STIB/212 (300 trypanosomes per kg of body weight). They were monitored for hematocrite, parasitemia and body weight for five months. The mRNA samples were harvested before infection, at the maximum of parasitemia, at the minimum of hematocrite and at recovery.

First, two total mRNA transcripts libraries were constructed from blood white cells of the N'Dama cattle: one at T0 just before the *T. congolense* experimental infection (T0L) and the other several days later, at the maximum of parasitaemia (MPL). Bioinformatic comparisons in the bovine genomic databases allowed us to identify 4763 sequences Tag, 2281 transcripts and 187 up and down-regulated genes. Among these differentially expressed genes, there were known genes (for instance IgM; IgG, class II BoLA-DQB up-regulated, BoLA class I genes down regulated), several ESTs and unknown functional genes.

These results will be compared to the one obtained from the Baoule and the two zebu cattle and will allow identification of the pool of genes involved in trypanotolerant or sensitive

character. The knowledge of all identified named or unnamed genes involved in the trypanotolerance control will allow to set up field marker assisted selections.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Tapping the World Wide Web for designing vaccines applicable for livestock diseases

C.C. Deocaris

Philippine Nuclear Research Institute, Commonwealth Avenue, Diliman Quezon City 1101 Philippines

E-mail: ccdeo@yahoo.com

The information superhighway, popularly known as the Internet, has become a pervasive influence in the health sciences as it democratically provides not just the most comprehensive genome, transcriptome and proteome information of organisms, including that of the human body, but also a collection of software that could analyze these genomic data for specific applications. Immune responses are generally based on the immunogenic proteins displayed on a pathogen and the process of deriving these immunogenic regions is vital for developing synthetic peptide vaccines. From the perspective of vaccinologists of the 20th century, immunogen mapping by fragmenting protein isolates and/or by random cloning of overlapping peptide fragments followed by monitoring immune response in a model animal are both cost- and labor-intensive. Such a BENCH-TOP experimental approach can only be successfully performed in laboratories running on massive funding. In the 21st century, vaccinologists can now tap publicly-accessed genomic databases and powerful computational tools to identify immunogenic regions and to design synthetic vaccines against any animal pathogen of known genomic data. Prediction of immunogenic regions can be divided into the identification of B- and/or T-cell epitopes. These regions have been defined using various algorithms, including the identification of charge and residue palindromes, MHC-binding motif density, proteosomal cleavage sites, among others. Identified epitopes were then strung by (less steric) glycine-bridges and their combination and permutations for multi-valency were analyzed. The final vaccine has to be both promiscuous as an immunogen at the same time stable structurally for optimal epitope mimicry. Interest in synthetic immunogenic peptides is increasingly growing due advantages over conventional vaccines, as these peptide vaccines are safer, they can be designed to induce defined immune responses and they can be synthesized in large quantities in high purity and they do not require a cold-chain. Furthermore, we have also developed an expert system, SYVAX ver.1.0, for hastening the simulating the combinatorial process of identified epitopes using secondary structural retention as screen. It is anticipated that this expert system for synthetic vaccine design will be a useful bioinformatic tool that could be very useful especially for animal scientists and veterinarians in developing countries who wish to construct vaccines via a DESK-TOP approach.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Complementing nuclear with DNA vaccine technologies for improving animal health: The Philippine experience

C.A.T. Villanueva[b], A.C.S. Maligalig[b], J.L.V. Relucio[b], M.E.K. Dacanay[b], E.A. Ramos[b], C. Cantor[b], M.S. Buenaventura[b], K.F. Pobre[b], R.G. Osorio[a], C.C. Deocaris[a]

[a] Philippine Nuclear Research Institute, Commonwealth Avenue, Diliman, Quezon City, Philippines

[b] National Institute of Molecular Biology and Biotechnology, University of the Philippines, Diliman, Quezon City, Philippines

Email: ccdeo@yahoo.com

Immunization with "naked DNA" (or DNA vaccines) coding for protein antigens can protect livestock against pathogens without the disadvantages associated with traditional vaccines. Advantages associated with DNA immunization are cheap production, heat stability, amenable to genetic manipulation, mimic viral infection, absence of requirement for a cold-chain, and no risk of reversion to pathogenicity. These gene-(expression/cloning) plasmid constructs have been amplified in a bacterial host, purified and administered into the target animal. The gene is expressed in the host and the antigen that is produced induces an immune response. We evaluated chicken egg-yolk polyclonal antibodies response (chIgY) by radioimmunoassay (RIA) after direct intramuscular inoculation of a plasmid expression vector pRc/CMV-HBs(S). Elevated levels of the polyclonal antibodies were observed after 6 weeks. Aliquots of chIgY were coated on beads for RIA and results were comparable with immunized sera. Antibodies for RIA sourced from chicken egg yolks and produced through DNA-mediated vaccination may be a cheaper and much less invasive alternative to serum-derived antibodies. Results from this preliminary experiment may help improve cost-effectiveness of RIA using DNA vaccine and chicken yolk antibody technologies.

For these vaccines however, virtually all recommended immunizations would require parenteral administration, which would be too tedious for this reason, alternatives to injections are being sought. The development and application of controlled and intelligent delivery depot systems for DNA vaccines have been studied. We have encapsulated pCMV-SPORT- β-gal, our reporter construct, in K-carrageenan-PVP-modified microspheres: IP20 (for stomach sensitive) and IP18 (for intestine sensitive) and chitosan microspheres. The DNA-loaded polymers were delivered as feeds to the model animals, *Oreochormis niloticus* (fish) and ICR mice. The gills, stomach, small intestine, spleen were then stained with X-gal to observe β-galactosidase activity. Intense staining was observed in the stomach regions with IP20 and chitosan, while minimal staining was observed with IP18. Spleen and other tissues did not express β-galactosidase activity. Our report is an evidence for successful gene delivery capabilities of radiation-synthesized microspheres in both fish and mouse models.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Molecular characterisation of filed isolates of African Swine Fever virus involved in infection persistence in Central Africa: with reference to the Democratic Republic of Congo

L.K. Mulumba-Mfumu[a], L.K. Dixon[b], P.J. Wilkinson[b], G.H. Hutchings[b]

[a] Central Veterinary Laboratory (CVL-Kin), Kinshasa, Democratic Republic of Congo

[b] Institute for Animal Health (IAH-P), Pirbright, United Kingdom

E-mail: *leopold.mulumba@caramail.com*

In order to investigate African Swine Fever virus strains under clinical situations, a field study was carried out focusing on virus identification through viral DNA detections. To achieve the virus DNA detection, both variable and constant regions of genomes of the ASFV isolates were used. Regarding the variable regions, the genes encoding j13L and CD2 like proteins were targetted; and for the constant region, the highly conserved gene encoding the VP72 major capsid protein was targetted too. VP72 gene is the most shared for most of ASFV isolates [1].

The study was achieved in the four following stages: - PCR; - PCR results confirmation; - Sequencing and; - relationship constitution, according to nucleotide sequences comparison between investigated ASFV isolates and some others from both, the African and the European continents. PCR was directly performed from a range of tissue samples including spleens, gastro-hepatic lymph nodes, kidneys, lungs, livers as well as blood clots. An amount of 468 organs was collected but, for PCR manipulations only 265 DNAs from 248 pigs were extracted. All the tested pigs did belong to 10 locations dispached in 2 groups: indigenous pigs, the so called local breeds or free-ranging animals from rural areas (1 critical location) and, improved breeds or overseas races of pigs from commercial farms (9 locations). The alone location for the indigenous pigs was a large market with a small-size abattoir where healthy pigs from different villages are usually gathered and slaughtered for commercial purpose of meat. It is very necessary to check the real status of these animals since their healthy status might be a ASF symptomless one. Basically pigs from villages in Africa are not only free-ranging but also feral animals that move from time to time to wildlife areas.

Primers for above mentioned ASFV genes were carefully selected, i.e. specific attention was payed to their lenght (29 to 32 nucleotides) as well as their GC %, due the fact that all the samples were originating from an endemic region thus, supposed to deal with low virulence strains of virus [2]. PCR manipulations were carefully performed according to the protocol described by Sambrook and amplicons were visualized after electrophoresis and the use of: Ethidium bromide, 1Kb DNA Ladder as scale, 1% Agarose gel in 0.5 x TAE buffer. The PCR results were confirmed by ELISA and IMP respectively on the basis of identification of the viral cytosoluble protein (in the relevant serums); and the viral protein 72 (VP72) in the tissues, using the ABC kit, notably for the sample selected for sequencing. A VP72 amplicon from a spleen sample, originating from a commercial farm challenged a couple of days back by an high mortality of pigs associated with Fever and the skin Hyperemia was selected for sequencing and relationship constitution. Nucleotide sequence of these PCR fragments was determined after cloning in a plasmid vector according to the protocol described by Promega Corporation (revised 4/00) / Part # TMO24 / page 8 and purification procedures were

performed with GIAGEN kits as described in the relevant books. The obtained sequences were compared using programmes from the GCG sequence analysis package.

African Swine Fever virus isolates DNAs were detected in the samples of pigs originating from 5 locations out of 10. All the targetted genes i.e. encoding the virus proteins P72, j13L, CD2 like were detected in at least 2 of the 5 positive locations, but not simultaneously in one sample. The VP72 gene was detected in all the 5 five locations, suggesting that it is the highly conserved gene in most isolates. The j13L gene was detected lenght in 3 locations. The CD2 like protein gene was not found directly in spite of primers lenght and GC rate, primers of genomic flanking regions were used in order to find this gene and as it was detected in 2 of the 5 positive locations. Interestingly a viral DNA (VP72 gene) was detected from a spleen of healthy indigenous pig, a local breed sow originating from the country side, suggesting a persistent infection [3], it might be either a case of an inapparent form of ASF as for the wild pigs [4], or a breed – based resistance but, other conditions are possible, i.e. a low virulence strain [2].

Following VP72 sequence comparison, a similarity was revealed with other isolates involved in outbreaks in at least five countries: Malawi, Uganda, Nigeria, Spain, and the Dominican Republic.

	Primer	Primer sequence (5' to 3')	Init. Denat Temp	Ann. Temp	Taq Poly	Frag. size	+ve Sites
	VP72 3 F	CTGTAACGCAGCACAGCTGAACCGTTCTG			*Pcia*		**5/5**
VP72	*VP72 4 R*	ATAGGATTAAAACCTACCTGGAACATCTCCG	94ºC	55ºC	*Mast*	500 bp	100%
	J13L 3 F	GGTTGGTTTTCAAATGTTGGCGAAAGTAG		55ºC	*Pcia*		**3/5**
j13L	*J13L 4 R*	CCATAAATTCTGTAATTTCATTGCGCCACAAC	94ºC	50ºC	*Trip*	700 bp	60%
	CD2v 1 F	GGGTTAACAATACGGTAATATTAC GTGGTG					**0/5**
CD2	*CD2v 2 R*	GATTCAGAGGGTGGTGGAATTTCTATTTC	94ºC	50ºC	Pcia	500 bp*	0%
	ep152 F 5'	CCAAAGCAGTTTAACTTTCTGTTAGACAGCGCA GAAGG			*Trip*		**2/5**
EP	*ep364R 5'2*	CATTGGATTATGTTGCATATCATGAAAATCGG	94ºC	50ºC	*Mast*	1700 bp	40%

PCR characterisation results according to the samples locations

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Use of DNA from milk tank for diagnostic and typing of bovine leukosis virus

R. Felmer, J. Zuniga, H. Floody, M. Recabal

National Institute for Agricultural Research (INIA), P.O. Box 58-D, Temuco, Chile

E-mail : rfelmer@carillanca.inia.cl

Bovine leukaemia virus (BLV) is an exogenous retrovirus distributed worldwide. Most BLV-infected cattle remain clinically normal during their lifetime, with only 1–5% eventually developing lymphosarcoma. However, up to one-third of BLV-infected cattle may develop persistent lymphocytosis (PL), a polyclonal expansion of infected B-lymphocytes [1]. In Chile the infection was first described in the early 80´s, and has since progressed slowly since then mainly because of a National Program implemented in the middle 90´s. Nevertheless, infection persists and there is a continue need for development of more sensitive tests that can be applied to control the spread of the disease.

We have been evaluating milk as a source of DNA that can be used for the rapid diagnosis and typing of BLV. In order to find seropositive herds we made a random screening of milk tank samples obtained directly from our milk quality laboratory. Samples were analysed by an indirect ELISA (BLV Svanova Biotech) according to the manufacturer instructions. Twenty three out of 76 milk tank samples analysed gave seropositive results to BLV antibodies with various degrees of reaction. To confirm the diagnosis through direct detection of proviral BLV DNA, leukocyte preparations from all 23 milk tank samples were prepared by an "in house DNA protocol". DNA samples were tested by nested PCR using primers specific for a highly conserved region of the *env* gene [2] and PCR conditions described elsewhere [3]. In samples from 15 herds BLV-env specific amplification products were detected (65%). The restriction analysis of these amplicons using the endonucleases *BamH* I, *Bcl* I and *Pvu* II, showed that 11 samples yielded the same pattern as first characterised by [4] in Australia. Three other isolates produced the same pattern as the Belgian variant [5] and no Japanese variants [2] have been found so far (Figure 1).

A wide variability in somatic cell count was observed in the analysed herd samples, which could explain the relatively low correlation found between ELISA and PCR [6]. Nevertheless, this procedure can be used not only as an aid in the diagnostic of BLV, but also in the typing of the virus after digestion with restriction enzymes which provides a means to better understand the epidemiology and distribution of BLV infection.

We are currently extending our studies to a higher number of herds and are carrying out sequence analysis of some of the viral DNA samples in order to build up a philogenetic tree.

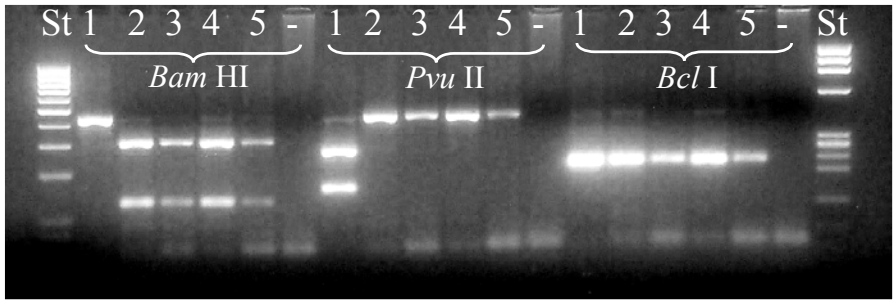

FIG.1. - 2% Agarose gel electrophoresis of Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplicons for BLV.

Lanes 1-5 are PCR amplicons for BLV, digested with *Bam* HI, *Pvu* II and *Bcl* I, corresponding to 5 different herds.
First and last lines correspond to molecular weight markers 1 Kb and Phi X-174 Hae III (Gibco BRL), respectively.
-: Corresponds to a negative herd sample.

Sample 1 showed a pattern similar to the Belgian subtype while samples 2-5 showed a pattern similar to the Australian subtype. No Japanese subtype have been found so far.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Molecular marker studies in riverine buffaloes for characterization and diagnosis of genetical defects

B.R. Yadav

Livestock Genome Analysis Laboratory, National Dairy Research Institute, Karnal–132 001, India

E-mail: bry@ndri.hry.nic.in

The buffalo is probably the last livestock species to be domesticated. Many of its genetical, physiological and behavioral traits are not yet well understood. There is a need for precise markers for the characterization of animals and breeds, diagnosis of diseases and causes of anatomical and physiological defects. Developments in DNA technologies have made it possible to discover a large number of genetic polymorphisms at the DNA sequence level for evaluation of the genetic basis for observed phenotypic variability. The present discourse deals with investigations on different molecular marker in buffaloes for identification of individuals, correct parentage and genetic defects.

Restriction Fragment Length Polymorphisms (RFLPs)

Restriction fragment length polymorphisms were studied in Murrah and Nili-Ravi buffaloes using conventional hybridization and PCR technique for polymorphism however, he found low heterozygosity and mostly monomorphic bands and may due to closed breeding policy and small population size of animals examined.

DFP revealed with genomic probes

The genomic probe Bkm and its derivative 2 (8) carrying 'GATA' repeats were found to produce DNA fingerprints. The probes also showed species-specific bands useful for identification of carcass or other unknown samples.

DFP revealed with oligonucleotide synthetic probes

DNA fingerprinting Nili-Ravi and Murrah buffaloes with five different oligonucleotide probes- $(GT)_8$, $(GT)_{12}$, $(GTG)_5$, $(TCC)_5$ and $(GACA)_9$ using five enzymes-*Alu*I, *Hinf*I, *Hae*III, MboI and *Eco*RI. All the probes gave multilocus hybridization patterns. The Probe $(GT)_8$, $(TCC)_5$ and $(GTG)_5$ gave polymorphic DNA fingerprinting. $(GTG)_5$ was the most polymorphic probe among all the five probes studied. The band patterns showed allelic frequency between 0.22 and 0.29, band sharing 0.45 and heterozygosity between 0.81 and 0.85 in Nili-Ravi and Murrah buffaloes.

Randomly amplified polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) technique studied in Nagpuri and Murrah breeds of buffalo. Polymorphic patterns revealed were used to find out band frequency, band sharing frequency, genetic distance, genetic identity index and mean average percentage (MAPD) in both the breeds (within and between). The average band sharing frequency within breed was 0.739 ± 0.032 in Nagpuri and 0.669 ± 0.035 in Murrah. The between breeds band

sharing was lower (0.490± 0.062) than the within breeds band sharing. The overall average genetic distance was 0.464 ± 0.15 between these two breeds. The genetic identity index was 0.632 ± 0.076 between Nagpuri and Murrah buffaloes. The RAPD fingerprint analysis showed that the average percentage difference (APD) value varied for each primer and MAPD for these two breeds was found to be 50.97 ± 6.15.

Microsatellite (Heterologous) primers:

Bovine microsatellite primers were studied on DNA samples of buffaloes (about 40 individuals of each breed) of Murrah and Nili-Ravi. For a total of 79 primers screened, 56 gave amplification product in buffalo and in control all the primers gave amplification. The observation showed that 70.89 % of bovine loci were conserved in the case of buffalo. Out of 56 conserved microsatellites loci 36 were polymorphic i.e. informative (36/56 =64.29%) and the rest were monomorphic. The numbers of alleles, their sizes, frequencies, heterozygosity and polymorphism information content (PIC) were calculated. The numbers of allele ranged from 2 (BM-044) to 6 (BMS-651) in Murrah and 2 (MB-077) to 5 (BMS-585) in Nili-Ravi. The size range of alleles at different loci varied from 104 (BMS-820) to 242 (MB-077) in Murrah and 100 (BMS-820) to 242 (MB-077) in Nili-Ravi. The heterozygosity of different microsatellite loci was calculated from allele frequency ranged from 0.47 to 0.77 in Murrah and 0.49 to 0.79 in Nili-Ravi. The average heterozygosity over different loci in Murrah and Nili-Ravi were 0.67 and 0.69 respectively. The range of PIC varied from 0.375 (MB-077) to 0.734 (BMS-651) for Murrah and 0.358 (MB-077) to 0.754 (BMS-585) for Nili-Ravi. The pooled PIC for Murrah and Nili-Ravi ranged from 0.371 to 0.746

Parentage determination: The knowledge of correct parentage is a prerequisite in breeding programmes. Highly polymorphic DNA fingerprinting markers are quite useful for this purpose. Different types of makers viz. genomic and oligonucleotide probes, RAPD and microsatellite markers were used in identification of parents in two sire families in each of the Nili-Ravi and Murrah buffaloes. Microsatellite primers were found more useful and particularly for verification of the semen used in artificial insemination.

Determination of freemartinism and other genetic defects: Individual specific DNA fingerprinting techniques were applied on twin born animals (XX/XY) chimaerism, anatomically defective and XO individuals. PCR-RFLP assay using sex chromosome (Y) specific primers enabled the identification of freemartin animal and other sex specific defects.

The genetic improvement of animals needs marker-based information, which depends on the choice of an appropriate marker system for a given application. Molecular markers serve as a potential tool to geneticists and breeders to evaluate the existing germplasm and to manipulate it to create animals as per desire and need of the society and conservation for posterity.

(The information was generated by various workers, viz. SHASHIKANTH, P.K. SHENDE, A. MITRA, DE, A. PANDEY, M.K. THAKUR and others in genome analysis laboratory).

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

G protein-coupled chemokine receptor, a host range gene suitable for phylogenetic grouping of the Capripoxviruses

C. Le Goff[a], E. Fakhfakh[b], A. Chadéras[a], G. Libeau[a], A. Diallo[c], E. Albina[a]

[a] CIRAD-EMVT, TA 30/G, 34398 Montpellier, Cedex 5, France

[b] IRVT, La Rabta, 1006 Tunis, Tunisia

[c] Animal Production Unit, FAO/IAEA Laboratories, A-2444 Seibersdorf, Austria

E-mail: genevieve.libeau@cirad.fr

The Capripoxvirus genus is comprised of sheeppovirus (SPV), goatpox virus (GPV) and lumpy skin disease virus (LSDV). The three separate diseases caused by capripoxviruses (CaPVs) in sheep, goat and cattle are most economically significant in large areas in Africa and Asia. CaPVs are generally considered to be host specific leading to outbreaks in the preferential host, even if experimental infections have shown that most strains can cause disease in more than one species. Sheeppox and goatpox exhibit similar clinical signs that can be confused with other exanthemas, i.e. orf. Lumpy skin disease is a subacute to acute cattle disease.

The CaPVs are serologically impossible to differentiate. Specific identification of the genus inside the Poxviridae family relies mainly on molecular tools rather than on classical serology. In the last few years, restriction fragment pattern analysis, cross-hybridisation and more recently the complete genome sequencing of the three viruses showed that grouping of isolates correlated with the animal species from which the viruses were isolated: SPV, GPV and LSDV are phylogenetically distinguishable through conserved genes responsible for host-range [1,2,3]. For taxonomy and evolutionary studies we have worked on a non-essential gene for the virus growth encoding an homologue of a G protein-coupled chemokine receptor (GPCR) described by Cao et al (1995)[4] on the genome of Kenya sheep isolate (KS1). The Q2/3L gene, known to be located in the terminal genomic region, is likely to affect the viral virulence [3]. This poxvirus-encoded gene affects the host immune response to viral infection because of its homology to mammalian chemokine receptors. We describe here its suitability for host range phylogenetic grouping.

The sequence analysis of the Q2/3L gene of KS1 vaccine strain led to the design of PCR primers to study the relationship among 23 CaPVs strains (including 13 virulent sheep isolates and 1 sheep vaccine strain, 5 goat isolates and 1 goat vaccine strain, and 3 bovine isolates and 1 bovine vaccine strain; 5 strain sequences were obtained from genbank). Alignment analysis of both the nucleotide and deduced amino acid sequence showed that one deletion was associated to the sheep strains and another to the cattle strains while these were absent from the goat strains. A representative phylogenetic analysis using the neighbour-joining method showed 3 tight genetic clusters suggesting a co-adaptation of the strains and their original host (Figure 1). Surprisingly, the KS1 strain was closer to the LSDV strain cluster and the goat vaccine was closer to the sheep group. These vaccine strains may in fact originate from bovine and sheep respectively. In the case of KS-1, the similarity with LSDV genome was recently evidenced [4].

These results suggest that the genomic mutations that occurred in the GPCR gene account for viral adaptation to combat the immune system in a specific host. This gene provides one starting point for the understanding of the genetic basis of the CaPVs host range specificity. A particularly valuable application of the delineated primers would be their direct use for disease epidemio-surveillance and differential diagnosis.

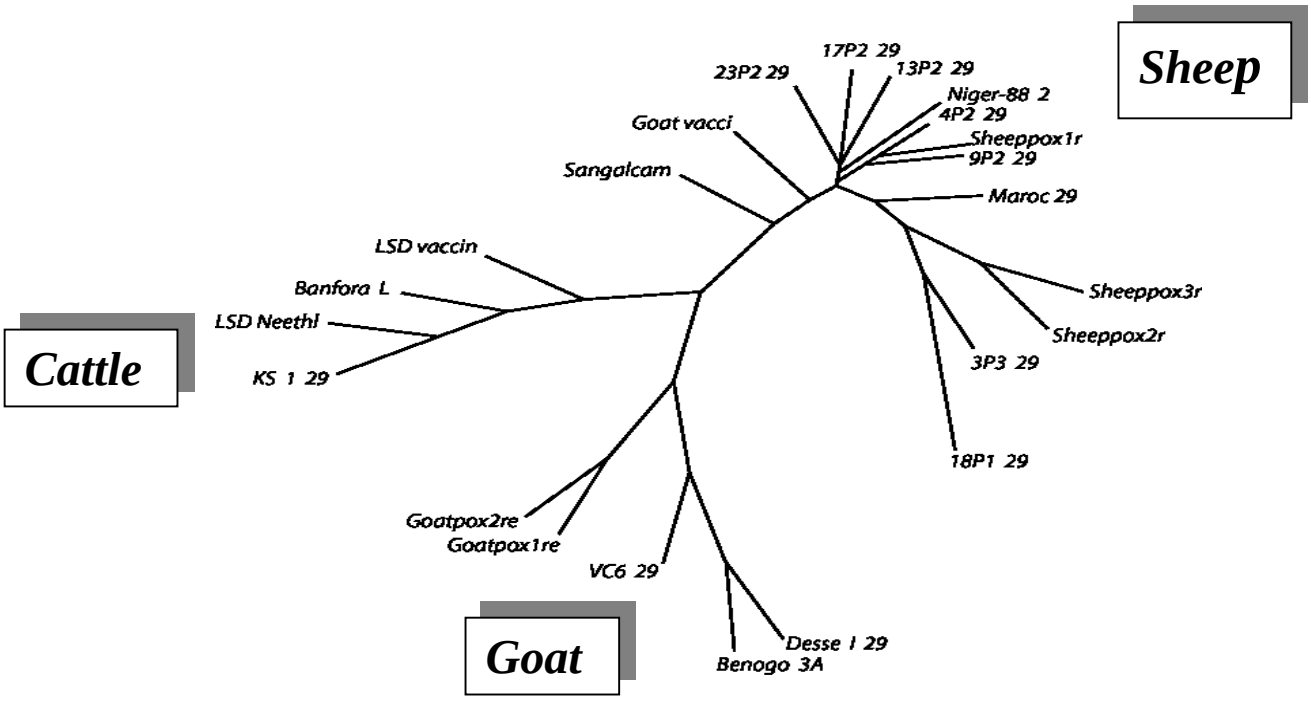

FIG.1. Phylogenetic tree of capripoxviruses based on the alignment of the GPCR gene. The alignment was performed by the DNA-DIST and NEIGHBOUR programmes of the PHYLIP.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

The use of gene-based technology to determine the prevalence of dairy and beef cattle with a natural resistance to bovine brucellosis in South Africa

L. Prozesky[a], G. Adams[b], E.H. Venter[a], T. Meiring[a], D. Verwoerd[a], P.D. van Helden[c]

[a]Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b]Texas Agricultural Experimental Station, Texas A&M University

[c]University of Stellenbosch, South Africa

E-mail: *lprozesky@op.up.ac.za*

Bovine brucellosis is a highly contagious disease caused by *Brucella abortus.* The principal manifestations of brucellosis are reproductive failure including mid- to late-term abortions or birth of unthrifty calves, infertility in cows and orchitis and epididymitis with frequent sterility in the male [1]. The disease has a high prevalence in southern Africa, especially in intensively farmed areas, and is the most important bacterial cause of abortion on the subcontinent [2].

Although cattle diseases can be partially controlled by the use of antimicrobials, pesticides, vaccines, isolation-and-quarantine, and test-and-slaughter policies, sustained losses due to infectious diseases continue to impede the livestock industry. For the past decade, the use of antimicrobials has been increasingly criticized because of the development of antimicrobial resistant pathogens and the dangers of residues in animal products used as food for human consumption. A balanced strategy in which drug use is minimized and the emphasis is placed on breeding disease resistant livestock must be considered. An area of recurrent interest in this regard is to increase the overall level of resistance in a herd by using selective breeding programs based on gene bases technologies.

"Natural disease resistance" refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization, of which the major component is heritable and, therefore, stably passed from parent to offspring. Because antibiotics cannot effectively cure bovine brucellosis, only three approaches are currently available to control the disease viz. a combination of vaccination, hygiene, and/or "test and slaughter". It was therefore decided to concentrate on the exploitation of natural resistance as a fourth important tool to control and eradicate the disease in South Africa.

It has been shown that natural resistance to brucellosis can be dramatically increased by selection and breeding of resistant animals. The frequency of natural resistance to brucellosis when challenging non-vaccinated cattle was 20%. Breeding a naturally resistant bull to naturally resistant cows increased the frequency of natural resistance in their progeny to 58.6% [3].

Bovine *NRAMP1*, the bovine homologue of the murine gene regulating the priming/activation of macrophages for antimicrobial and tumoricidal activity, has been identified as a major candidate gene determining resistance/susceptibility to bovine brucellosis and tuberculosis. It

was decided to employ a probe for this gene in South Africa to determine the prevalence of resistance in dairy cattle in an area with a high incidence of natural brucellosis in both brucella infected and brucella free herds. Furthermore, the prevalence of natural resistance was determined in a local improved beef cattle breed (Bonsmara) and compared to the prevalence in an indigenous Ancoly breed in Rwanda.

It is believed that by applying gene-based technologies to identify bovines with natural disease resistance, we can effectively reduce the incidence of brucellosis in cattle herds, providing new approaches to sustainable agriculture and safer pre-harvest foods.

Experimental work is in progress and the results will be presented at the symposium.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

The use of polymerase chain reaction for rapid diagnosis and differentiation of para- and ortho-pox virus infections in camels

A.I. Khalafalla[a], M. Buettner[b] , H.-J. Rziha[b]

[a] Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, Shambat 13314 Khartoum North, Sudan

[b] Department of Immunology, Federal Research Centre for Virus Diseases if Animals, Paul-Ehrlich Str.28, D 72076, Tuebingen, Germany

E-mail: virosat@sudanmail.net

Camels are important livestock resource uniquely adapted to hot and arid environment. Two camel diseases are of economic importance; Camel Pox (CP) caused by an orthopoxvirus and Camel Contagious Ecthyma (CCE) caused by a Parapoxvirus. Clinically CCE is indistinguishable from CP, especially when both diseases co-exist in the same locality. The current traditional methods for laboratory diagnosis of both diseases are not reliable, time consuming or not available in countries where these diseases are endemic.

Nucleic acid hybridization techniques based on the polymerase chain reaction (PCR) are now used for detection and characterization of many viruses including Poxvirus. The aim of this investigation is to develop a rapid and reliable technique to separate CCE from CP and to overcome costly and time- consuming traditional techniques.

A number of 22 CCE-suspected and 4 CP-suspected skin scabs collected from camels in the Sudan as well as 3 skin scabs collected from sheep affected with Contagious Pustular Dermatitis (Orf) were used. As a reference, MR I strain (Parapoxvirus) and Vaccinia Elstree strain (Orthopoxvirus) were included. Skin scabs were cut into small pieces and homogenized. Scab homogenates were either used directly in the PCR or purified through a 36% sucrose cushion. Viral DNA was extracted from skin scabs using a commercial DNA Isolation kit (Gentra Systems). Accordingly, 3 PCR methods were followed; 1) using DNA prepared directly from skin scabs (PCR 1); 2) scab homogenates were added to the PCR minus the polymerase and then the reaction heated at 100^{o}C as described by Ireland and Binepal [1] (PCR 2); 3) purification of the poxvirus in scab homogenate and virus pellets added to the PCR reaction and then treated as in 2 (PCR 3). In order to compare the PCR methods with traditional techniques for diagnosis, a small volume of each sample was examined by negative contrast electron microscopy (EM). Portion of the same samples were also inoculated onto bovine embryonic oesophageal cells (KOP) and Vero cells for virus isolation.

For the amplification of parapoxvirus-specific DNA by PCR, the primers and reaction conditions were used as described by Sullivan et al. [2]. For the amplification of orthopoxvirus-specific DNA were used as described by Meyer et al. [3].

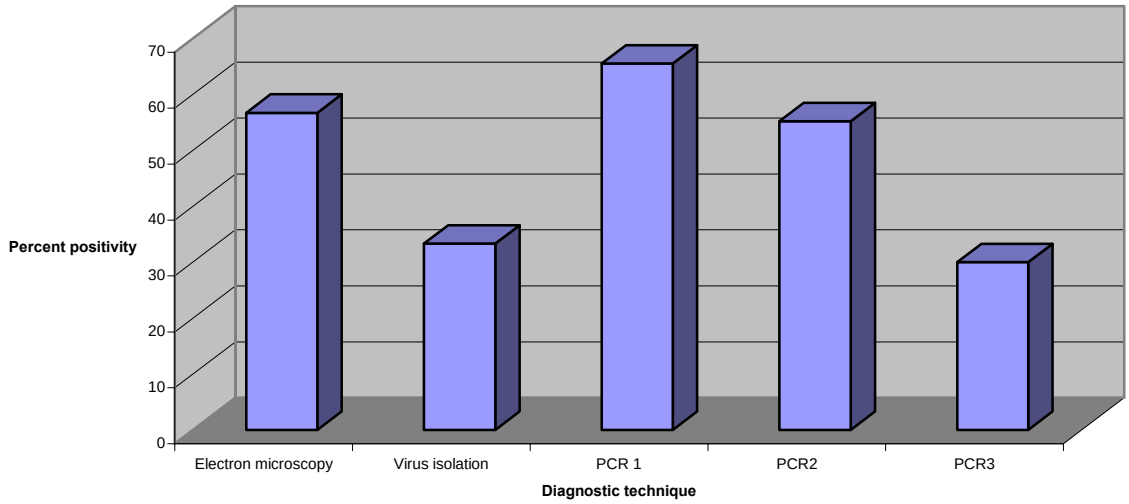

FIG.1. Percent positivity of skin scab samples tested by different diagnistic techniques

Results are summarized in Figure 1. The size of the amplification products exactly correspond to those obtained with the reference parapoxvirus (MR I strain) and the reference orthopoxvrus (Vaccinia strain). A total of 8 skin scab samples were negative by all diagnostic techniques used. Method 1 in which viral DNA was extracted directly from scab samples followed by PCR proved to be superior and more sensitive than the other two methods. The results showed that the diagnostic sensitivity of this PCR method is greater than traditional diagnosis techniques of electron microscopy and virus isolation in cell culture. Well-equipped laboratories with cell culture and electron microscopy facilities are not needed and results can be obtained within 24 h of sample receipt in small laboratory.

The PCR test described in this report is a valuable addition to the current methods for diagnosis and differentiation of para- and orthopoxvirs infection in camels.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Development of a new live rough vaccine against bovine brucellosis

D.J. Comerci[a], J.E. Ugalde[b], R.A. Ugalde[b]

[a] Grupo Pecuario, Unidad de Aplicaciones Tecnológicas y Agropecuarias, Centro Atómico Ezeiza,Comisión Nacional de Energía Atómica, Buenos Aires, Argentina

[b] Instituto de Investigaciónes Biotecnológicas, Universidad Nacional de Gral. San Martín, Parque Tecnológico Miguelete, INTI Ed. 24, San Martín, Buenos Aires, Argentina

E-mail: dcomerci@iib.unsam.edu.ar

Brucella abortus S-19 is the most commonly used attenuated live vaccine to prevent bovine brucellosis. The vaccine induces good levels of protection in cattle, preventing premature abortion. Although *B. abortus* S-19 is the most used vaccine in eradication campaigns worldwide, it has two mayor problems: (i) it produces abortion when administered to pregnant cattle and is fully virulent for humans and (ii), the presence of smooth lipopolysaccharide interferes with the discrimination between infected and vaccinated animals during immune-screening procedures.

In our laboratory we have previously cloned, sequenced and disrupted the gene coding for the enzyme phosphoglucomutase (*pgm*), responsible for the interconversion of glucose-6P to glucose-1P. The mutant does not synthesize the sugar nucleotide UDP-glucose and/or ADP-glucose and thus is unable to form any polysaccharide containing glucose, galactose or any other sugars whose synthesis proceeds through a glucose-nucleotide intermediate. The mutant has a rough phenotype, is avirulent in mice but retains the ability to multiply inside HeLa cells, although it shows a delay of the exponential intracellular replication. These characteristics prompt us to evaluate the potential use of this strain as a new live rough vaccine. We generated an unmarked deletion mutant of *pgm*. Western blot analysis of purified lipopolysaccharide from *Δpgm* indicated that it is devoid of O-antigen, however Δ*pgm* whole cell extracts contained detectable amounts of O-antigen with a MW of 45 kDa, indicating that mutant strain is able to synthesize O-antigen but incapable to assemble a complete LPS probably due to the presence of an altered core structure.

When administered intraperitoneally in Balb/C mice, the number of viable Δ*pgm* recovered from spleens were, at all tested times, significantly lower than those inoculated with the parental virulent strain S2308 and was completely cleared at 8 weeks p.i., thus indicating a severe virulence reduction. These results indicate that even at high doses, strain Δ*pgm* is cleared from the animal in a short period of time.

The antibody response against O-antigen was evaluated using Fluorescence Polarization Assay with FITC-coupled O-antigen as a tracer. Mice receiving S2308 developed antibodies to the O-antigen that reached its maximal value at 49 days p.i (152.71±27.65 mP). In contrast, Δ*pgm*-vaccinated mice failed, as the saline control mice, to elicit antibodies against O-antigen at any tested time (92.33±3.66 mP and 92.20± 7.10 mP at 49 p.i. respectively). These results indicate that the O-antigen present in Δ*pgm* is incapable to elicit a detectable specific antibody response.

It is well established that for intracellular pathogens like *Brucellae*, cellular immunity plays a central role in protection. To investigate the cellular immune response induced by Δ*pgm,* we analyzed the proliferative splenocytes response of vaccinated and non-vaccinated mice upon stimulation with heat-inactivated S2308 whole cells. At 8 weeks post inoculation, splenocytes recovered from mice vaccinated with Δ*pgm* proliferated upon stimulation in a specific manner, in contrast to the non-vaccinated control group (60,142 ± 7,443 c.p.m. *vs* 20,855 ± 2,541 c.p.m., *P<0.001*). All immunized animals responded equally to the non-specific mitogen Concanavalin A. The spleen cells from Δ*pgm* vaccinated animals were induced to secrete high levels of IFN-γ (112.0 ng/ml) after stimulation. A significant induction was also observed upon stimulation with ConA (53.8 ng/ml). In contrast, the splenocytes from PBS-inoculated control animals only released IFN-γ upon stimulation with ConA. IL-4 was not detected in the supernatants of splenocytes obtained from both immunized and non-immunized animals. These results indicate that vaccination with Δ*pgm* induce a classical ThI cellular proliferative response.

In order to examine the protection induced by Δ*pgm,* a vaccine-challenge experiment was performed. Groups of five mice were vaccinated intraperitoneally with 10^7 CFU of Δ*pgm,* saline or 10^5 CFU of B19. Eight weeks post-vaccination animals were challenged with 10^5 CFU of strain S2308. Protection was defined as the difference between the numbers of viable bacteria recovered from spleens of immunized mice compared to those receiving saline. Vaccine efficacy was expressed as $\log_{10}$ units of protection. Δ*pgm* generated a significant protection 2 and 4 weeks post challenge, with 2.25 and 1.93 protection units, respectively. As expected *B. abortus* strain S19 induced also a significant protection at 4 weeks (1.78 protection units).

These results, together with the ability of the mutant to generate a strong cellular Th1 response without eliciting specific O-antigen antibodies, emphasize the potential use of this mutant as a new live vaccine for cattle.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Molecular characterization and phylogenetic study of NDV isolates from recent outbreaks in Uganda

M.O. Otim[a], M. Bisgaard[b], H. Christensen[b], P. Jorgensen[c], K. Handberg[c]

[a] Livestock Health Research Institute, LIRI, P. O. Box 96, TORORO

[b] Institute of Veterinary Microbiology, The Royal Veterinary and Agricultural University Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark

[c] Danish Veterinary Institute (DVI), Hangøvej 2, DK-8200 Århus N, Denmark

E-mail: *moo@kvl.dk*

Molecular techniques were used to characterize sixteen Newcastle Disease (ND) Virus (NDV) isolates from ND outbreaks in chickens in Uganda in 2001 and to evaluate ND epidemiology. Virus isolation and was made out in SPF eggs. RNA was extracted from the virus isolates using QIAgen kit.

Reverse and forward primers covering the cleavage site of the fusion (F) protein gene and a specific HN gene segment were designed and used in a single tube Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

The resultant complimentary DNA fragment products were visualized on agarose gel and extracted using QIAgen kit.

The purified amplified F and HN gene products were purified by microspin column tehnique. The PCR primers were used to sequence the products in BigDye Terminator cycle sequencing.

The cycle sequencing products were precipitated by ethanol/Na-acetate method and loaded onto acrylamide gel for analysis in the ABI 377 automated DNA sequencer (Perkin Elmer Applied Biosystems).

Comparative genetic and phylogenetic tree analyses were performed on the HN genes of the isolates and 17 NDV strains selected from the GenBank. ClustalX 1.81 and phylip software were used for gene alignment and the final phylogeny was produced by neighbour-joining method.

Results showed that all the Ugandan NDV isolates were closely related. F gene cleavage site sequence analysis had the amino acid sequence ^{112}RRQKRF^{117} at the C-terminus of the F2 protein and F (phenylalanine) at residue 117. Figure 1 shows the amino acid sequence at the C-terminus and around the cleavage site.

There is therefore a pair of basic amino acids R, arginine and K lysine at residues 116 and 115 respectively and a phenylalanine, F at residue 117 as well as a basic amino acid R, arginine at residue 113 indicating a high virulence [1,2] for the NDV isolates from Ugandan ND outbreaks.

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            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|

                     70         80         90        100        110        120

Pal/03/05    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/04/07    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Sor/05/09    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/04/06    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/01/03    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/02/08    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/04/08    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATASG GRRQKR↓FVGA

Pal/02/06    -IVKLLPNMP KDKEACARAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/03/21    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/01/02    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/04/05    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Sor/06/09    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Sor/01/04    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Sor/08/14    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Pal/05/15    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA

Tor/02/22    -IVKLLPNMP KDKEACAKAP LEAYNRTLTT LLTPLGDSIR RIQESATTSG GRRQKR↓FVGA
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FIG.1. Alignment of predicted amino acid sequences surrounding the fusion protein cleavage site. Basic amino acids are shown in bold. All the 16 Ugandan NDV isolates have the same amino acid residues RRQKRFVG around the cleavage site. ↓ indicates cleavage site.

The ^{112}RRQKRF^{117} motif is majority/consensus sequence around the F2/F1 cleavage site.

All the NDV isolated from Uganda were highly virulent and none of them was related to the vaccine strain.

Upon phylogenetic analysis, all isolates formed separate clades from the currently known 8 genotypes suggesting that they are a novel genotype, unrelated to those that have caused previous outbreaks worldwide.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Gene discovery in *trypanosoma vivax* through GSS and comparative genomics

A.M.R. Dávila, L.T.A. Guerreiro, S.S. Souza

Instituto Oswaldo Cruz, Rio de Janeiro, Brazil

E-mail: davila@fiocruz.br

Trypanosoma vivax is a hemoparasite affecting livestock industry in South America and Africa [1,2]. According to Seidl et al [3] more than 11 million cattle evaluated in more than 3 billion dollars are found in the Pantanal region of Brazil and other lowlands in Bolivia. According to the same authors, if the outbreak reported in Poconé-MT (Center-East of Brazil) had gone untreated, the estimated losses would have exceeded US$140,000 on the seven ranches, $200 million in the Pantanal and $700 million regionwide.

Despite the high economic relevance of the disease caused by *T. vivax*, few researches on its molecular characterization has been made as compared with human trypanosomes as *T. brucei* spp and *T. cruzi*. The main reason is the difficulty to grow the parasite into laboratory rodents and "*in vitro*". Very few (West African) strains have been adapted to laboratory rodents. Furthermore, most field isolates cannot be characterized by tools as RAPD, since parasitemias are usually very low making difficult the separation of parasites from animal blood for posterior extraction of parasite DNA [4]. These characteristics have limited the research on *T. vivax* during the last decades, consequently very few markers have been described for its molecular characterization. A search in Genbank showed that there are only 22 entries for *T. vivax* confronted with nearly 98289, 38577, 23507 available for *T. brucei*, *T. cruzi* and *Leishmania*, respectively. *T. vivax* (molecular) biology is also little understood, even considering major differences as mechanical transmission in South America and both cyclical and mechanical transmission in Africa.

In a consultation with several experts on genomics, it was emphasized that *T. vivax* and *T. congolense* are underepresented species in the molecular parasitology and genomics age, then they should be considered to have their genome sequenced [5]. In order to discovery new markers to be explored in the molecular characterization of *T. vivax*, we decided to do the sequencing of the ends of 80 GSS. For that purpose a semi-normalized genomic was constructed. Basically, *T. vivax* genomic DNA from the ILDat2160 cloned stock (kindly provided by Dr. Noel Murphy) was partially digested with Sau3A restriction enzyme, cloned into the BamHI site of the pUC18 plasmid and transformed into *E. coli* DH5α. The library was then probed with the major repetitive regions described for *T. vivax* in the literature: mini-exon, 18S, 5.8S, satellite DNA and a gene coding for an antigen. Colonies that were negative with the probes were selected and checked for inserts. From colonies presenting inserts size of 1.5-3Kb, 80 were randomly selected to have their ends sequenced.

Our preliminary results show that ~ 30% of the sequenced GSS has no significant similarities to sequences in the Genbank, representing potential species-specific markers. *vivax*. Once the ends of the 80 GSS have been sequenced, some markers will be chosen to obtain their complete sequences, and explored for the molecular characterization of *T. vivax*.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Pathogenicity and immunogenicity of the reassortant attenuated strain R566 of Rift Valley fever virus in sheep

Y. Thiongane[a], P. Lena[b], M. Moustapha Lo[a], B. Sall[c], V. Martin[d], P. Formenty[e], P.Vialat[f], M. Bouloy[f]

[a] Institut Sénégalais de Recherches Agricoles, BP 2057, Dakar, Senegal

[b] INRA Rétrovirus et Pathologie Comparée, 50 Av T. Garnier 69007 Lyon, France

[c] Direction de l'Elevage, 67 Avenue Pasteur, Dakar, Senegal

[d] Food and Agriculture Organization (FAO), Animal Health Service, Viale delle Terme di Caracalla, Rome, Italy

[e] World Health Organization, 20 avenue Appia, Geneva, Switzerland

[f] Unité de génétique moléculaire des bunyaviridés, Institut Pasteur, 25 Rue du Dr Roux, 75724, Paris cedex 15, France

E-mail: thiongane@sentoo.sn

Rift Valley Fever (RVF) is an emerging zoonosis producing exploding outbreaks in ruminants, and humans cases, in Africa, Madagascar and the Arabic Peninsula. The Rift Valley Fever Virus (RVFV) is a *Phlebovirus* which possesses a single strand RNA genome composed of 3 separate segments, allowing the generation of reassortants by exchange of segments between parental strains. Here we show that the reassortant strain R566, selected to accumulate attenuation markers on all 3 segments, is both immunogenic and non-pathogenic after experimental inoculation to Sahelian sheep.

Increasing doses of the R566 strain, ranging from 10^1 to 10^6 plaque forming units (pfu) were administered subcutaneously to six groups of four animals kept in a mosquitoes-proof facility in Senegal. As low as 10^4, and higher doses, was enough to induce RVFV neutralizing antibodies (Nab) titers higher than 1/10 in all four animals. These Nab became detectable 21 days after inoculation, whereas no clinical signs were detected with any of these doses. Three non-inoculated animals kept in contact as sentinels remained seronegative during the all observation period, indicating that seroconversion was the result of R566 inoculations.

In order to examine the pathogenicity of the R566 strain, the minimal dose inducing Nab, i.e. 10^4, was inoculated to seven pregnant ewes during the first, second, third and fifth month of gestation. This vaccine regimen was immunogenic, non pathogenic, and non abortogenic in all 7 animals.

All the pregnant ewes which received the R566 attenuated strain delivered healthy lambs and developed RVFV Nab titres>1/10, three weeks after inoculation. Nab titers decreased progressively below 1/10 after an average period of 120 days. All three lambs born from ewes inoculated at the fourth and fifth month of gestation quickly acquired colostral antibodies.

Our study highlight the safety and immunogenicity of the live attenuated strain R566. These properties make R566 a promising veterinary vaccine candidate against Rift Valley Fever. Further investigations are urgently required to establish the minimal dose providing protection to animals challenged with a virulent strain of RVFV and the duration of the protection. Moreover, this future study should consider the genetic stability of the vaccine after *in vivo* passage in sheep and mosquitoe vectors transmitting naturally the disease.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Comparison of immunocapture and RT-PCR techniques for the detection of peste-des-petits-ruminants virus (PPRV) in eye and nose swabs from infected animals.

D. Sy[a], K. Tounkara[b], A. Diallo[c], G. Libeau[d], A. Diarra[a], B. Kamissoko[a], A.P. Traore[a], S. Sidibe[a], C.F. Simbe[a]

[a] Laboratoire Central Vétérinaire, BP2295, Bamako, Mali

[b] OUA/IBAR/PACE, P.O. Box 30786, Nairobi, Kenya

[c] Animal Production Unit, FAO/IAEA Laboratories, A-2444 Seibersdorf, Austria

[d] CIRAD-EMVT, Programme Santé Animale, TA 30/G, Campus International de Baillarguet, 34398 Montpellier, Cedex 5, France

E-mail: dieneba_sy@hotmail.com

Peste des Petits Ruminants (PPR) is a highly contagious disease of domestic and wild small ruminants. It constitutes a major constraint on production in areas where it is endemic. Classically, it is characterised by fever, nasal and ocular discharges, diarrhoea, respiratory distress, mucosal erosive lesions and death in 40-80% of acute cases. All these clinical signs, apart from the respiratory symptoms, are very similar to those of rinderpest (RP). The causal agents of both diseases are viruses which belong to the *Morbillivirus genus.* Described for the first time in 1942 in Côte d'Ivoire [1], PPR was considered for a long time as a disease of West African countries. The current knowledge on its epidemiology shows that this is no longer true, nor it is a solely African disease since it is widespread in countries lying between the Sahara and the Equator, in the Middle East and in South-West Asia [2]. These data indicate that PPR existed undetected in most of the known endemic areas for a long time. It was overlooked because of the similarity of clinical signs to rinderpest as indicated above and also to pasteurellosis for the bronchopneumonia. The current knowledge of the disease has grown up quickly once specific diagnostic tests became available in the 1990's: serological diagnosis in the competitive format [3] or for antigen detection by immunocapture [4] cDNA probe [5] and the amplification (RT-PCR) technique for the nucleic acid detection [6]. While the immunocapture (ICE) can detect virus up to $10^{0.6}$ TCID50 of virus in 50µl of sample, the limit of detection of the RT-PCR is estimated to 0.001 TCD50/ml.

During a study to analyse the pathogenicity of some PPRV isolates, we have compared the efficiency of the ICE test, antibody-based antigen detection, and RT-PCR, with the RT-PCR, nucleic acid-based detection technique, to detect the shedding of the virus by the infected animals.

For the study, Sahelian goats were inoculated IM with PPRV Guinea (isolate from Guinea Conakry) or PPRV C.I. M7 (isolate from Côte d'Ivoire). For the PPRV Guinea strain, the virus was detected by ICE in the nasal and lachrymal swabs from day 1 to 6 after infection. However, the RT-PCR, it was possible to demonstrate the excretion of the virus in the swab until day 8 after infection. With the PPRV CI M7, no virus was detected by ICE in swab after 2 days. But the RT-PCR, virus excretion could be demonstrated up to day 4 post-inoculation.

In conclusion, our study has demonstrated that goats can excrete the PPRV very soon after infection by intrasmuscular route. Apparently the amount of the excreted virus is depending to the strain and the time after infection. The RT-PCR can detect the virus excretion in a longer time than can do the immunocapture technique.

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TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

Gene-based technology on characterisation of a avirulent thermostable vaccine strain I-2 of Newcastle disease *v*irus used in rural areas of developing countries

P.N. Wambura, H.M. Msami

Animal Diseases Research Institute, P. O. Box 9254, Dar Es Salaam, Tanzania

E-mail: *cvltemeke@twiga.com*

I-2 is an avirulent, thermostable strain of Newcastle disease virus (NDV) of Australian origin, used as a vaccine for village-based flocks of chickens in developing countries. There have been many studies on the biological properties of strain I-2, but none until now on the molecular basis of these properties. A single tube RT-PCR technique was conducted and generated 387 bp and 300 bp cDNA fragments encoding a portion of F_0 gene of NDV strain I-2, respectively. The amplicons were directly sequenced by the dye-terminator cycle method. The resulting nucleotide sequences were used to deduce amino acid sequences surrounding the cleavage site of F protein. This was compared with sequence for other NDV strains. The cleavage-activation site of strain I-2 had a pair of basic residues followed by a single basic residue with an intervening glutamine (^{112}**R**KQGR^{116}) whereas the corresponding site of other avirulent strains has a sequence of ^{112}**G**KQGR^{116}. The obtained sequence motif for strain I-2 was unique amongst the lentogenic strains of NDV by having a substitution of arginine (R) for glycine (G) at position 112 at the C-terminus of F_2 protein. At the N terminus of F_1 protein, strain I-2 had a sequence pattern of ^{117}LIG^{119}, which was similar to other lentogenic strains and not the ^{117}FIG^{119} motif of virulent strains. On the basis of these findings, it was concluded that strain I-2 is an avirulent strain having a non-basic residue (position 115) at the cleavage activation site and leucine (L) at position 117 of the N-terminus of the fusion inducing domain. It is noteworthy that the sequence pattern of strain I-2 at the cleavage activation site was different from that of emerging virulent strains causing clinical outbreaks of Newcastle disease (ND) in Australia. The determination and analysis of amino acid sequences provides useful information on NDV. This information may contribute towards the understanding of molecular epidemiology of ND clinical outbreaks and determination of the suitability of using strain I-2 as a vaccine to control ND.

TOPIC: Gene-based technologies applied to pathogens and host-pathogen interactions

The CENTAUR network contribution to the gene-based technology: Dissemination of information, international collaboration and training

K. Hruska, K.J. Wojciechowski

Veterinary Research Institute-VRI, Brno, Czech Republic

E-mail: hruska@vri.cz

The CENTAUR Network (FAO Established Veterinary Biotechnology/Epidemiology Network for Central and Eastern Europe) is designed for cooperation and information of workers in state and regional veterinary directorates, veterinary practitioners, teachers from veterinary schools, advanced students, politicians specialized in agriculture, economy, science management, workers from diagnostic and research laboratories, breeders and other interested parties. It contributes to the use of the Internet, to the improvement of communication in English, to deepening of co-operation between workers with similar interests and to the better link with international organizations and internationally recognized centres of excellence. Formally, 23 countries officially belong to the CENTAUR network. However, in reality nearly one thousand CENTAUR network members are from the all continents. The membership is free and everybody can profit from all information offered.

The CENTAUR is creating a protective belt for Central and Eastern European Countries especially those with the "economies in transition" regarding the devastating Transboundary Animal Diseases such as bluetongue, foot and mouth disease and other vesicular diseases, rinderpest, peste des petits ruminants, contagious bovine pleuropneumonia, sheep and goat pox, classical swine fever, African swine fever, BSE/TSE, as well as other priority diseases and supports the prevention of a possible risk of biological terrorism. CENTAUR is based on the voluntary cooperation of countries concerned. The main task is to achieve compatibility of the disease control standards namely for better disease and contamination diagnosis and vaccine production, the modern computerized veterinary epidemiology and food safety and consumer protection with the OIE and EU international requirements. It is contributing to the accession of 8 Eastern European countries to the EU in 2004 and some other countries at a later stage. CENTAUR maintains in continuous link with the FAO Animal Health Related Networks like EMPRES, RADISCON, EUFMD, FAO Library, Rome and the AGROWEB and WAICENT Networks.

Biotechnological Component was created during preliminary 1991–1995 FAO consultancy missions and training meetings in Czech Republic, Hungary, Slovakia and Poland and the FAO Technical Cooperation Programme Project TCP/RER/4551 (T): 1995–1997. An extension of the CENTAUR network should take place to cover Balkan, Baltic and other Eastern European Countries as Ukraine, Byelorussia, and Russia.

Epidemiological Component. The FAO Assessment Project Formulation Mission to Croatia, Slovenia, Romania and Bulgaria took place in January 1999. It showed (similarly to the Biotechnological component) a lack of compatibility with the OIE and EU standards of infectious diseases control. The new TCP Regional Project: TCP/RER/0066 (T): 2001–2003 has been implemented to cover Albania, Bosnia-Herzegovina (the Federation and Republika Srbska), Bulgaria, Croatia, FYR Macedonia, Moldova, Romania, and Turkey. The consultancy missions were implemented to Moldova and Bosnia and Herzegovina. The

workshops in Albania, Turkey and FYR Macedonia promoted: computerized epidemiology TAD info epidemiological software and application of molecular epidemiology.

Food Safety and Consumer Protection Component (initiated by VRI, Brno since 2002) contributes to harmonization of the legislation, improve the technology both for production and distribution, the control of food borne diseases using the molecular methods, the control the pollution of the environment, prevention of contamination of feeds and food, and improvement of animal health and welfare.

Dissemination of Information available from the FAO, FAO-IAEA, OIE, EU, WHO, and other different sources using e-mail messages distributed to the 27 field specific groups of free-registered network members worldwide takes place through the VRI, Brno. About 500 informations are issued annually, listed in an electronic bulletin CNFI (CENTAUR Newsletter Flash Information) covering gene-based technologies and molecular methods applied in biotechnology for better diagnostics, vaccine production, application of molecular epidemiology, food hygiene and consumer protection, emergency diseases and bioterrorism, veterinary administration, education, epidemiology and scientific information. CENTAUR website offers also news, basic information for the network members, on-line free registration, archive of information distributed with instant updating, papers and articles, reports, reviews and opinions, biographies of the esteemed persons in the field, supercourse PowerPoint lectures and internet workshops (i.e. on Good Research Practice). The close cooperation was established with the FAO David Lubin Memorial Library, Rome. The cooperation with related AGROWEB and WAICENT Networks as well as with the SEUR FAO Sub-regional Office in Budapest was initiated by the VRI Mission in 2002.

Towards Extension of the CENTAUR. The feasibility study mission also related to the extension of CENTAUR took place in June 2001 to Lithuania, Latvia, Estonia and St. Petersburg (Russia). In May/June 2003 during the Mission to Ukraine new institutions and scientists in Russian Federation, Byelorussia, Ukraine and Tajikistan were identified. The missions were implemented in cooperation with Warsaw Agricultural University-WAU. For the geographical integrity Serbia and Montenegro as well as the EU Countries: Finland and Greece are invited to join CENTAUR.

Catalytic role of CENTAUR. contributed to establishing of the Interfaculty Studium of Biotechnology at the WAU, Poland, exchange of scientists, cooperation among biotechnologists in the regions, organization of the annual international Biotechnology Seminars (since 1998) by the Warsaw Agricultural University and upgrading of university curricula. It has been promoting biotechnology as the key tool for reduction of famine and malnutrition in the world (International Dialogue & Universalism Conferences/Congresses in Poland, by the Institute of Philosophy, Warsaw University, 2003), regular exchange of scientific information, training, scientists and links with International Organizations and Centers of Excellence.

CENTAUR web page (news, registration, information, newsletter, articles, reports, index, who's who, supercourse: http://centaur.vri.cz