



### NRL Contacts

**Antimicrobial  
Resistance  
Zoonoses  
(salmonella)**  
Dr M Gutierrez

**Listeria  
Staphylococci  
Milk & Milk Products**  
Ms B Hickey

**Ecoli (VTEC)**  
Dr L Scott

**Parasites**  
Dr T Murphy

**TSE's**  
Dr P Collery

**Residues/Chemical  
Elements**  
Dr C Mannion

**Pesticide Residues**  
Mr M Hickey

**Campylobacter**  
Dr J Egan

**Animal Proteins**  
Dr J Choiseul

## 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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## NRL Parasites

### **Pilot Study of high risk water supplies show wildlife may be the major source of *Cryptosporidium* contamination.**

The Summer issue of the NRL Newsletter featured the opening of a *Cryptosporidium* Reference facility at Backweston by Minister Coveney and outlined progress on the development of various molecular typing methods for this organism. There is little known about the prevalence of *Cryptosporidium* species in Irish water supplies or the source of any contamination. There are now over 20 recognised species of *Cryptosporidium* but only 3 species pose a significant risk to public health; *Cryptosporidium hominis*, *C. parvum* and *C. cuniculus*. Current water testing methods for *Cryptosporidium* in Ireland do not distinguish between species of public health concern and those that have no known risk to public health. Molecular methods now established in the NRL in Backweston are currently being used for speciation of *Cryptosporidium* oocysts detected in water supplies.

Over the past 10 months a pilot water sampling project has been underway for drinking water supplies on the EPAs remedial action list to identify the genotype and likely source of *Cryptosporidia* contamination. Samples were collected monthly and to date 115 samples have been analysed. While *Cryptosporidium* oocysts were detected in 59 (49.5%) of the samples the levels recovered were too low in 31 of these samples to allow genotyping of isolates. Of the 21 isolates genotyped to date preliminary results have shown that only 2 contained oocysts likely to cause infection in humans. Most contaminants were likely to have resulted from a variety of wildlife sources. Sampling and analysis will continue for a further 12 months and results should give a better insight into the origin and risk associated with *Cryptosporidium* in public health supplies. This will provide vital information for catchment management, risk assessment and public health initiatives in control of this parasite in Irish drinking water.

# Towards the Development of a National Reference Facility for *Cryptosporidium*

Carolyn Read, Jenny Pender, Tom Murphy,  
John Egan, Grace Mulcahy and Theo de Waal.

A Collaboration between the EPA, UCD and DAFF



## Project Objectives

- Establishment of a *Cryptosporidium* reference facility located at Central Veterinary Research Laboratory, DAFF, Celbridge, Co Kildare
- Survey of *Cryptosporidium* monitoring in Irish drinking water supplies
- Detection of *Cryptosporidium* In Water- US EPA Method 1622
- Genotyping of *Cryptosporidium* oocysts from slides using emerging methods
- Pilot scheme focusing on detection and genotyping of *Cryptosporidium* oocysts in supplies on the the EPAs Remedial Action List



## Progress to date

- Survey of *Cryptosporidium* monitoring in Irish drinking water supplies complete and available on request.
- USEPA method 1622 for the detection of *Cryptosporidium* oocysts in water has been established in the laboratory and validation is near completion.
- Routine genotyping of *Cryptosporidium* oocysts off slides from method 1622 by PCR-Sequencing has been used successfully for the pilot scheme samples (Xiao et al., Ryan et al.)
- Emerging methods using real time PCR followed by high resolution melt curve (HRM) analysis (DiGiovanni et al.) are being established.
- Pilot scheme sampling and analysis commenced in February 2011- 9 months completed.

## Preliminary Pilot Scheme Results

Pilot scheme Details	Samples	<i>Cryptosporidium</i> spp. detected	No. Samples	Possible source
Samples submitted	91	<i>C. ubiquitum</i>	5	Wildlife
Total positive by USEPA1622	40	<i>C. andersoni</i>	6	Cattle
DNA Extracted	29*	<i>C. parvum</i>	2	Cattle/Human
Genotyped	17**	<i>C. bovis</i>	1	Cattle
		<i>C. muris</i>	1	Mouse
		<i>C. andersoni/C. bovis</i> mixed	1	Cattle
		<i>C. ubiquitum/C. xaoi</i> mixed	1	Wildlife/Sheep

\*DNA not extracted from slides containing only 1 oocyst

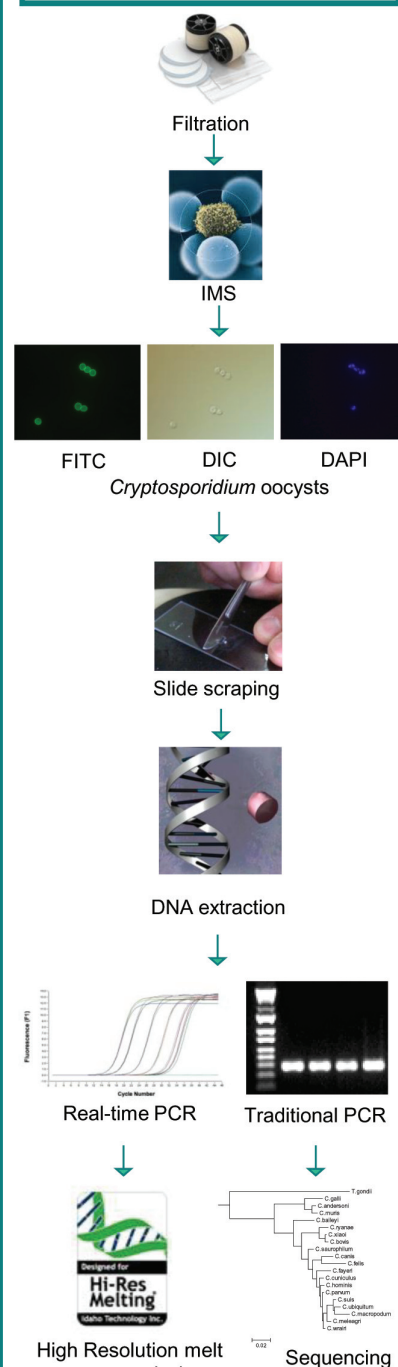
\*\*Genotype was not determined from some slides containing less than 5 oocysts

## Future of Laboratory:

- Accreditation assessment by INAB due in March 2012
- Commence genotyping on oocysts from slides referred from water testing laboratories
- Consultation with stakeholders to establish a model for future sustainability of a national reference facility for *Cryptosporidium*



## Method for Detection and Genotyping



## References

- DiGiovanni et al, Water Research Foundation, Web Report #4099.  
Xiao et al, 2001, Applied and Environmental Microbiology, Mar 2001, p. 1097-1  
Ryan et al, 2003, Applied and Environmental Microbiology, July 2003, P. 4302

## NRL Parasites

### Report of the 6<sup>th</sup> Workshop of the EURL for Parasites, ISS, Rome.

**NRL Representative:** Pat Kearney

This 2 day workshop was attended by representatives of NRLs from all EU Member States (MS) states. Also in attendance were representatives from Norway, Croatia and Serbia. The workshop reviewed the EURL Proficiency Tests (PT's), discussed NRL ring trials organised in their own countries and included presentations on foodborne parasitic zoonoses including *Trichinellae*, *Echinococcus*, *Anisakidae* and *Leishmania*.

**Bibiana Janackova (DG Sanco)** gave a general overview of the role of the EURL/NRL network. She stressed the need for *Trichinella* testing laboratories to be accredited by December 2013 - a requirement arising from Article 12 of Regulation 882/2004. She informed the audience that the EURL had achieved an A rating in the recent assessment of EURL's. A new antibody method for the detection of *Trichinella* sp. antigens has been validated by the EURL and she anticipates that Regulation 2075/2005 will be amended to include this method.

Many NRLs gave short presentations on the epidemiology of foodborne parasitic zoonoses in their countries. *Trichinella* was associated with 39.5% of all EU outbreaks of food borne origin in 2009. Romania reported 146 human cases and Serbia 111 human in 2010. Spain reported an outbreak of 6 cases in 2011 in which 1 person died. Interestingly, while no *Trichinella* cases have been reported in Portugal since 1966, a serological survey of hunters showed 5.8% of them has antibodies to the organism suggesting that the infection may be under diagnosed.

A round table discussion followed regarding PT's for the detection of *Trichinella* larvae in meat samples. The main points of issue were the preparation of ring trials by the NRLs and the performance of the testing laboratories in these trials. In most MS, some of the testing laboratories are accredited however not all laboratories are involved in the PTs. Different approaches are taken by the NRLs in preparation of ring trials, for example the type of sample used (digestive fluid v spiked samples in meat), a partial or full digest of the meat and the use of dead v live larvae. The NRLs had different views on the frequency at which these tests should be carried out and there was no standard evaluation of results with some countries using a qualitative evaluation while others used a quantitative one. It was felt that a quantitative evaluation was a better way to monitor laboratories performance. There was also a general discussion about testing laboratories paying for PT's provided by the NRLs, biological safety if large numbers of larvae are used to prepare samples, poor training records kept by testing laboratories and the advantages and disadvantages of reducing the number of testing laboratories in a country. Overall it was

evident that there is no harmonisation of results or consequences for failure of proficiency tests in MS. Guidelines for these issues are expected from the International Commission on Trichinellosis in 2012.

**Dr. Gianluca Marucci (EURL)** presented a report on the validation of the latex agglutination test to detect *Trichinella* species in pork muscle. This work was carried out at the EURL and four involved NRLs in Austria, Belgium, Estonia and Sweden. The Trichin-L Antigen test kit, based on latex agglutination, is produced by Bio-Rad. The strengths of the test kit highlighted were: ease of use; positive results can be obtained with samples spiked with only one larva; good reproducibility; reduced time needed to obtain results after digestion; no specialised training required to recognise *Trichinella* larvae; if the homogenate is properly treated the DNA can be concentrated to identify *Trichinella* species using multiplex PCR. The weaknesses of the kit were identified as: detergents used to clean equipment can result in false positive results; the filter crushing and antigen solubilisation procedure can be negatively influenced by the operators manual ability; the digestion protocol is not very robust and the blending instructions in the test kit must be strictly followed as minor changes could impact on the result. The overall conclusion of the validation was that the Trichin-L test kit meets the requirements for the accurate detection of *Trichinella* larvae in pork samples. A discussion on the *Trichinella* latex agglutination test chaired by Dr. Bibiana Janackova (DG SANCO) followed. The EURL provided the Commission with the validation report on the method and DG SANCO recommends the amendment of Chapter 11 of Annex 1 to Regulation 2075/2005 to include the Trichin-L test kit by BIO-RAD.

**Dr. Tomas Romig (Universitat Hohenheim)**, an invited speaker, gave a presentation on the Epidemiology and Control Programs for *Echinococcus multilocularis*. He outlined the increased prevalence in Europe since 1973, the risk to domestic dogs from infected foxes and the importance of preventative treatment of dogs. The role of dogs and cats in the epidemiology of alveolar echinococcosis in humans is unclear however he reported an increase in the annual incidence of alveolar echinococcosis in Switzerland from 0.1 between 1990-2000 to 2.6 in 2001-2005. He concluded that while eradication of *E. multilocularis* is not possible, a drastic reduction in prevalence could be achieved with the wide spread baiting of foxes and stray dogs with anthelmintic drugs. This would require a long term political commitment.

The role of the raccoon dog in the transmission of *E. multilocularis* was outlined by **Astrid Sutor (NRL Germany)**. This dog was first introduced into Europe, mostly Ukraine in the period 1929 to 1945. It has since spread to most parts of mainland Europe and was detected in Spain in 2008. Studies suggest that the raccoon dog can promote the spread of this parasite.



**Dr Teresa Audicana Berasategui (Hospital Santiago Apostol, Vitoria- Gasteiz Spain)** gave a presentation on Anisakidae worms and outlined their life cycle, diagnosis and the allergic reactions caused by this parasite. In Spain 8% of acute urticaria episodes are due to *Anisakis* allergy.

**Dr. Laura Rinaldi (University of Naples)** gave a presentation on the FLOTAC system for the diagnosis of intestinal parasites. It is a flotation based technique that can detect all parasite stages present in faecal samples, eggs, larvae, oocysts and cysts. Validation has shown this system to be robust and is now used in over one hundred laboratories worldwide.

**Dr. Luigi Gradoni (EURL)** gave a presentation on the epidemiology of zoonotic leishmaniasis in the EU and stated that epidemiological data suggested that infection is spreading northward within Europe. The incidence of cutaneous leishmaniasis is under reported because of its benign nature and misdiagnosis. Similarly canine leishmaniasis is also underreported in Southern Europe.

**Dr. Franck Boue (ANSES, France)** presented the main results on the use of the Segmental Sedimentation and Counting Technique (SSCT) for the diagnosis of *E. multilocularis* infections in foxes. This method is a very useful and reliable technique for large epidemiological studies particularly in areas where the endemic prevalence of *E. multilocularis* is low or unknown and is much quicker than the gold standard method.

The *Trichinella* Proficiency Test on the digestion method has been confirmed for 2012. It was suggested to reduce the number of samples from 10 to 5. Two other PTs on the identification of *Trichinella* larvae and on the detection of *Echinococcus* adult worms in intestinal contents have also been confirmed.

## NRL *Salmonella*

### *Salmonella* Typhimurium isolated from a further duck farm in November.

*Salmonella enterica* serovar *Typhimurium* phage type DT8 was an uncommon isolate in humans but since September 2009 it has been isolated from human food poisoning cases both in Ireland and the UK. There were 34 confirmed human cases in Ireland up to April this year with some further cases occurring in subsequent months. The outbreak is linked to consumption of contaminated duck eggs and although duck eggs form only a small part of total egg sales there has been a significant growth in sales in recent years.

Following the outbreak DAFM has undertaken a significant programme of testing in the main duck farms in Ireland. Testing of duck farms began in 2009 and in 2010 a total of 54 farms from 21 counties were visited and 312 samples grouped in 93

different submissions were submitted to the NRL for testing. These included environmental samples, i.e. faeces, faecal swabs, dust, fluff, post-disinfection swabs, as well as eggs, feed and water from the farms. Results showed the presence of *S. Typhimurium* in 24 farms from 14 counties; the positive samples being boot covers, dust and faeces. Isolates from 22 of these locations were confirmed as phage type DT8 or DT30, which is closely related, confirming the presence on these farms of the outbreak strain. The investigation into the source of the outbreak strain in duck farms pointed towards ducklings imported from the UK.

To date in 2011 testing has been conducted in a further 18 duck farms. Four sites have been confirmed as positive for *S. Typhimurium* with units ranging in size from 20 ducks to 2000 and distributed in Cavan, Meath, Kildare and Carlow. Restrictions are immediately issued on the sale of eggs from infected farms.

Up to 2010 duck farms were outside the mandatory *Salmonella* control programme required for poultry production in EU MS. In 2010, SI 565 was enacted to make it mandatory on all duck farms selling eggs to have a *Salmonella* control programme in place. The FSAI undertook an extensive intervention programme, producing leaflets and information notes on all aspects of *Salmonella* for consumers and for producers and distributing them via butchers, retailers and farmer markets. Similarly, Safefood produced information for all Island distribution. The ongoing sporadic cases highlight the difficulty on efficiently identifying contaminated flocks and communicating with small scale local producers.

## NRL *Campylobacter*

### Report of the 6<sup>th</sup> Workshop of the EURL *Campylobacter*, Uppsala, Sweden, 4 - 5<sup>th</sup> October 2011

**NRL Representative:** Dr Montserrat Gutierrez

There were representatives from 27 MS and Norway, FYROM, Croatia, Bosnia Herzegovina and Turkey. **Klaus Kostenzer (DG Sanco)** indicated that the EURL *Campylobacter* had achieved an excellent result in the evaluation of EURLs.

**Anca Stoicescu (EFSA)** presented the *Campylobacter* results from the 2009 EU Summary Report on Trends and Sources of Zoonoses and Zoonotic agents and on Antimicrobial Resistance. In humans *Campylobacter* continued to be in 2009 the most reported gastrointestinal bacteria with a notification rate of 45.6 per 100,000 population and 4% more reported confirmed cases than in the previous year. Approximately one third of the cases were characterised at species level, showing *C. jejuni* in 90% of these cases. Poultry meat still appeared to be the most important food-borne source of *Campylobacter* since

the occurrence of the bacteria remained at high level at 28% in poultry meat. In contrast the detection in fresh pig meat and bovine meat was 0.6 and 0.5%, and it was only occasional in other food commodities including milk or cheese. In animals the prevalence was in general high for broiler flocks and pig herds while it was lower for cattle herds. *C. jejuni* was the most commonly isolated bacteria in broilers and cattle while most pig isolates were found to be *C. coli*. There was a large variation on the levels reported by different countries and the presenter acknowledged the fact that the conclusions of the report are limited by the small number of countries reporting data in some of the categories and by the variations in the monitoring programs. In the case of food-borne outbreaks caused by *Campylobacter*, there were 333 reported in the EU by 16 MS in 2009, however only 16 of them were verified. These outbreaks affected 102 people with 9 hospitalisations and one fatality.

Eleven MS and Iceland contributed with data on *Campylobacter* AMR in humans compared to 16 MS and 2 non-MS countries reporting data on antimicrobial resistance (AMR) in *Campylobacter* from animals and food. While the data coming from human isolates differed substantially among countries regarding methodology, antimicrobials tested and interpretation criteria, the monitoring in animals and food isolates was harmonised as recommended by EFSA. In general it was observed a high level of resistance towards fluoroquinolones and tetracyclines and a low level towards macrolides in isolates from humans and animals and food. *C. coli* were more resistant than *C. jejuni* and porcine *C. coli* even showed high resistance to erythromycin.

**Klaus Kostenzer (DG SANCO)** summarised the EFSA opinion on possible control options for *Campylobacter* in broiler meat production that was published in April 2011. Although data for source attribution in the EU are limited and epidemiology may vary among regions, it is considered that handling, preparation and consumption of broiler meat may account for 20 to 30% of human cases of *Campylobacteriosis* while 50 to 80% may be attributed to the chicken reservoir as a whole. However there is considerable underreporting and one has to bear in mind that there are multiple pathways to human exposure, that travelling is a reported risk factor and that there is a gap on knowledge on the role of certain reservoirs such as pets and wild birds. Therefore it is recommended to establish active surveillance of *Campylobacteriosis* in all MS, to obtain a representative collection of isolates from humans and reservoirs and to develop research on *Campylobacter* virulence and ecology and the role of immunity on human infection.

A quantitative microbiological risk assessment model was used to estimate the impact on human infections due to the presence of *Campylobacter* spp. in broiler meat and the effect of different interventions. With approximately 9 million cases per year in the EU27, the disease burden of *campylobacteriosis* and its sequelae is 0.35 million disability adjusted life years (DALYs)

per year and a total annual cost of €2.4 billion. The public health benefits of controlling *Campylobacter* in primary broiler production are expected to be greater than controlling it later in the food chain. The main interventions that showed risk reduction at farm level were the use of fly screens, the restriction of the slaughter age to a maximum of 28 days and the discontinuing of thinning. After slaughter the use of hot water, chemical carcass decontamination with lactic acid, acidified sodium chlorite or trisodium phosphate, and the freezing, cooking or irradiation all showed several levels of risk reduction.

The model quantified that reductions of public health risk of 50% and 90% respectively would be obtained if targets of 25% or 5% of flock prevalence were achieved. Alternatively the same reductions would be obtained if all batches that are sold as fresh meat would comply with microbiological criteria with a critical limit of 1000 or 500 cfu/gram of neck and breast skin respectively.

**Ingrid Hansson (EURL)** gave an overview of an outbreak of *Campylobacteriosis* in Sweden in early 2011 that was associated with the consumption of raw milk. This outbreak affected 12 cases, all of them relatives to the milk-producing farmer. Three different strains of *C. jejuni* were isolated from 10 cases, 9 had consumed raw milk and one had direct contact with another case with gastroenteritis. Isolates from milk were obtained by testing the milk filter and the comparison with the human isolates allowed the matching with one of the human strains by using PFGE. Although raw milk cannot be sold in Sweden the consumption of raw milk is common in some farming areas and to assess the risks associated to this practice the EURL is undertaking a project to test milk filters for the most common food-borne pathogens.

Dr Hansson also presented the results of the PT no. 8 for the detection, enumeration and speciation of *Campylobacter* in minced meat samples that was conducted in April 2011. The participants were requested to test the samples according to ISO 10272 Parts 1 and 2 and to incorporate additional steps such as enrichment of 24 h in Preston broth and Bolton broth; while the method of speciation was not dictated by the EURL. Bolton broth incubation for 48 h as per ISO method gave the best results for all the samples with the exception of the 2 samples that contained *Campylobacter* in the presence of ESBL *E. coli*, in which case Preston broth incubation was better. There was adequate performance of all the laboratories in the detection part of the PT, with most laboratories detecting all the positive samples correctly. However 2 laboratories underperformed in the enumeration part and six failed to carry out correctly the speciation of *Campylobacter* species.

**Boel Harbom (EURL)** presented the preliminary results of a study that compared the real time PCR iQ-Check *Campylobacter* kit from BioRad with the traditional culture detection using ISO 10272 in its application with matrices other

than food, i.e. caeca, neck flaps, carcass rinses and sock samples. The rapid method was faster than culture and provided comparable results in the matrices studies, although some samples required to be repeated to dilute them due to the presence of inhibitors. On the other hand the PCR method does not differentiate among *C. jejuni*, *C. coli* and *C. lari* although it detects the 3 species because they are all labelled with the same dye.

**Enne de Boer (RIVM, The Netherlands)**, on behalf of the working group on *Campylobacter*, provided a synopsis of the state of play of the revision of ISO 10272 Parts 1 and 2 for detection and enumeration of *Campylobacter*. Among the proposals being progressed the most significant are:

- The introduction of a new testing regime for samples containing high background count of non-*Campylobacter*, e.g. raw chicken, raw meat and raw milk: Preston broth incubation for 24 h at 41.5°C followed by isolation in mCCDA without a second selective media AND direct plating in mCCDA from sample homogenate. The rest of samples continue to be tested as per current method.
- For enumeration to plate initial suspension and decimal dilutions according to ISO 7218:2007, so not in duplicate. Also to carry out inoculation of 1 ml of initial suspension on a large or on 3 small agar dishes in duplicate when low counts are expected.
- For confirmation deletion of the "Aerobic growth at 41.5°C and the change from "Microaerobic growth at 25°C" to "Aerobic growth at 25°C". Also the microscopic examination is permitted directly from colonies on blood agar or mCCDA and the suspension in Brucella broth is not needed.
- For identification deletion of the antibiotic sensitivity tests for nalidixic acid and cephalotin because of the increased resistance of some species for these antibiotics. Addition of a recommendation to use alternative confirmation/identification tests such as PCR, immunological tests, microarray, etc.
- As an alternative to the microaerobic atmosphere incubation it is proposed to allow the incubation of the enrichment in tightly closed containers filled with enriched broth and having a reduced headspace, but only after the laboratory has proven evidence that the correct microaerobic conditions are created.

Further work is necessary to establish (i) if Preston or Bolton broth select for different *Campylobacter* species and (ii) if the use of filter bags should be introduced so that skin or meat pieces could be separated from the enrichment broth after mixing. Finally an ISO 10272-4 is being progressed for samples from primary production (animals and their environment).

Once the standard is revised interlaboratory studies will have to be carried for Parts 1 and 2 before the revision is adopted. These studies are planned to start at the end of 2012 or beginning of 2013.

**Hilpi Rautelin (University of Uppsala)** presented her investigations on severe *Campylobacteriosis* in Finland. A striking finding is that although most patients were not immunocompromised there was a high incidence of bacteraemia. She studied the relation that the presence or absence of virulence factors and AMR has with clinical manifestations and with risk factors such as association to travel or domestic infection. For example gamma-glutamyl transpeptidase is associated to the clinical sign of presence of bloody stools, or in her observations severe diarrhoea is more associated to antibiotic susceptible strains of *Campylobacter jejuni*. The investigation also included the use of an in vitro model using cell cultures to identify immune response pathways.

**John Rodgers (Animal Health and Veterinary Laboratories Agency, UK)** gave an update on *Campylobacter* research and control activities in the UK under the following topics:

- a) A National Prevalence Survey in broiler flocks covering the 3-y period 2007-2009 has shown a prevalence of 79.2%, with a breakdown in species of 75% *C. jejuni* and 25% *C. coli*. A clear association exists between slaughter age and previous thinning, with the prevalence being 50% in batches of birds presented at slaughter at <36 days old and not previously thinned, and going up to 99% in those birds >46 days and previously thinned. Risk also associated to summer months and to recent mortality in flock.
- b) Use of Multi Locus Sequence Typing (MLST) to assess diversity within broiler flocks and to assess whether sampling strategy influences the diversity. Twenty batches of broilers were intensively sampled at slaughter comparing the diversity between taking individual and pooled caecal samples and between testing one isolate per sample or up to 5 isolates per sample. The results proved that 70% of the flocks contained multiple STs, with one flock showing 7 different types. The testing of individual samples of caeca and the collection of more than one isolate per sample increased the number of clonal complexes, the MLST types and the unassigned types. However on most occasions the typing of one single isolate in the pooled sample provided the predominant ST of the flock and it is considered a good method to be used for carrying out surveys.
- c) Genetic diversity of *Campylobacter* using MLST: A total of 890 successfully characterised strains by MLST were obtained. As usual the diversity was low among *C. coli*, with isolates showing type ST-828, while there was a large

diversity among *C. jejuni* with ST-257 being the most prevalent. There were no significant differences among 37 companies checked and all of them but 2 exhibited the most prevalent type ST-257. No significant differences were observed either on the types isolated from caecal or neck flap samples, and many types were also common to humans, although types ST-573 and 661 were over-represented in broilers while ST-257 and 45 were in poultry meat. Another interesting point was the dominance of *C. jejuni* among humans compared to the most proportionate ratio in broiler samples.

- d) Matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry to identify *Campylobacter*. This is a new technology used for confirmation and speciation of *Campylobacter* and its main advantages are that it is quick and can be carried out directly from a colony, even taken from the selective agar stage.
- e) A joint government industry initiative is on the way in the UK to work towards the achievement of a voluntary target of less than 1,000 cfu/gram of *Campylobacter* in at least 81% of chickens by 2013. The progress on the achievement of this target will be independently monitored.

## NRL *E. coli*

### Report on the 6<sup>th</sup> Annual Workshop of the EURL *E. coli*, Rome, Italy, 4<sup>th</sup> November 2011.

**NRL Representative:** Dr Montserrat Gutierrez

The workshop provided an opportunity to discuss the results in proficiency testing organised by the EURL and to disseminate information in the topic of *E. coli*, in particular the O104 outbreak. There were representatives from the NRLs of 25 MS and from Norway, Switzerland and Turkey and observers from Russia and Egypt.

The morning session was covered by six speakers from different organizations that had contributed to the investigation of the *E. coli* O104:H4 outbreaks in Germany and France. No German representative attended this workshop.

**Dr Johanna Takkinen (ECDC)** provided an overview of the activities on the outbreak at the EU level. She described the epidemiology of the German outbreak and listed the epidemiological studies that were carried out to determine its source. The first notification to the Robert Koch Institute (RKI) occurred on the 19/05/11 when Hamburg notified 3 HUS cases in children. RKI soon found that there were many other cases with the same characteristics and by Sunday 22 May 2011 ECDC was involved in the outbreak investigation. There were 6

case-control and 4 cohort studies carried out, all completed before the 08/06. On the basis of the results of the RKI first case-control study of the 25/05 a warning on the risks of salad consumption was issued, especially tomatoes, cucumbers and lettuce. A second case study of RKI on 29/05 confirmed the results of the first case study. It was not until early June that sprouts were highlighted as the vehicle when the results of a recipe-based restaurant cohort study were issued. The statistically significant relationship between sprout consumption and disease had a relative risk of 14.2 with all 31 cases in the cohort study capable of being explained by sprout consumption. It is considered that the sprout consumption was easily forgotten by the people interviewed as part of the case-control studies as they are not very obvious components of the dishes eaten.

The unusual epidemiological profile of the HUS outbreak in Germany and the large number of cases was very worrying and attracted a lot of media interest and political pressure. A total of 855 cases of HUS and 2987 cases of STEC gastroenteritis were notified in this outbreak, with 53 deaths, compared to a baseline of approximately 200 cases and 1 death per year for all EU. The proportion of women was 68% among HUS cases and 58% among cases with acute gastroenteritis. The median age was 42 (0-91) years among HUS cases and 46 (0-100) years among gastroenteritis cases. Only 2% of HUS cases were <5 years old. Cases were reported from all 16 federal states however highest incidences were recorded in the five northernmost states. Before the second outbreak in France, there were 83 STEC and 54 HUS cases in 15 other countries, mostly Sweden and Denmark.

Case numbers peaked on 22/05, around the time the outbreak was detected, and the last outbreak case had disease onset on 04/07. The incubation time was longer than normal, average 8 days. The time taken from diarrhoea to HUS was also longer than normal, 5 days on average. This is considered to be due to the peculiarities of the outbreak strain. Also interesting is the fact that there was no significant person-to-person transmission, although asymptomatic carriers, mainly children, were observed.

**Valentina Rizzi (EFSA)** gave a presentation on the source of the outbreak (Trace back/Trace forward investigations) and on scientific advice provided. EFSA contributed to the investigation of the German and French outbreaks, coordinated a European Task Force on the topic and published both a report and a risk assessment. This was the first time EFSA was involved in assisting with an outbreak investigation.

Once the cohort study identified the sprouts as the source of the outbreak and after the French outbreak had occurred, the traceability of the sprouts showed that fenugreek seeds were the only ones capable of causing both the German and the French outbreaks. The seeds were traced back to a consignment of 15 tons that came from Egypt at the end of



2009. EFSA issued a recommendation to stop growing and eating raw sprouts that lasted until early October. Interestingly the outbreak strain has never been isolated from samples of seeds and sprouts despite repeated analysis and this is explained by low contamination levels, insufficient sampling volumes, heterogeneous contamination and specific physiological conditions of the seed matrix.

**Dr Flemming Scheutz (WHO International *E. coli* and *Klebsiella* Centre)** outlined his centre's involvement in the study of the outbreak strain. This strain belongs to serotype O104:H4, is vtx2a positive, eae negative and has substantial antimicrobial resistance, including ESBL properties. It causes attaching and effacing lesions characteristics of STEC strains, but it also has enteroaggregative characteristics of EAggEC strains, and this unusual combination of EAgg and VTEC could possibly be phagetype mediated. It has 4 types of fimbria to attach to the intestine; it causes inflammation of epithelia and produces toxins. Another unusual characteristic of the strain that was exploited for its diagnosis is its ability to agglutinate with K9.

Queries promptly sent to relevant organisations revealed *E. coli* O104 was a rare serotype only occasionally reported. It had only been observed 10 times before the outbreak in the EU and Norway, some of the cases could be traced back to Egypt, Turkey and Tunisia. In particular O104:H4 had only been seen once in France in 2004 and once in Finland in 2010, although the strains were not the same than the outbreak strain. Exhaustive comparison studies of the outbreak strain with related strains indicated that this strain had evolved naturally. Looking back at the events Flemming pointed out at deficiencies in the reporting system that allowed a considerable delay before any alert of the outbreak happened. Also the incorrect notification of the cucumbers as the cause of the outbreak on the basis of a STEC result before the strain was fully characterised. Lastly he pointed out the necessity for monitoring HUS associated *E. coli* types of any serotype.

**Dr Alfredo Caprioli (EURL)** outlined the role of the EURL in the outbreak. The EURL contribution was the development of methods of analysis, the distribution of materials and the organisation of proficiency testing. The EURL adapted, validated and distributed an isolation method for O104, including the RT-PCR and circulated DNA of the outbreak strain using the NRL network. A proficiency test for isolating VTEC from naturally incurred seeds was organised and involved 8 NRLs, surprisingly none of the laboratories including the EURL, were able to detect the positive sample correctly, what was attributed to heterogeneous or low levels of contamination. The EURL was also part of a FVO mission team to Egypt and was involved on the analysis of samples from Egypt.

**Kris De Smet (EU Commission)** described the role of SANCO in the crisis and possible developments in the legislation on food hygiene and other initiatives. Different economic burdens on foot of the outbreak were presented:

Coga-Cogeca estimated more than €812m losses for farmers in the first few weeks. The Russian market alone is worth €600m a year. The Commission is incurring expenditure as a result of exceptional measures to the fruit and vegetable market for promotion and communication with a budget of approx €250m.

The Commission imposed, after MS approval, an import ban on certain seeds and beans from Egypt. Following which there was a FVO mission carried out in this country and the ban was partially lifted for the importing of green beans with the ban on seeds continuing until the end of March 2012. He pointed at possible future consequences of the outbreak that are being contemplated such as a changes in legislation in the hygiene of sprout production, the development of a database of pathogens from humans and food, a specific programme of Better Training for Safer Food on the topic of Food Borne Outbreak investigations, and the creation of a Health Secure Initiative with a legal basis to ensure that the controls on the public health side are of a certain minimum and standardised to allow better management, preparedness and coordination among different sectors and administrations both intra and inter-countries. More specifically there is discussion in Europe at the moment of the need of new microbiological criteria for seeds and/or sprouted seeds, notwithstanding the difficulties associated with the detection on this type of matrix, and the fact that this problem could be resolved with the introduction of decontamination strategies such as the use of irradiation or quick heating of the seeds before sprouting.

**Estelle Loukiadis (NRL France)** described two French EHEC outbreaks in 2011. The first one was first notified on the 14/06 involving 5 HUS cases in the Lille region that was traced back to frozen minced beef from a supermarket brand. The product was withdrawn from the market and analyses were conducted resulting on the isolation of VTEC strain O157:H25. Contamination of other meat products was proven. This outbreak accounted for a total of 19 HUS cases.

The second outbreak was declared on the 22/06 in the Bordeaux region and was caused by the same strain that caused the German outbreak. A common food exposure was identified as the human cases attended an open-day event at children's community on 8 June 2011 in which sprouts had been consumed. Overall 15 cases were found, with 9 HUS and no deaths.

**Dr Geraldine Smith (NRL UK)** gave a presentation on the HPA validation of a RT PCR from the EURL for confirmation and detection of VTEC in faecal human samples. The method comprises 2 duplex RT PCRs for virulence and intimin genes and the validation demonstrated that it was capable of detecting all the strains tested, both O157 and non-O157, including the subtypes vtx1a, b, c and d as well as vtx2 a, b, c, d, e and g, however it did not detect vtx2f. This method was applied to 214 diarrhoea specimens sourced from human cases that originated in people returning from Germany or their in-contacts. Out of 7 positive PCR samples obtained a total of 5

outbreak strain cases were identified, all of which originated in people that had returned from Germany.

**Rosangela Tozzoli (EURL)** presented results of the 6<sup>th</sup> joint PT on VTEC and *vtx* genes typing. This PT was organised in collaboration with the WHO International *E. coli* and *Klebsiella* centre and involved 80 participating laboratories, of which 29 were NRLs. A new nomenclature has been adopted to divide the *E. coli* virulence genes in types 1 and 2 and subtypes *vtx1* a, c and d and *vtx2* a, b, c, d, e, f and g. As these subtypes express different degree of pathogenicity it is important to be able to distinguish them. The main goal of this study was to evaluate the performance of laboratories carrying out a new set of PCR protocols established to differentiate the virulence genes subtypes. Five strains were distributed and participants were requested to determine the O group and the subtypes 1a, 1c, 1d, 2a, 2c and 2d. The results showed better performance for the detection of *vtx1* subtypes with 32 false positive results associated to the detection of *vtx2* subtypes.

**Gaia Scavia (EURL)** presented results of the 7<sup>th</sup> PT on the detection of VTEC in vegetables. This study required the detection of VTEC in 3 spinach samples according to the proposed ISO method that requires PCR from the enrichment buffer to detect *vtx* and *eae* genes, followed by PCR for determination of serotype, subsequent isolation of *E. coli* by using immunomagnetic separation and selective plating and confirmation of suspect colonies by PCR. One of the samples was spiked with O157 *vtx1*+, *vtx2*+ and *eae*+, a second sample with O145 *vtx1*+ and *eae*+ and the third one did not contain *E. coli*. 41 laboratories participated of which 26 were NRLs and 22 of them were EU-NRLs. Regarding the results obtained among the NRLs, most of the results were correct, only 6 laboratories providing incorrect results.

**Pina Fratamico (USDA)** outlined the regulatory and methodological aspects of USDA's policy to combat VTEC. She gave a comprehensive presentation on the current state of affairs of STEC in the US. Compared to infections caused by O157, the non-O157 infections are less severe but more prevalent, the noticeable increasing trend in their incidence being partly attributed to enhanced testing and awareness. 81% of non-O157 belong in decreasing order of prevalence to O26, O103, O111, O121, O45 and O145. Although they are not often recognised as part of outbreaks, O111 has been more associated with outbreaks than other serotypes. The most frequent transmission routes in outbreaks are person-to-person and foodborne with a diversity of food commodities being involved, e.g. dairy, leafy vegetables, beef, pork, game meat and fruits/nuts. Cattle are considered the main reservoir, but other ruminants and non-ruminants are also carriers. Recently the results of a survey conducted across the US of VTEC in minced beef have been published and they showed that about 24% of samples screened positive for virulence genes and 7% yielded STEC isolates but only 0.2% of samples contained pathogenic STEC.

Pina Fratamico also described the complexity of virulence genes and pathogenicity factors in STEC and emphasised the differences observed in the severity of their different combinations. Also the problems associated with the isolation methods, an area in which she is currently working trying to enhance the FSIS method MLG.5B, which is similar to the draft standard ISO/IEC 17043. In particular her group is involved in the development of immunomagnetic separation dynabeads for O121 and O45, selective agars for isolation of STEC strains and latex reagents for unusual serotypes.

The Food Safety and Inspection Service consider O157 and non-O157 adulterants in certain raw beef products and testing by the food industry of all batches of these products for the presence of non-O157 strains will complement the current testing for O157 early next year. There will be some cost benefits from the program.

**Camilla Sekse (NRL Norway)** gave a presentation on the occurrence of *E. coli* O145 in Norwegian sheep flocks. O145 is considered important in Norway since 2009 when an outbreak occurred in a child centre. A survey of 620 sheep flocks conducted between 2006 and 2007 based on RT PCR showed a high isolation rate for VTEC in faecal samples with 29% of flocks positive.

**Stefano Morabito (EURL)** outlined a proposal for a repository of molecular typing data for VTEC from animal and food sources. The EURL proposes to establish a database in the style of PulseNet to which the NRLs contribute with non-human strains. This initiative would complement a similar one by the ECDC for human strains. It is still in early stages but it is envisaged that at a later stage the EURL will be involved in training for molecular typing techniques and proficiency testing to ensure the quality of data fed into the database by the contributing laboratories. The problem of the profile assignment was recognised.

The EURL announced that it plans to organise a proficiency testing in 2012 for the isolation of VTEC in either water or seeds.

## NRL Coagulase Positive *Staphylococci*

**Report on the 5<sup>th</sup> Annual Workshop of the EURL Coagulase Positive *Staphylococci* (CPS), Paris.**

**NRL Representative:** Ciara O'Dowd

27 NRLs from 25 EU MS and from Norway were represented at the meeting. Representatives also attended from EC/DG SANCO, EFSA and JRD/IRMM-Geel. A wide range of topics

were discussed including regulatory control issues, epidemiological reporting, reference materials for SE (Staphylococcal enterotoxins), proficiency testing on SE detection and enumeration, VIDAS SET2 detection studies, CPS enumeration and detection, SE strain typing and epidemio-surveillance.

**Leena Rasanen (DG DANCO)** presented the process of evaluation of the EURLs for DG SANCO. The outcome for EURL CPS was satisfactory. The use of alternative methods satisfying Article 5 of Regulation 2073/05 in official controls was still under consideration by EU legal services. This topic will be discussed in the revision of Regulation 882/2004 on official controls.

**Valentina Rizzi (EFSA)** outlined the activity of the Unit on Biological monitoring and the data collected during 2007, 2008 and 2009. From 2007 to 2009, 11 to 14 MS reported the presence of SEs in food (cheeses and dairy products). Around 10% of the food-borne outbreaks in the EU (2007-2009) were likely caused by bacterial toxins. The largest proportion of verified outbreaks caused by staphylococcal toxins (21.6 %) was attributed to cheese, followed by mixed or buffet meals (15.9 %). Closer collaboration between veterinary/food and public health sectors is crucial for obtaining good quality zoonotic data.

**Reinhard Zeleny (JRD/IRMM-Geel)** gave a presentation on considerations and challenges for production of food microbiology certified reference materials (CRMs). It included requirements for their production, their use and the specific issues in food microbiology. A project had commenced with the EURL on the production of SEs in food as CRM. Work has been carried out on cheese and cooked ham (frozen or lyophilised) matrices.

NRLs from Italy, Poland and the Netherlands gave presentations on the organisation of PT trials for the networks of official control laboratories in their countries.

**Majbritt Karliskov Moos (FVST, DK-NRL)** spoke about an SE outbreak that occurred in 2010 in Denmark, where 150 persons fell ill. A sandwich was the source of contamination. A CPS strain was isolated and the presence of SEA and SEB confirmed. A *Bacillus cereus* strain was also detected.

**Bertrand Lombard (EURL)** presented the amendment of the EN ISO 6888-1 method. Data was presented at the 2010 meeting of ISO/TC34/SC9 following an EU RL study and a decision was taken to prepare an amendment that includes an optional confirmation step using the RPFA stabbing method. The EURL will conduct an experimental study in order to assess the impact of sub-sampling on the measurement of uncertainty and on the representativeness of the analytical results. This study, aims to harmonize the different sub-sampling practices and get more representative results with a lower measurement of

uncertainty. Naturally contaminated samples from various origins will be used in the study:

**Frédéric Auvray (EURL)** gave an overview of different molecular methods used to detect or enumerate **Staphylococcus aureus**; using both commercial and in-house methods, including real-time PCR. Only a few methods have been described for direct quantification of CPS. New sample preparation methods seemed to be promising but needed to be further assessed.

**Sabrina Dermouche (EURL)** presented the progress of the study on iq-PCR conducted in the EURL. This method produced good results, except for some cross-reactions with SEE. The sensitivity of the method may allow using iq-PCR, without the dialysis step for SEA. This study will be continued.

**Anne-Laure Pruffer (EURL)** presented the results of the intralaboratory validation study of Ridascreen SET Total and Vidas SET2 kits performed on food products other than milk and milk products. This study on 102 samples and 4 different types of food matrices globally, gave satisfactory results, both in terms of sensitivity and specificity. In order to fully validate the use of the Ridascreen SET total kit for milk and milk products, the study had to be performed according to EN ISO 16140.

She also introduced a project on mass spectrometry to detect SE which was conducted in collaboration with the EURL and the French Alternative Energies and Atomic Energy Commission (CEA), Grenoble. The method under development enables detection and quantification of SEs in all types of food matrices using labelled Protein Standard Absolute Quantification peptides (PSAQ). The production of PSAQ was optimized and validated at CEA in 2010 and will be transferred to the EURL later in 2011, where the method would be implemented and validated. IRMM offered to collaborate with the EURL on this topic.

**Isabelle Papinuaud-Mutel and Anne-Laure Pruffer** both presented the results of an interlaboratory study of Ridascreen SET Total in milk products. Only 175 results were statistically exploitable but this did not satisfy the requirements of EN ISO 16140 (240 results required at least). This study also pointed out the possible lack of ability of some participants to use correctly this kit. The EURL underlined the need of training on the use of the kit, to be organized by the kit manufacturer (R-Biopharm). Once this training would be performed, the EURL would organise complementary tests to complete the interlaboratory validation study (target: early 2012) in order to fully validate the kit. They also reported the results of the interlaboratory study performed on both VIDAS SET2 and Ridascreen SET Total kits for matrices other than milk and milk products: For DC (Dialysis-concentration) + Vidas SET2: sufficient results were obtained for statistical analysis and the outcome was very satisfactory (sensitivity and specificity of 100%), thus full validation was achieved for kit for use in European Screening Method (ESM). For DC + Ridascreen SET

Total: not enough statistically exploitable results (227) were obtained, so there is was a need for training and additional interlaboratory study but the temporary use may be included in ESM.

Jacques-Antoine Hennekine (EURL) gave an update on the CEN Mandate, which had been signed at the end of 2011 by EC. The aim of the mandate is to validate a method on SE detection in food, to become an EN ISO Standard.

**Benjamin Felix (EURL)** presented the data on a number of topics. The results of a PT trial on se gene detection organized in 2010 were discussed. EURL are continuing with an ongoing project on the implementation of a Real-Time PCR protocol for the detection of 13 se genes. The outcome of a study where 163 strains were typed by biotyping, PFGE and spa-typing was presented. PFGE remained the best technique with a higher discriminatory index compared to spatyping, but a combined approach could help to better discriminate profiles. The EURL has commenced a project to build a European strain collection, which would contain strains isolated from foods implicated in staphylococcal food-borne outbreaks (SFBOs) from different countries and from various origins (food, animal carriage).

## NRL Antimicrobial Resistance

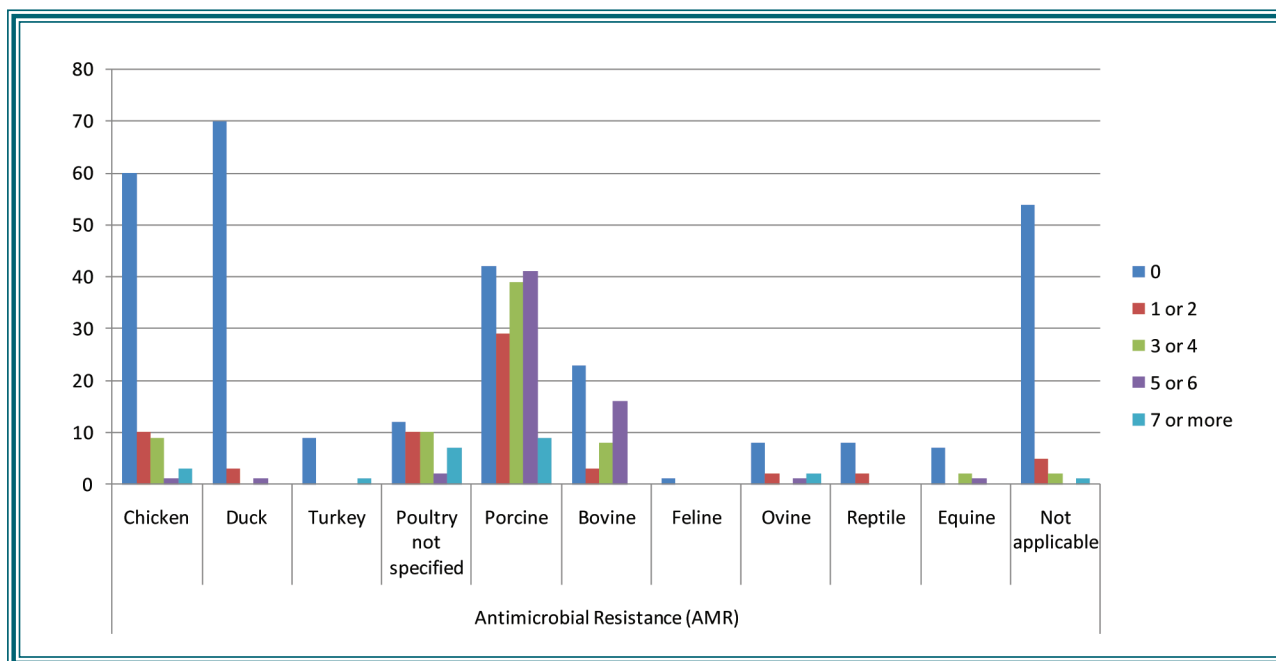
### Update on presentations.

**Dr John Egan and Dr Montserrat Gutierrez** gave overview presentations on the Antimicrobial Resistance (AMR) situation in the veterinary and food animal production sectors to the Relay Workshop at Ashtown on 10th November and to DAFM's Veterinary Inspectorate Seminar in Backweston on 23rd and 24th November. Highlighted at these presentations were the ongoing national and international efforts to tackle the growing concerns on AMR. Food animals in Ireland remain free of MRSA which is a significant concern in some MS but vigilance is required to ensure that detected resistant clones (e.g. ESBL Salmonella) do not become established in food animals as is the case in some other countries. Pathogen reduction programmes for Salmonella have been very successful in the poultry sector and efforts continue with progressing control of this organism in the pig sector and of *Campylobacter* in poultry. NRL data for 2010 (Figure 1) shows that most AMR in Salmonella is found in isolates recovered from the pig sector. Information on AMR levels has to be coupled with data on antimicrobial usage to assist in targeting controls. The Irish Medicine Board, as part of a European effort to collect information on antibiotic usage in animals, recently published data showing that 93.2 and 91.1 tonnes of veterinary antimicrobials were used in Ireland in 2009 and 2010 respectively. Tetracyclines, penicillins, aminoglycosides and trimethoprim/sulphonamide drugs accounted for 88% of the

veterinary antimicrobials used during 2010 with cephalosporins, fluoroquinolones, macrolides and other antimicrobial classes accounting for the remainder. Medicated premix and other oral dosage forms continued to be the main route of application (approximately 67% of total consumption for 2010 and 69% for 2009).

DAFM and the NRL are also contributing to EU efforts at addressing the AMR issue. In recent days the European Commission's Action plan against the rising threats from Antimicrobial Resistance (COM 2011, 748) has been published. As the increasing resistance to antimicrobial drugs represents one of the major emerging threats to human health it stresses the need for a holistic approach in line with the "One Health" initiative. The Commission proposes to put in place a 5-year action plan to fight against AMR based on 12 key actions:

- Strengthen the promotion of the appropriate use of antimicrobials in all Member States.
- Strengthen the regulatory framework on veterinary medicines and on medicated feed.
- Introduce recommendations for prudent use in veterinary medicine, including follow-up reports.
- Strengthen infection prevention and control in healthcare settings.
- Introduce of a legal tool to enhance prevention and control of infections in animals in the new Animal Health Law.
- Promote, in a staged approach, unprecedented collaborative research and development efforts to bring new antimicrobials to patients.
- Promote efforts to analyse the need for new antibiotics into veterinary medicine.
- Develop and/or strengthen multilateral and bilateral commitments for the prevention and control of AMR in all sectors.
- Strengthen surveillance systems on AMR and antimicrobial consumption in human medicine.
- Strengthen surveillance systems on AMR and antimicrobial consumption in animal medicine.
- Reinforce and co-ordinate research efforts.
- Survey and comparative effectiveness research.



**Figure 1: *Salmonella* strains of different animal origin and number of antimicrobials to which they are resistant**