Activities of National Reference Laboratories (NRL’s)

Introduction

In 2006 following the designation of a number of additional Community Reference Laboratories (CRL’s) by EU, Member States were required under Article 33 of Regulation 882 / 2004 to designate one or more National Reference Laboratory (NRL) for each CRL. The Departments of Health and Children and Agriculture and Food, as the Irish Competent Authorities, assigned these NRL functions to a number of laboratories including those within the Backweston Laboratory Campus.

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Article 33 of EC Regulation 882/2004 on Official Controls tasks NRL’s with many duties including:

- coordinating, for their area of competence, the activities of official laboratories responsible for the analysis of samples;

- where appropriate, organising comparative tests between the official national laboratories and ensuring an appropriate follow-up of such comparative testing;

- ensuring the dissemination to the competent authority and official national laboratories of information that the Community Reference Laboratory supplies;

- providing scientific and technical assistance to the competent authority for the implementation of coordinated control plans;

- being responsible for carrying out other specific duties provided for in accordance with the procedure referred to in Article 62(3), without prejudice to existing additional national duties.

Official microbiological controls on food across the production chain are undertaken by laboratories under both the control of the Department of Agriculture, Fisheries and Food and the Department of Health. While there has been ongoing interaction and exchanges in information between all the laboratories, it has been recognised that some formal coordination was desirable. The FSAI organised this meeting and presentations were given by each of the sectors outlining the work done in the various laboratories, the data generated and the technical and other issues that needed to be addressed. It was agreed that the NRL’s would undertake additional coordination of test methods used for food safety controls and the proficiency testing used by laboratories. Participants agreed that coordination meetings should be held annually.
NRL Parasites

Update on Echinococcus multilocularis and proposals to harmonise EU Regulation 998/2003 on pet travel

Echinococcus multilocularis is a fox tapeworm and among the world’s most lethal zoonoses. Although foxes are the maintenance host, dogs are highly susceptible and appear to be the main source of infection for humans in endemic areas. Humans are aberrant hosts for the metacestode stage of the parasite. Once an intermediate and aberrant host ingests the tapeworm eggs, the oncospheres migrate via the circulatory system to the liver where they develop into multilocular or alveolar cysts. Alveolar echinococcosis in humans is of considerable public importance because mortality can be up to 100% if undiagnosed or untreated. The infection has a long incubation period (circa 5-15 years) and clinical symptoms, which are invariably vague and not typically related to the disease, occur in the late phase when the parasite has already infiltrated a large part of the liver. There have been four cases human echinococcosis diagnosed in Ireland in recent years, two in 2008, one in 2009 and one in 2010. It is believed that the affected individuals were non-nationals.

The parasite is found throughout the northern hemisphere including Germany, France and Switzerland and has spread to Belgium, Czech Republic, Italy, Luxembourg, The Netherlands, Poland, Slovak Republic and Hungary. It is thought that the spread of E. multilocularis is due to foxes migrating from endemic zones. The main risk factor associated with human infection in endemic areas is a high prevalence of the parasite in both wildlife and domestic pets. It is estimated that there are at least 150,000 foxes in Ireland and previous surveys have found no indication that the parasite is present in the country.

The absence of this parasite in Ireland and some other EU Member States (MS) was recognised in EU Regulation 998 / 2003 which allowed countries free of the parasite (Ireland, UK, Sweden, Finland, Malta) apply more stringent national rules on pet imports. As the EU Commission has been anxious to harmonise the rules for pet movements it was necessary for Ireland and the other MS requesting additional controls on pet imports to undertake a surveys of foxes for evidence of the parasite. These surveys were undertaken by the DAFF veterinary laboratory service in 2009 and 2010 and involved examination of the intestines of almost 600 foxes to give a 95% confidence limit for identifying one positive animal in a population of over 100,000 at a disease prevalence of 1%. The sedimentation and counting technique, considered the “gold standard”, was selected as the method of choice with a sensitivity and specificity of 100%. Following stripping of the intestinal mucosa the intestinal contents were suspended in phosphate buffered saline and subjected to four cycles of washing and sedimentation after which the cleared sediment was examined in aliquots of 5-10ml. The study results confirmed the absence of E. multilocularis from foxes in Ireland.

A new regulation permitting the pre-movement treatment of dogs travelling to listed MS claiming an Echinococcus - free status will apply from 1 January 2012. Before travel to one of the four MS currently listed – Finland, Ireland, Malta and the United Kingdom – dogs will have to receive a specific treatment administered by a veterinary surgeon which must be documented in the animal’s passport. The animal will then be able to travel from 24 hours to five days after the treatment. To remain on the list of Echinococcus - free countries, MS must introduce surveillance programmes and report the findings to the Commission once a year.

NRL Residues of Veterinary Drugs

Report on the Annual Workshop of the EURL for Residues of Veterinary Drugs, Berlin, Germany, 3rd - 6th May 2011

NRL Representative: Maryjean Shanahan

The aim of this workshop was to discuss various applications for residue analysis and performance criteria for multi-residue analytical methods, review proficiency test results, discuss laboratory accreditation and flexible scope, evaluate national monitoring plans and evaluate hair as a matrix for the determination of B-agonists. Current topics at EU level were also outlined. The 4-day workshop was divided into both practical and theoretical parts with a total of 35 participants from EU NRLs and 4 participants from candidate and affiliated countries in attendance.

Wolfgang Radeck (EURL) gave an overview of 2010 National Residue Control Plans (NRCPs) and 2009 results for group A5 substances (B-agonists). He noted that the absolute minimum requirements for monitoring B-agonists are clenbuterol, brombuterol, ractopamine, zilpaterol and salbutamol; however isoxsuprine should also be included due to the occurrence of non-compliant results in recent years. In 2009 there were two non-compliant bovine samples, one each for isoxsuprine and clenbuterol. The mean number of B-agonist compounds being monitored by MS is 12.7. Urine and liver are the matrices which are mostly screened and if hair is screened on-farm it should always be examined in conjunction with another matrix due to the difficulties differentiating between external contamination and misuse. Lung has been introduced as a new substitute matrix for B-agonists in one MS and possibly should be considered by others as studies have shown its persistence in this matrix which is longer than in other tissues. The majority of
official laboratories are using LC-MSMS (liquid chromatography-tandem mass spectrometry) technology for screening and confirmatory analyses. Finally he suggested that screening methods used in the past are only suitable to a limited extent.

Thijs Meijer (NRL, The Netherlands) presented the results of an animal study to investigate the possible source of isoxsuprine residues in the hair of cows and their calves. Isoxsuprine is a registered veterinary medicine in the EU and can be used for relaxation of the uterus during caesarean sections in cows. However there is also the potential for it to be used as an illegal growth promoter and previously it had been detected in the hair of veal calves. This study concluded that isoxsuprine treatment of cows prior to a caesarean section can result in residues in cow's hair until 3 months at levels up to 50µg/kg and calf hair until 2 months of age at levels up to 4µg/kg. Future work will explore its persistence in milk.

Jack Kay (VMD, UK) hosted a discussion group on the establishment of appropriate performance criteria for multi-residue analytical methods. Commission Decision 2002/657/EC, concerning the performance of analytical methods, applies to single analyte methods only and the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) have established an electronic working group to draft performance criteria for multi-residue analytical methods. It is hoped that this guidance, when finalised, will be incorporated into CD 2002/657/EC. Questions posed included whether the current 5% false positive / false negative criteria, and performance standards for trueness, precision and ion intensities, for single analyte methods could be safely extended to multi-residue methods. It was concluded that multi-residue laboratory data is required to answer these questions.

Joachim Polzer (EURL) hosted a discussion group on laboratory accreditation and the approval process. He outlined the legal basis for accreditation, Regulation (EC) No 882/2004 and Regulation (EC) No 765/2008, outlined the purpose of the European Co-Operation for Accreditation (EA) and explored the concept of flexible scope according to ISO/IEC 17025. Historically accreditation has generally been based on fixed scopes of accreditation which provides for a certain degree of flexibility as limited extensions to the scope can be done at any time throughout the assessment cycle. In 2008 the EA published its requirements for the accreditation of flexible scopes which allows methods to be added within defined limits of the scope on the basis that the competency of the laboratory to develop and validate methods has been positively evaluated. A survey by the EA confirmed that most accreditation bodies (ABs) offer accreditation on flexible scopes. However this approach does not necessarily result in a reduced cost and can be demanding initially and on an ongoing basis. Flexible scope is more suitable for activities where changes are frequent. It was suggested that guidance from the EA on which accreditation approach to take could help laboratories, however it was decided that it is better for each laboratory to discuss with their respective ABs in order to make the best decision. Finally it was agreed that flexible scope offers a solution in emergency situations, e.g. during a food crisis, when there is time pressure for an accredited laboratory to have reliable results and should be mentioned in the EA guidance document on the accreditation of flexible scopes.

The practical session finished with practical work in small groups in which a multi-residue method for the determination of B-agonists in hair was demonstrated by Beate Matthes (EURL). Washing and extraction steps were performed on 1 gram of uncut hair and the importance of not discarding the combined ethanol and water phases during washing was highlighted. A complete method procedure was circulated including details of instrument conditions required for LC-MSMS, HPLC-MS (high performance liquid chromatography-mass spectrometry) and HPLC-TOF (high performance liquid chromatography-time of flight).

Petra Gowik (EURL) opened the theoretical session by outlining topics for discussion e.g. NRCP evaluations, proficiency test (PT) evaluations, nitroimidazoles in feed, and hair testing for residue control etc. The planned EURL 2011 work programme was also presented e.g. long-term stability studies for all substance groups, research and identification of unknown compounds, optimisation of a method for diclofenac (non-steroidal anti-inflammatory drug - NSAID) in milk, development of multi-residue methods by LC-TOF (liquid chromatography-time of flight) etc. It was noted that the activities of the EURL/NRL Berlin over the previous 12 year has involved method development, training courses, and supply and shipment of standards.

Frank Swartenbroux (DG Sanco) spoke about the developments in the field of residue control and noted that work on the review of Council Directive 96/23/EC is ongoing. He stated that risk-based monitoring has increased the workload for some MS, as in addition to completing their annual NRCP numbers, sample numbers can increase where specific risks are identified. He questioned the need for the establishment of reference points for action (RPAs) for all banned compounds, instead suggesting that it should only be necessary in order to ensure the functioning of controls. The establishment of RPAs was also explored in terms of linking them with maximum residue limits (MRLs) and minimum required performance limits (MRPLs). It was concluded that there is plenty of work to be carried out on implementing measures for residue control.

Dr. Frank Hamann (EURL) reviewed the application of hair testing for residue control in food-producing animals. The major practical advantage of hair testing compared to urine testing for drugs is its larger surveillance window i.e. whereas urinalysis provides short-term information on an individual’s drug use, long-term histories are accessible through hair analysis. The hair sample should be taken as close to the skin as possible at this...
is the region of least variation in growth rate and should be stored in the dark at room temperature. Washing of hair is important as it not only removes contamination but helps identify those that are externally contaminated as distinct from those that are positive due to administration. He concluded that screening of hair works as it offers the broadest detection window (≥6 months) and should be combined with a matrix for confirmation with a comparable window of detection e.g. retina.

**Dr. Kathrin Schmidt** (NRL, Berlin) presented a method for the analysis of steroid esters in bovine hair. The occurrence of natural hormones e.g. testosterone, oestradiol, boldenone, nortestosterone can present a problem when discriminating between endogenous origin and illegal treatment, and also when establishing threshold values. Steroid esters (synthetic in origin) may be administered to cattle by injection or pour-on treatment. Due to rapid hydrolysis they are hardly detectable in urine and plasma but may be incorporated into hair and their detection in hair confirms illegal treatment with natural hormones. In conclusion the use of hair as a matrix for testing is a promising tool to confirm illegal treatment with natural hormones as they can be detected up to months after treatment; however the location of hair sampling relative to the injection site seems to be critical.

**Joachim Polzer** (EURL) discussed the development and validation of a suitable method for the determination of nitroimidazoles in feed. Pigs and poultry are the target species for nitroimidazole treatment and as all nitroimidazoles are banned or not licensed, cross contaminations are highly unlikely. The LC-MSMS method developed was shown to be suitable for 8 compounds, however further optimisation is required as one compound, carnidazole, could not be detected reliably. He also evaluated 2010 NRCPs noting that most MSs employ multi-residue methods for the detection of nitroimidazoles; however some countries are not achieving the recommended concentrations or are using inappropriate matrices. Despite the recommended matrix for poultry being plasma or retina an evaluation of the 2010 NRCPs revealed that 7 MSs use liver or muscle as matrix which makes it difficult to reveal a misuse of nitroimidazoles. He concluded that MSs should pay special attention to pigs and poultry, including eggs, due to the highest misuse potential which can be expected. The misuse potential in bovine, ovine and caprine, including milk, can be assumed to be lower and for aquaculture some misuse can be assumed as they can be used in ornamental fish. The absolute minimum requirements for monitoring nitroimidazoles are dimetridazole, metronidazole, ronidazole and the hydroxyl metabolites, HMMNI and MNZOH in porcine, poultry and eggs.

**Wolfgang Radeck** (EURL) introduced the new limits, maximum residue limits (MRLs) and maximum levels (MLs), and related regulations which are now in place for coccidiostats both as feed additives in ruminant growth promotion and veterinary drugs in the treatment of coccidia. He noted that although Regulation 124/2009/EC states that foodstuff shall not be placed on the market where it contains a coccidiostat at a level exceeding the established ML, the finding of a significant value below the ML should trigger investigations to confirm it is present as a result of unavoidable carry-over and not illegal administration. Directive 2009/8/EC establishes MLs for unavoidable carry-over in non-target feed. MLs are not to be considered as MRLs but are based on an EFSA evaluation and performance of analytical methods. When evaluating 2010 NRCPs he highlighted that some MS mistake MLs for MRLs. The EURL absolute minimum requirements for monitoring coccidiostats are diclazuril, lasalocid, maduramycin, monensin, narasin, nicarbazin, robenidine and salinomycin, and were covered completely by 16 MS in 2010. Finally a review of results from 2005-2009 showed nicarbazin to be the most commonly detected coccidiostat and the majority of MS are using LC-MSMS technology for screening and confirmation.

An evaluation of the 2010 EU-RL proficiency test for NSAIDs in milk was presented by **Manfred Stoyke** (EURL) and showed that 29 out of 40 laboratories did not participate successfully due to a failure to include the basic NSAIDs. All laboratories used LC-MSMS technology and overall results of the PT study concluded that some laboratories still show a need for development as regards training and use of suitable analytical methods. An evaluation of MS NRCPs for NSAIDs noted that selecting the appropriate matrix for testing is important for human nutrition especially children i.e. muscle or milk and some matrices (plasma and urine) do not allow for conclusions with regard to compliance with MRL values. A minimum of 8 NSAIDs should be monitored per MS. Some MS do not analyse milk while others only analyse milk for 1 or 2 analytes.

The workshop concluded with a discussion, by **Wolfgang Radeck** (EURL), on multi-residue screening methods in relation to targeted, semi-targeted or unknown screening, instrumentation and legislative requirements of Commission Decision 2002/657/EC and Council Directive 96/23. He noted that fast triple-quad MS (mass spectrometry), TOF, QTOF (quadrupole time-of-flight), Orbitrap and Qtrap (quadrupole ion trap) are proper instruments for data acquisition in multi-residue screening analysis, however consideration must be given to which compounds to validate and combining substances within a group with similar chemical-physical properties.
NRL Residues of Animal Origin

Report on the Annual Workshop of the EURL for Antibiotic and Dye Residues, Fougères, France, 9th - 10th June 2011

NRL Representative: Lynda Harman

Eric Verdon (EURL) welcomed all 43 participants to the annual workshop for antibiotic and dye residues. The participants were mostly from EU MS with three visitors from Latin American countries and one representative from the European Commission. Topics covered included proficiency testing schemes, method development, validation, stability studies and current developments in Community residue control legislation.

Eric Verdon also gave an overview of methods currently under development and validation in the EURL e.g. methods for the determination of sulphonamides and tetracyclines by LC-MSMS (liquid chromatography - tandem mass spectrometry) in honey. He noted that further work is required on tetracyclines by LC-MSMS in meat as difficulties were encountered with accurate quantification in the proficiency test for multi-antibiotic residues in pig muscle in 2010. He compiled data regarding the screening and confirmatory methods implemented in EU 2010 monitoring plans for the substance group B1 (Antibacterials). This data was extracted directly from the DG-SANCO residues website. It was noted that some MS appear to be using inhibitor tests as confirmatory methods which is incorrect and possibly due to input errors. In general member states use a relatively large number of techniques for screening methods whereas the number of techniques employed for confirmation has decreased dramatically.

In a further presentation Eric Verdon gave an overview of carbadox and laquindox monitoring in EU MS. Although previously used for growth promotion, prevention and treatment of swine dysentery and enteritis in pigs, it has been banned in the EU since 1999 (EC Regulation 2788/98) but is still approved in the US. Quinoxaline-2-carboxylic acid (QCA) is used as the marker metabolite. The last EURL PT for this compound in 2006 encountered difficulties with preparation of the metabolite and there were not enough participants for relevant information to be gained. Recent work in North America has identified desoxy carbadox (DCBX) (carcinogenic and genotoxic) as an alternative and relatively stable biomarker however further work needs to be done to demonstrate the presence of DCBX even when QCA is absent.

Valerie Gaudin (EURL) presented 2 validations on the evaluation and validation of commercial immunological kits for the screening of various antibiotic residues in honey; the first using various commercial immunological kits (ELISA kits and receptor tests) and the second using two commercial microbiological inhibition tests. In conclusion several tests in the first validation performed in parallel; however time and money were factors to consider. In the second validation results were promising; however there were concerns with a high false positive rate and inadequate detection capabilities.

Matthew Sharman (NRL, UK) discussed veterinary drug residues in honey and issues relating to detection and control. Although a cascade MRL can be used for honey as it is a minor species, the difficulty for veterinarians is setting suitable withdrawal times when treating hives to ensure no risk to consumer health. With greatest variation in analyte residues observed between hives depletion studies need to examine more variables e.g. geographical location (especially with varying climatic conditions), number of hives and method of treatment of hives.

Matthew Sharman also spoke about the FERA approach towards the testing of dyes in fish. He showed how the list of analytes to be detected is constantly growing as new dyes are used. Interestingly other sources of contamination (paper towels and marker pens) can occur, giving rise to false positive results, e.g. fish stored in supermarket overnight wrapped in green paper towels tested positive for Malachite Green dye.

Dominique Hurtaud-Pessel (EURL) described a method for analysis of Methylene Blue and its possible degradation products in fish tissue using LC-MSMS. Although not regulated in food fish in the EU, Methylene Blue is widely marketed for treatment purposes in ornamental fish. The conclusions of the study were that more work needs to be done to improve quantitation, the use of internal standard will be investigated, and also that Methylene Blue seems to be slowly absorbed but rapidly eliminated from fish muscle.

Régine Fuselier (EURL) presented the results of the proficiency test (PT) for multi-antibiotic residues in pig muscle. This PT had 46 participants; 22 NRL’s and 24 regional French laboratories. The matrix was derived from pigs held on the EURL farm, two pigs were used as blanks and three were injected intramuscularly (enrofloxacin, sulfadimethoxine and oxytetracycline). Although some participants encountered difficulties accurately quantifying oxytetracycline, the main conclusion from this PT was that 70% of the NRL’s analyse correctly for the compounds tested.

Régine Fuselier also gave an evaluation of data from the proficiency test (PT) for chloramphenicol and dye residues (Leuco Malachite Green and Leuco Crystal Violet) in trout. Both proficiency tests were performed using spiked minced fillet of trout. In the chloramphenicol PT one blank and two spiked samples were provided. Out of 46 participants there were 5 unsatisfactory and 2 questionable Z-scores. In the dye residues PT one blank and two spiked samples (one of each spiked with Leuco Malachite Green and Leuco Crystal Violet) were provided. Out of 29 participants there were three...
questionable or unsatisfactory Z-scores. Overall it was concluded that the NRLs responsible for chloramphenicol and dye residues in aquaculture products are very efficient in the ranges tested. It was also announced that Anses-Fougères have achieved accreditation as a proficiency test provider by COFRAC (French accreditation body). A proficiency test for tetracyclines in spiked and incurred porcine muscle is scheduled for early 2012.

Murielle Gaugain (EURL) described the evolution of the targeted LC-MSMS screening method for antibiotic residues in meat and milk. Previously the method, which involved 2 different extraction procedures, screened for 60 analytes from different families. The method has been improved in a few ways. Firstly new compounds have been added and secondly time windows during data acquisition have been added which has achieved increased sensitivity. The method is run as a semi-quantitative method i.e. if a sample has a concentration greater than 0.5 MRL it is sent for confirmation. It is hoped that further development will lead to a single extraction and acquisition method for all compounds except for aminoglycosides.

Murielle Gaugain also presented details of a stability study for various antibiotics in solution and in matrix. The initial aim of this study was to assess the stability of spiked matrix samples so that QC samples for the LC-MSMS method could be prepared, stored and used for analysis for a specified length of time. The secondary aim was a stability study of stock solutions and an opportunity to determine better conditions for dissolving antibiotic standards. The conclusions reached in the study were that further investigation is required for some aminoglycosides and macrolides in matrix, no differences were observed between storage at -20°C and -70°C except for penicillins and that the variation in precision of methods e.g. LC-MSMS vs LC/UV methods, can influence what criteria to be established at start of study. It was noted also that to ensure the quality of a stability study as many factors as possible should remain constant and where possible use the same batch of standards throughout the study. Finally the value of collecting stability data from all NRLs to compile a database was proposed which will be made available via the Fougères website.

Désiré Laza (NRL, Belgium) described the validation of a multi-residue analysis method for macrolides in muscle and kidney. This method is fully validated for confirmation of 5 macrolides in bovine and porcine muscle. The scope could be enlarged to include more macrolides and validation work is ongoing in porcine kidney.

Dominique Hurtaud-Pessel (EURL) gave an overview of advances in EURL screening approaches with the introduction of a LTQ-Orbitrap system (linear ion trap Orbitrap). This technology was used to develop a screening method for antibiotic residues in muscle. It has a high sample throughput and aims to avoid false negative results. Full scan acquisition is used and a sample is detected by the presence of an exact mass at the correct retention time. Sulphonamides, macrolides, beta-lactams, tetracyclines, quinolones and lincosamides can be detected using a generic sample preparation. A further SPE (solid phase extraction) step is required for aminoglycosides. This method has no limitation in the number of compounds to be searched for but the limitation lies in the sensitivity of the detection.

Linda Stolker (NRL, The Netherlands) spoke about the identification of ‘unknowns’ in feed by microbiological screening and LC-ToF-MS (liquid chromatography - time of flight - mass spectrometry). At times the active substance in samples screened by microbiological methods could not be identified using targeted LC-MSMS methods. RIKILT have developed a procedure to identify the unknown active compounds. This procedure includes sample concentration, sample fractionation, determination of active fractions and finally identification of the active substance. In this study a screening procedure for ‘unknown’ samples was developed by using microbiological screening and LC-ToF-MS identification. Final confirmation of the identity must be checked by comparison with a reference standard.

Georgi Stoev (NRL, Bulgaria) gave a quantitative assessment of the contribution of high resolution mass spectrometry (HRMS) to the reliability of compound confirmation. He questioned the reliability of the identification/confirmation of the analyte detected by HRMS and calculated the probability of a wrong result. His conclusion was that when the molecular mass of an analyte increases, the number of possible elemental composition possibilities grows exponentially, and as a result the employed mass resolution must be increased adequately.

Eric Verdon briefly reviewed the validation criteria for FS-HRMS (full scan high-resolution mass spectrometry) according to Decision 657/2002/EC for both screening and confirmatory methods. Although more multi-class multi-residue targeted methods are now being developed, there are no criteria established for these multi-residue methods. Established criteria apply to single analyte methods. However, at an international level CCRVDF guidelines for multi-residue methods are currently being drafted and may be adopted in a future review of Decision 657/2002/EC.

Martin Brandtner (NRL Austria) and Eric Verdon discussed potential biomarkers for the detection of Nitrofuran abuse. It was concluded that Semicarbazide is not an unambiguous marker for nitrofurazone abuse and since 2005 various alternatives have been proposed by different laboratories.

In conclusion the workshop agreed a need to review Commission Decision 2002/657/EC and its joint documents, Sanco/2004/2726/rev4 of December 2008, and Guideline for validation of screening methods of January 2010, a need to have more validation criteria specific to multi-residue methods, clarification and harmonisation of CC calculation and its use for banned compounds and the establishment of an EU-RL database compiling data regarding methods, stability studies etc. from NRLs.