Multidisciplinary approach to the control of Campylobacter

Campylobacter is the most common cause of infectious intestinal disease in humans in the UK and elsewhere and poultry meat is regarded as one of the main sources of infection. Increasing interest in the development of national Campylobacter control programmes prompted a VLA and Bristol University project, funded by Defra, to take a multidisciplinary approach involving a survey of farmers, the development of novel molecular tools and epidemiological studies.

Farmer survey

A small group of conventional and organic poultry farmers was asked about their current general management and disease control measures and what would promote or hinder their uptake of new measures and their view on Campylobacter and its control.

What control measures do you currently use and why?

What are the constraints of adopting these measures?

What would encourage you to adopt these measures?

What do you view Campylobacter and its control?

The conclusions drawn from the survey showed that the adoption of a control package by a farmer depended on many factors including cost, perceived benefits, self-interest, risk, and the international complexity of the package. Other factors influencing farmers' behaviour included company assurance schemes and market demands to produce high numbers of specific weight chickens at different times. Farmers had a good level of understanding of biosecurity but felt constrained by factors outside their control such as low margins, market-led demand for supply, and sub-contractors they employed.

The ideas and opinions from the farmers interviewed have been used to produce a structured questionnaire that will be used in a larger survey later in 2004.

Molecular tools

As part of the approach to develop practical control measures for broiler flocks against Campylobacter, it was important to take a detailed look at the possible sources and routes of transmission.

Potential sources and routes of campylobacter infection

One aim of the study was to develop a molecular method to specifically and retrospectively detect Campylobacter strains that had colonised the broilers from the broiler house environment. A real-time Polymerase Chain Reaction (PCR) based on Fluorescence Resonance Energy Transfer (FRET) hybridisation probes specific for the C jejuni isolated from the chickens was developed as a method of detecting different strains.

The nucleotide sequences of Campylobacter flagellin genes vary considerably. A short variable region (SVR) between positions 450 and 500 is flanked by regions of conserved sequences. Hybridisation probes specific for the C jejuni isolated from the chickens was developed as a method of detecting different strains.

The results were very encouraging and demonstrated that the development of a rapid, real time PCR based, specific detection system was feasible. This assay may have multiple molecular epidemiological applications and initially will be used to identify the main sources and routes of transmission of the strains that first colonised broiler flocks. This information will enable the development of targeted biosecurity control strategies.

Epidemiological studies

Preliminary, large scale and extensively reared (organic/free-range) broiler farm studies are being conducted. Preliminary - longitudinal sampling on three farms has provided a ‘proof of principle’ for the proposed molecular approach.

Large scale field - longitudinal sampling and retrospective molecular testing of environmental samples to identify the main sources of the first colonising Campylobacter strain in flocks.

Extensively reared flocks - monitoring of successive poultry meat is regarded as one of the main sources of infection.

VLA held its 9th Annual Conference in August 2003 at Nottingham University based on the theme of our mission statement - “safeguarding public and animal health through world class veterinary research and surveillance of farmed livestock and wildlife”

Plenary sessions included the opening address by Mark Addison, Defra’s Director General of Operations & Service Delivery on the progress of Defra since its creation just over two years ago. There was also a presentation by Pat Troop, the Chief Executive of the newly formed Health Protection Agency (HPA) outlining their role and responsibilities for protecting human health and highlighting the importance of HPA’s collaborative projects with VLA on a variety of zoonoses.

Strategies for enhancing veterinary surveillance in both the Netherlands and the UK were also presented and this theme was carried through to the VLA Christmas Reception for our customers, Ruth Lyons, Head of Defra’s Veterinary Surveillance Division, spoke about the new data collection software, Rapid Analysis and Detection of Animal-related Risks (RADAR), and how it was moving from a concept into reality. Graham David, Endemic Diseases Programme Manager, presented the VLA’s perspective on the future for veterinary surveillance and our considerable contribution to Defra’s overall strategy.

This issue of Insight has been born out of a series of discussions, held at our Annual conference, which emphasised the diversity of VLA activities. The articles range from the development of molecular diagnostic techniques to national disease surveys.
The DNA microarray chip technology offers a new way for biologists to understand the complexities of a living organism. It provides a tool for analysing the whole genome, whether it is bacterial or eukaryotic, as long as the genome sequence is known for that organism.

### Comparative genomic indexing of enteric pathogens

#### E.coli microarrays

Field isolates of E.coli from seven different serotypes representing enterohaemorrhagic E.coli (EHEC) and enteropathogenic E.coli (EPEC) pathotypes were hybridised against an E.coli K-12 microarray. The resulting data enable the strains to be grouped into EHEC, EPEC or ‘other’ based on their chromosomal relatedness, compared to the more common grouping of strains produced from partial and whole-genome shotgun sequencing projects. The microarray hybridisation was able to identify the presence or absence of genes found in E.coli K-12, allowing for the rapid identification of strains.

#### Salmonella microarrays

Field and clinical isolates from Salmonella enterica subspecies I were hybridised against a microarray chip with the 23 unique genes from Typhimurium. Salmonella strains were separated into separate variable regions of the chromosome which harbour Typhimurium-specific genes.

The regions of genomic differences identified by microarray in both E.coli and Salmonella serotypes may provide useful markers for the development of new PCR-based methods to differentially diagnose pathogenic isolates. However, the analysis of the whole genome using microarray technology in its current form is expensive and time consuming due to the volume of data generated for analysis. Consequently, it is impractical for use as a diagnostic test.

### Leptospirosis testing at VLA

Leptospirosis, also known as Weil's disease, is an infectious and contagious bacterial disease of wild, farm animals and many wild-life species. In cattle, it can produce an abortion rate of up to 30% when it occurs during the final trimester of pregnancy. The sources of infection are most often urine containing leptospires that contaminate pastures, drinking water or food.

VLA's main interest in leptospirosis is the disease in sheep, as it can cause significant economic losses in sheep flocks. Detection of the disease requires both clinical and laboratory techniques to confirm the diagnosis.

#### Microarray Hybridisation using E.coli pathogenic strain vs laboratory K-12 - red spots for control, green spots for test and yellow spots where the gene was present in both strains.

#### Microarray technology

As part of VLA's 'Bionomics Initiative', we have been using microarrays for comparative genomic indexing to detect the presence and absence of genes in both field and clinical isolates compared to a strain whose genome sequence is known. Whole genome microarrays of Escherichia coli and Salmonella strains have been used to compare the genomic differences between natural isolates from various serotypes.

The microarray technology provided a rapid method to differentiate between pathogenic and non-pathogenic strains of E.coli and Salmonella. It enabled the identification of unique genes from Typhimurium that were present in the tested strains, allowing for the rapid identification of pathogenic isolates.

### Differential Diagnosis of Foot and Mouth Disease in Sheep

Among the many practical problems during the 2001 pan-Asian type O Foot and Mouth Disease (FMD) outbreak, accurate diagnosis of the disease by clinical examination of sheep alone often proved difficult. This was due to the often mild disease course and the similar appearance of common endemic diseases such as interleukin dermatitis and oral (enteropathogenic) infection, but was further complicated by the presence of oral lesions of unknown causes. Interleukin ulceration of the oral cavity had largely gone unrecognized and consequently unreported before the epidemic, but large numbers of sheep were subject to a more detailed examination of their results and test during the outbreak. These oral lesions were colloquially named CHMAGO (Cuir Mouchis et Gums-Occulte Disease). Incredibly, some of these cases led to the slaughter of flock as suspect FMD.

As a consequence, Defra commissioned VLA to analyse the clinical data from the suspected sheep outbreaks and develop methods to differentiate FMD from other diseases on clinical grounds. The regions of genomic differences identified by microarray in both E.coli and Salmonella serotypes may provide useful markers for the development of new PCR-based methods to differentially diagnose Foot and Mouth Disease in sheep.

VLA veterinarians, together with Defra colleagues, were central to the initial identification of the problems with the clinical diagnosis of FMD in sheep. New diagnostic parameters for FMD and enteropathogenic oral ulcers have been defined and a dichotomous key for the diagnosis of FMD has been established.
VETERINARY LABORATORIES AGENCY

Identical samples were compared in three tests, two western blots and the Platelia assay:

Mycoplasma species samples

The western blots would allow visualisation of extracted proteins that were immunoreactive to the antibodies used; whilst the Platelia assay would confirm previously obtained survey results. The samples for each test were tested in a non-identical way using the BIO-RAD Platelia kit that was slightly modified to allow for the use of differing Proteinase K concentrations.

Molecular typing of other exotic mycoplasma species such as M. bovis, M. mycoides subsp. mycoides capri and M. mycoides subsp. mycoides capri has also been carried out and a high degree of genetic variability has been demonstrated. Strains from different geographical areas have shown widely differing genetic profiles.

Future research into endemic mycoplasma species has focused on Mycoplasma bovis, a primary cause of bovine pneumonia, arthritis and mastitis and associated with keratoconjunctivitis, otitis, meningitis and by the survival of the N-terminus and core protein epitopes (see Figs 1 & 2). Each WB system was categorised separately.

Using these methods, a comparison was made between African strains of contagious bovine pleuropneumonia (CBPP), Scrapie is a disease found in sheep and goats and a member of the Transmissible Spongiform Encephalopathies (TSEs) which include bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids and variant Creutzfeldt-Jakob disease (vCJD) in humans. Although BSE, CWD and vCJD are relatively new diseases, scrapie has been around for several hundred years.

VLA was asked to conduct a national survey to determine the prevalence of scrapie infection. This was a legal obligation in all EU countries, and the minimum requirement was to use a rapid test followed by a confirmatory test. We collected additional samples to enable us to evaluate whether alternative approaches were better, but this report relates to the challenges that were presented by the basic legal obligations.

As part of the survey, samples from 29,201 sheep from abattoirs were analysed for the presence of scrapie protein (PrPSc) using the BIO-RAD Platelia ELISA method and confirmed by immunohistochemistry (IHC). A further 21,429 samples were tested using the Prionics Check Western Blot (PCWB) with additional IHC on obese, selected lymph nodes and tonsils.

In addition, a total of 2,213 animals found dead on farms or dying in transit to the abattoir were tested using PCWB, IHC and Electron Microscopy to identify the presence of scrapie associated fibrils (SAF).

Testing was carried out and all samples were genotyped to examine the intra-strain variations at codons 136, 154 and 171 on the prion protein (PrP) gene.

Of the total of 29,201 samples tested using the BIO-RAD Platelia ELISA, 52 were found to be positive but only 24 of these could be confirmed by IHC and/or PCWB testing; 28 samples were confirmed negative. These were obviously a concern, but previous experience of using the ELISA suggested that the levels of PrPSc digestion (used in the tests to eliminate normal PrP and to leave only disease specific PrPSc) was probably a contributing factor.

A project to ascertain the diagnostic status of the unconfirmed positive samples was carried out using a BIO-RAD Western blot (WB) confirmatory test was initiated. This test, recently developed for the detection of ovine scrapie, was modified at VLA to measure selected bands of protease digestion. Various concentrations of Proteinase K were tested to determine the bands used in the BIO-RAD Platelia ELISA, the confirmatory WB method (1%4) and more traditional Western blotting methods (1%4) were used to study the effect of increased proteolytic activity and the sensitivity of the WB to protease digestion. Tests were also carried out with no Proteinase K, as a control.

Two separate antibody formulations developed by BIO-RAD were used to detect the prion protein using Western blotting to identify different regions of the extracted proteins and to establish which part of the disease was not detected in the reactions to increased Proteinase K digestion. Two further antibody formulations were raised against the PrP protein to examine the effect of the different Protease K concentrations.

The BIO-RAD Platelia assays confirmed the previously obtained diagnostic results except for one positive result in the confirmed group and one in the unconfirmed group. Neither of these samples showed banding between 5 and 100 kDa in either western blot when treated with Proteinase K.

All banding between 5 and 100 kDa in the BIO-RAD WB was noted and only those without any banding in this region were identified as scrapie negative.

The banding patterns showed remarkable variation but could be categorised into sub-groups determined by Proteinase K sensitivity of the prion protein and by the survival of the N-terminus and core protein epitopes (see Figs 1 & 2). Each WB system was categorized separately.

Of the 47 negative control samples, 45 showed no banding between 5 and 100 kDa when treated with any of the attempted Proteinase K concentrations, therefore identifying them as scrapie negative by WB. However, the results for all of the 47 confirmed and unconfirmed positive samples showed various banding in the same region but with varying digestion with the lowest concentration of Proteinase K, which suggests the presence of a protein with some resistance to Proteinase K in these animals.

An Unusual ‘somewhat non-specific’ Prion Protein

Identical samples were compared in three tests, two western blots and the Platelia assay:

In conclusion, this study has demonstrated the presence of partially Proteinase K resistant PrPSc in vivo when using WB on samples that were identified as unconfirmed positives by the Prionics ELISA test. While it explains why traditional Western blotting failed to confirm the ELISA results, it does not offer much help in explaining why they were also negative by immunohistochemistry.

Most important: these findings do not infer any disease association or TSE infectivity in the cases involved - only that there appears to be an unusual and previously unidentified isomer of the prion protein present.

Further studies are planned on emerging similar samples from the Scrapie Surveillance Scheme which are now categorised as ‘UNCLASSIFIED’.

Molecular epidemiology of Mycoplasma species

The Mycoplasma form a genus of bacteria many of which are pathogens of animals and birds. They cause many ailments such as pneumonia, mastitis, genital disorders, keratoconjunctivitis and respiratory disease.

Research at VLA into the development of molecular typing techniques for Mycoplasma species is designed to monitor the distribution of pathogens for both outbreak investigations and epidemiological surveillance. It may also enable us to identify strains that are more virulent and associated with more severe disease symptoms and trace and control outbreaks of exotic strains should they enter the UK.

Most molecular typing techniques are based on examining segments of DNA for strain-specific variations (polymorphisms) in the DNA sequence. The profiles of DNA bands are analysed using a computer-based programme to determine any similarity between specific strains and to ascertain whether they originated from the same source. Several well-established typing methods have been used at VLA including Pulse Field Gel Electrophoresis (PFGE) and two Polymerase Chain Reaction (PCR) based methods: Random Amplification of Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP). We have also employed Denaturing Gradient Gel Electrophoresis (DGGE), a technique more commonly used for the detection of human genetic mutations and microbial ecology, which had not previously been used to type Mycoplasma strains.

Using these methods, a comparison was made between African strains of contagious bovine pleuropneumonia (CBPP), Mycoplasma bovis, a primary cause of bovine pneumonia, arthritis and mastitis and associated with keratoconjunctivitis, otitis, meningitis and the survival of the N-terminus and core protein epitopes (see Figs 1 & 2). Each WB system was categorized separately.

Future research into endemic mycoplasma species has focused on Mycoplasma bovis, a primary cause of bovine pneumonia, arthritis and mastitis and associated with keratoconjunctivitis, otitis, meningitis and the survival of the N-terminus and core protein epitopes (see Figs 1 & 2). Each WB system was categorized separately.

Future research will focus on whether there is any variation in virulence and whether these strains can be genetically distinct groups of strains.

Dairy sheep flocks in Portugal which are highly susceptible to Mycoplasma infections...
A pig, a bug and a programme

At least 17,000 people a year in Britain are affected by Salmonella infection, which may lead to a very unpleasant enteritis - or worse! Salmonellae in humans is most often associated with Salmonella Enteriditis (65% of cases) which are particularly associated with poultry, or S. Typhimurium (13% of cases) which has a wide range of hosts including cattle, sheep, pigs and poultry.

In 2000, results of a VLA survey of abattoirs showed that 25% of pigs slaughtered in the UK tested positive for Salmonella and the most common serotype was S. Typhimurium. This caused some concern to Defra, the Food Standards Agency and the UK pig industry since although this bacterium is well-adapted to the environment in pigs, it could be a potential source for human infection. To monitor the level of infection, the British Pig Executive (BPEX) launched the Zoonoses Action Plan (ZAP) Salmonella Monitoring Network, based on a Danish scheme, whereby on-farm samples taken from herds in the abattoir are tested by BSA to detect antibodies to common Salmonella serotypes.

At the same time, VLA undertook an epidemiological research programme, commissioned by Defra, to discover the extent of Salmonella infection on British pig farms and to develop and test simple control methods that could be adapted by the farmer. Over 300 farms were surveyed and less than 10% of pigs tested positive. This was very encouraging, since it suggested that practical, economical and effective control measures could be achieved.

VLA also worked with a group of 22 farmers to trial strategies for improving hygiene and biosecurity. This led to some interesting discussions including some strong opinions about how best to deepen a tract. Eleven of the farms adopted an extensive strategy and the remainder continued with the same management, with a view to comparing the results.

At the end of the trial, the spread of infection was lower on the farms using the extensive strategy, but the strategy had little effect on the level of Salmonella at the start of the 'treatment' period which lasts approximately 20 weeks to six months of age. We also found that every farm infected did not go on to infection. This is very encouraging since it suggests that the strategies may be effective in controlling Salmonella in the future.

PulseNet?

PulseNet is a US national molecular subtyping network for foodborne disease surveillance (www.cdc.gov/pulsenet), which was initiated in 1996, since when it has proved to be an important network for the detection of outbreaks caused by the most important foodborne pathogens, including salmonellosis and Escherichia coli 0157. VLA is part of this network.

The network is led by the Center for Disease Control & Prevention (CDC) in Atlanta, and the participants are State and County public health laboratories and their counterparts in the United States, State's Department of Agriculture and the Food and Drug Administration. Serotypes from humans and food are typed using pulsed-field gel electrophoresis (PFGE) using a standardised protocol, and images of the PFGE profiles submitted electronically to the CDC are compared with profiles submitted by other laboratories. Comparison is made in real-time with sophisticated image analysis software (BioNumerics). Currently, more than 40,000 PFGE profiles of foodborne pathogens have been entered into the central database and although this is expensive, the network is considered very cost effective.

In Europe, development of this type of database has been slower. The surveillance of foodborne infections has historically been a national matter and there is little tradition for collaboration between the medical, food and veterinary authorities - although VLA is now doing this in the Health Protection Agency (HPA). There is no detailed standardisation of molecular typing methods.

A group of scientists led by Dr Peter Gerner-Smidt at Statens Serum Institut (Copenhagen) have started what can be considered as 'PulseNet Europe', a similar to the one operating in the USA. Initially, the network will be set up to hold standardised PFGE data of zoonotic Salmonella, verocytotoxigenic E. coli and Listeria monocytogenes but in time, more organisms and typing methods will be added to the network. Bacterial strains from humans, food and animals will be characterised on a real time basis enabling efficient detection and investigation of outbreaks of foodborne infections.

The method will be compatible with those used by the PulseNet USA, PulseNet Canada and PulseNet Asia-Pacific, thereby enabling detection and investigation of foodborne outbreaks involving more than one continent. Reference centres from all over Europe have been invited to join the project.

BSI, BSE and risk assessment

Bovine Spongiform Encephalopathy (BSE) is a fatal disease in cattle characterised by spongiform tissue that develops in the brain. As one of the group of diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases, BSE is a fundamental part of any BSE risk assessment estimating the quantity of ‘Infective Agent’ contained in the pathogenic source under consideration.

Attempts to understand the epidemiology of BSE have been hindered by the long incubation period and the variation of individual clinical presentation. These two factors imply that, as a quantitative measure, BSE risk assessment is most effectively based on mathematical models.

The BSE pathogen is generally perceived as a modified prion protein (PrP). It is accumulated in a spongiform tissue that develops in the brain. As one of the group of diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases, BSE is a fundamental part of any BSE risk assessment estimating the quantity of ‘Infective Agent’ contained in the pathogenic source under consideration.

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This process generates a statistical framework for deriving the individual and societal risk to be associated with each exposure pathway, by a variety of quantitative approaches. This provides the risk manager with a tool for assessing the significance of risks, which in turn provides vital input into decision making.

Risk estimation - using data from different sources, the proportions of the ‘Infective Agent’ flow through the ‘event tree’ and are estimated.
Testing for the presence of the nucleic acid (RNA or DNA) sequences that make up the genome of pathogens is becoming an increasingly common strategy to aid in the diagnosis of disease. These so-called ‘molecular tests’ provide an alternative to more traditional tests and may be faster, more sensitive and specific.

A ‘nested PCR’ for the detection of Bovine Viral Diarrhoea Virus

**Brucellosis**: a new era of vaccines

Brucellosis is a zoonotic bacterial disease of considerable social and economic importance and is described by the Food and Agricultural Organisation (FAO) and the World Health Organisation (WHO) as ‘the most widespread zoonosis in the world’.

The disease is caused by bacteria of the genus *Brucella* and affects a diverse range of host animals from economically significant livestock to companion animals, wildlife and ultimately man. In ruminants, brucellosis infection is characterised by subclinical abortion but in humans, infection is characterised by an undulant fever and a variety of unpleasant side effects. The incidence of human disease is linked to occupational exposure to the organism or the ingestion of contaminated meat and dairy products.

Brucellosis is almost eradicated from the cattle herds of northern and western Europe but in Mediterranean countries, the cause of brucellosis in sheep and goats, remains a severe problem. The disease is responsible for important economic losses through interference with breeding programmes and reduction in milk yield. It also hampers control of bovine brucellosis and poses a considerable public health threat as a virulent zoonosis. It has become apparent that vaccination will have to be an essential facet of any disease control or eradication programme.

Historically, successful vaccines against intracellular pathogens such as *Brucella* have consisted of live attenuated strains of the organism. The immunological memory is sustained ... persistence and transmission, pathogenicity in immunocompromised or inappropriate hosts and reversion to virulence.

The project is generating encouraging results. We have been able to produce protection against a subtly yet complex intracellular pathogen using a simple plasmid-based vaccine encoding a single antigen among the potential myriad of proteins expressed by the pathogen during natural infection. The vaccine is non-infectious and does not contain the *Brucella* LPS antigen and thus should not compromise diagnoses. The next step is to find a way of harnessing the protective effect of our vaccine in a single dose Liposome formulation vaccine. Such preparations are currently under investigation.

There is still a way to go in the search for the perfect vaccine, but we have made a number of steps toward our goal.
**Dying swans**

Post-mortem examinations at VLA Bury St Edmunds of whooper swans from a wetland wildlife reserve in East Anglia revealed necrotic enteritis. *Clostridium perfringens* alpha toxin was detected in the intestines and histological examinations supported the diagnosis of clostridial enteritis. Similar cases have been reported from other areas of the country.

**VLA experts in demand**

As the Office International des Epizooties (OIE) laboratory for Transmissible Spongiform Encephalopathies, VLA experts were asked to assist in the confirmation of the first case of BSE reported in the USA. Prestained histological slides were examined on Christmas Day at VLA Weybridge from a cow imported into the USA from Canada.

**‘Avian flu’ in the Far East**

As the Community Reference Laboratory for the European Union (EU) and an OIE reference laboratory for avian influenza, VLA scientists have been in discussions with the EU and Defra on all aspects of the virus outbreak in chickens in the Far East. Also diagnostic reagents have been supplied to a number of countries in the region to help control the highly pathogenic virus.

**Budgerigar mystery virus**

VLA identified a mystery virus, which has been decimating the UK budgerigar population and causing movement restrictions of the birds, as a reovirus. These are extremely complex and have been associated with hepatitis, diarrhoea and mortality in several parrot species. Having identified the virus, most samples now investigated demonstrate the reovirus-like agent, which has not been the case until now. Further studies are on-going.

**First time for six years**

VLA Langford has isolated Salmonella Paratyphi B variant Java (S.Java), for the first time in cattle for six years. Samples were received from a group of calves in Somerset but as yet no disease associated with this strain has been reported elsewhere, nor has there been any human isolates reported in people associated with livestock infection during the period.

**Botulism**

VLA Shrewsbury reported a severe outbreak of botulism in a group of in-calf heifers at pasture. The cattle were in a field where manure from the farm’s own broiler sheds had been spread. The Food Standards Agency has been informed and voluntary animals movement restrictions have been agreed by the farmer to protect the food chain.

Street market in the Far East