EUROPEAN SCIENCE FOUNDATION
SETTING SCIENCE AGENDAS FOR EUROPE

Exploratory Workshops Scheme

Standing Committee for the European Medical Research Councils (EMRC)

SCIENTIFIC REPORT

ESF Exploratory Workshop on

Clostridium perfringens Induced Disease In Domestic Animals: Learning From Human Medical Science And Biotechnology For Understanding Animal Disease

Ghent, Belgium, 22 - 24 October 2007

Convened by:

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1. Executive summary

Clostridium perfringens strains cause a variety of economically significant diseases in domestic animals. These diseases include several enteric syndromes such as necrotic enteritis in poultry, bovine and ovine enterotoxaemia and lamb dysentery. In these syndromes in various animal species different *C. perfringens* toxins can be involved. Although many disease entities in various species have been known for years, some have emerged spectacularly in recent years, including enterotxaemia in calves and especially necrotic enteritis in broilers. A major problem currently is necrotic enteritis in poultry. In Europe, antimicrobial growth promoters, used to increase weight gain in broiler chickens, are banned from poultry feed since January 1, 2006. In Scandinavian countries, national policies lead to the ban of antimicrobial growth promoters already years ago. This was almost immediately followed by health problems in broiler flocks, with most remarkable an epidemic of subclinical and clinical *C. perfringens* infections (necrotic enteritis). Also in other countries veterinarians report a sudden increase in necrotic enteritis in broilers, making *C. perfringens* a very important emerging pathogen in the veterinary field. The *C. perfringens* infections in poultry may present as acute clinical disease or subclinical disease. The acute form of the disease leads to increased mortality in the broiler flocks. This can account for 1% losses per day, for several consecutive days during the last weeks of the rearing period. In the subclinical form, damage to the intestinal mucosa caused by *C. perfringens* leads to decreased digestion and absorption, reduced weight gain and increased feed conversion ratio. Moreover, it is shown that cholangiohepatitis occurs in the subclinical form of *C. perfringens* infection and that there is an increased number of condemnations at processing due to these liver lesions. Colonization of the chicken gut by *C. perfringens* has thus increased recently, and since also enterotoxin positive strains have been found in the chicken gut and as a consequence also on carcasses, it can be expected that the probability for transmission to humans and thus the induction of type A food poisoning may increase. *C. perfringens* induced food poisoning is thus possibly an emerging disease.

Although it is believed for more than 20 years that the alpha toxin is the most important virulence factor for necrotic enteritis in poultry, novel data reveal that this is most likely not the case. Alpha toxin production of isolates from broilers suffering from necrotic enteritis is not higher compared with other isolates from healthy chickens. Even more, an alpha toxin knock-out mutant of a virulent field strain was still able to cause necrotic lesions in broilers. However, vaccination studies with an alpha toxoid have shown partial protection, indicating a certain (secondary?) role of the toxin. It is also unclear whether other toxins, adhesion factors or virulence factors can play a role, making a scientific-based control of the disease problematic. Alpha toxin producing isolates from bovine haemorrhagic enteritis and human gas gangrene do not cause disease in broilers, and alpha toxin producing isolates from normal microbiota of broilers also do not cause disease or lesions in an in vivo model, in contrast to outbreak isolates. It seems thus to be the case that there is some kind of host-specificity in the *C. perfringens* infections, in which some animal species are more vulnerable to certain strains, harbouring a specific genetic background. It is currently unknown which *C. perfringens* genes or proteins are involved in the host specific behaviour of *C. perfringens*. It is difficult to establish control measures without a thorough knowledge on the pathogenesis of the disease.

Thus necrotic enteritis, either subclinical or clinical, is an important and emerging problem in the poultry industry, and the lack of understanding of the pathogenesis and the lack of effective non-antibiotic control measures in the field resulted in the organization of the ESF workshop. The aim of the ESF workshop was to bring together biotechnologists, human and veterinary medical scientists to exchange information and discuss innovative interdisciplinary research on the pathogenesis of the disease in broilers. The European Commission already published some draft calls for collaborative projects and one of these draft calls (FP7-KBBE-2008-2B, topic 2008-1-3-01) contains an interesting topic related to the topic of the ESF workshop. This topic used the ban on growth promoting antibiotics as basis to start a search for alternative strategies to prevent animal disease and food safety problems. This needs to be based on a sound understanding of the interaction between the microbiota and the gut physiology.

The program of the workshop was divided in two sections. On October 23rd the participants presented their expertise by giving up-to-date reports on different aspects of C. perfringens induced diseases in both animals and humans. On October 24th the participants discussed the possibilities to submit a proposal for FP7 based on the above mentioned topic. After a presentation of the general structure of such a proposal, the details and workpackages of such a proposal as well as the partners and SME to be involved was discussed. Thus as main outcome of the ESF workshop, an intention to write up a detailed proposal for FP7 was generated. The participants agreed to write up their possible contributions to such a proposal and to send the text to the convenors of the ESF workshop. The convenors would then compile all these suggestions into a proposal for FP7, call FP7-KBBE-2008-2B, topic 2008-1-3-01. Hence the ESF workshop fulfilled all aims for which it was organized. It gave the opportunity to bring together scientists from different fields of the C. perfringens research, and the combined technologies and knowledge will be used in a collaborative research proposal.

2. Scientific content of the event

The first day of the workshop presentations were given by different experts in order to give an overview of all aspects of necrotic enteritis in broilers caused by C. perfringens, human food poisoning by C. perfringens, action of C. perfringens toxins and molecular approaches to identify virulence genes of C. perfringens. Below a summary of the content of the presentations is given. The data presented in these presentations were used in the second day of the workshop, at which the setup of a FP7 proposal was discussed.

Filip Van Immerseel opened the ESF workshop with a short introduction. He briefly introduced all toxins and enzymes of C. perfringens that are potential virulence factors. He explained that the agent can induce of lot of different diseases in different animal species, and that for most diseases the pathogenesis is not clear. While for some disease it is known which toxins are relevant, and while for some disease good vaccines are available, both are not for poultry. Furthermore, due to the EU ban on antimicrobial growth promoters it is clear that much more problems have arisen with subclinical and clinical necrotic enteritis. Currently the problems are still to some extent under control since anticoccidials with antimicrobial activity are used in-feed. Filip Van Immerseel presented the aims of the ESF workshop and explained that is was attempted to bring together biotechnologist, human and veterinary medical scientists to exchange information and discuss innovative interdisciplinary research on the pathogenesis of the disease. The focus of the ESF exploratory workshop should be on the initiation of follow-up research activities. He explained that this should be done both by discussing and establishing immediate collaboration on some specific topics and by discussing a EU Framework Program 7 initiative. The ESF workshop would thus in this way explore the possibilities of high quality collaborative research efforts at European level in order to form the basis of a multidisciplinary platform that can compete with research groups worldwide.

Simon Hardy gave a presentation on human food poisoning by C. perfringens, i.e. by entertoxin positive C. perfringens. He told the group that in Norway, *C. perfringens* was the most important cause of foodborne outbreaks until 1992. More recent data from the UK show that *C. perfringens* is one of the most frequent aetiological agent in outbreaks (second only to salmonellae) as well one of the most frequent causes of death. Similar results are observed in Australia. The outbreaks are typically observed in commercial catering events. Notification of food poisoning is mandatory in the UK but not the organism, thus *C. perfringens* is almost certainly under-reported. However, recent data suggest the incidence is falling, a finding also observed in Norway. Simon Hardy explained that in contrast to *E coli* 0157, *Salmonella* spp. and *Campylobacter* spp, *C. perfringens* has no public image. Combined with perceived low academic interest, the organism poses significant problems in attracting research funding. This is one of the main reasons the ESF workshop was organized and a FP7 proposal will be applied for.

Miia Lindström briefly presented some data on the molecular epidemiology of C. perfringens bacteria, harbouring the enterotoxin gene. In an introduction, she discussed how food poisoning outbreaks can occur. When preparing food using higher temperatures vegetative cells are destroyed but spores can survive, and temperature abuse (slow cooling or storage at too high temperature) can lead to spore

germination and rapid growth. These cells will then arrive in the gut, and sporulate. During sporulation the enterotoxin is produced, leading to intestinal damage. Miia Lindström presented that the enterotoxin gene (cpe) can be located on the chromosome and on plasmids. Chromosomal cpe carrying strains are food poisoning strains, while strains carrying cpe on plasmids are not, but these strains can cause sporadic and antibiotic-associated diarrhoea. The reason for these differences is the fact that chromosomal cpe containing isolates are able to grow at lower and at higher temperatures. The group of Miia Lindström developed a technique to enumerate cpe-containing C. perfringens strain in faecal samples and food using a hydrophobic grid membrane filter colony hybridization technique. The technique is much more sensitive than plating or other techniques that were used in the past. Miia Lindström presented some results from their own research; as expected, strains isolated from human food poisoning outbreaks mostly contained the chromosomal cpe gene, but also a rather high number of plasmid cpe strains were detected. Thus she concluded that both chromosomal and plasmidial cpe genotypes are involved in C. perfringens type A food poisoning. They also could detect these strains in healthy humans that thus are a reservoir and vehicle for these strains. Interestingly, plasmidial genotypes were quite commonly found in persons with antibiotic-associated diarrhoea and babies with Sudden Infant Death Syndrome post-mortem.

Richard Titball gave an overview of the mechanisms of action of C. perfringens virulence factors (toxins and enzymes), with focus on the alpha and epsilon toxin. The gene encoding the alpha-toxin (cpa) is present in all strains of C. perfringens, and the purified alpha-toxin has been shown to be a zinc-containing phospholipase C enzyme, active towards phosphatidylcholine and sphingomyelin. The alpha-toxin is hemolytic as a result of its ability to hydrolyze cell membrane phospholipids and this activity distinguishes it from many other related zinc-metallophospholipases C. Recent studies have shown that the alpha-toxin is the major virulence determinant in cases of gas gangrene, and the toxin might play a role in several other diseases of animals and man as diverse as necrotic enteritis in chickens. In gas gangrene the toxin appears to have three major roles in the pathogenesis of disease. Firstly, it is able to cause mistrafficking of neutrophils, such that they do not enter infected tissues. Secondly, the toxin is able to cause vasoconstriction and platelet aggregation which might reduce the blood supply to infected tissues. Finally, the toxin is able to detrimentally modulate host cell metabolism by activating the arachidonic acid cascade and protein kinase C. The molecular structure of the alpha-toxin reveals a two domain protein. The amino-terminal domain contains the phospholipase C active site which contains zinc ions. The carboxyterminal domain is a paralogue of lipid binding domains found in eukaryotes and appears to bind phospholipids in a calcium-dependent manner. Immunization with the non-toxic carboxyterminal domain induces protection against the alpha-toxin and gas gangrene and this polypeptide might be exploited as a vaccine. Richard Titball explained that the epsilon- (ε) toxin is produced by C. perfringens types B and D, but not by types A, C or E. C. perfringens types B and D have a limited host range, being mainly isolated from sheep, occasionally from goats and cattle, and rarely from man. The bacteria cause lamb dysentery and pulpy kidney disease in sheep, with mortality rates as high as 100%, and are of great economic importance wherever animals are raised intensively. Richard Titball's group described the molecular structure and showed that it is structurally related to the relatively non-toxic pore-forming haemolysin, aerolysin. Immunization of mice with a nontoxic mutated epsilon toxin resulted in the induction of a specific antibody response and immunized mice were protected against epsilon toxin. Richard Titball ended his presentation with a brief description of the action of the beta toxin.

Magne Kaldhusdal presented an overview of all aspects of necrotic enteritis in broilers. He argumented that the significance of necrotic enteritis is increasing since the use of growth-promoting in-feed antibiotics is banned in the EU, and will probably be even more increasing if the use of in-feed anticoccidials is abolished. The disease is most common in broiler chickens, young replacement broiler breeders and young meat turkeys. The agent can be introduced into the flock via the hatchery, via a contaminated farm environment, and via personnel, equipment or feeds. Spread within a flock kept on litter predominantly takes place via the faecal-oral route. Important epidemiological factors influencing the disease risk include the birds' access to faeces (floor type), intestinal coccidia, level of specific immunity, feed composition, feed structure and feed processing, and management. Magne Kaldhusdal presented that the pathogenesis of necrotic enteritis is incompletely understood and that the presence in the intestine of *C. perfringens* alone is not sufficient to induce illness. The presence of some factor causing damage to the intestinal mucosa is necessary to cause disease. Magne Kaldhusdal gave an

overview of the disease forms and lesions produced by the bacterium. The clinical course of the disease may vary from acute illness with high mortality, to a sub-clinical form that is detectable only as impaired production results. The latter form is probably the most important because it is most common. The characteristic gross lesion of necrotic enteritis is a pseudomembrane attached to the intestinal mucosa, primarily the small intestine. The characteristic microscopic lesion of necrotic enteritis is aggregations of large, Gram-positive rod-shaped bacteria surrounded by necrotic tissue delineated from viable tissue by a zone of granulocyte infiltration containing pyknotic cell nuclei. The most common liver lesion in broilers associated with *C. perfringens* is cholangiohepatitis, which is characterised by lesions in the extrahepatic and intrahepatic segments of the biliary tract. Kaldhusdal concluded that up to now in-feed antimicrobials has been the main means of disease control, but this practice is questioned, and there is a strong need for alternative preventive measures. Kaldhusdal finished his talk by stating that so far no single alternative measure with similar efficacy as in-feed antimicrobials has been found.

Filip Van Immerseel and **Anouk Lanckriet** presented the use of molecular biological tools that can be used to identify virulence genes involved in C. perfringens induced diseases. First Filip Van Immerseel gave an example why these tools can be useful. He presented in detail some research results of his group and others showing that alpha toxin, already for more than 20 years commonly accepted to be a major factor in necrotic enteritis in poultry, is most likely not the main virulence factor in broiler necrotic enteritis. Alpha toxin production of isolates from broilers suffering from necrotic enteritis is not higher compared with other isolates from healthy chickens. Even more, an alpha toxin knock-out mutant of a virulent field strain was still able to cause necrotic lesions in broilers. However, vaccination studies with alpha toxin have shown partial protection, indicating a certain (secondary?) role of the toxin. It is also unclear whether other toxins, adhesion factors or virulence factors can play a role, making a scientific-based control of the disease problematic. Alpha toxin producing isolates from bovine haemorrhagic enteritis and human gas gangrene do not cause disease in broilers, and alpha toxin producing isolates from normal microbiota of broilers also do not cause disease or lesions in an in vivo model, in contrast to outbreak isolates. It seems thus to be the case that there is some kind of host-specificity in the *C. perfringens* infections, in which some animal species are more vulnerable to certain strains, harbouring a specific genetic background. It is currently unknown which *C. perfringens* genes or proteins are involved in the host specific behaviour of *C. perfringens*. It is difficult to establish control measures without a thorough knowledge on the pathogenesis of the disease. Thus there is urgent need to identify virulence factors (e.g. toxins) that are primary factors involved in induction of necrotic lesions in the gut of broilers. Filip Van Immerseel first presented the use of comparative phylogenomics. This technique uses DNA micro-arrays and is able to compare full genomes of strains from different sources (animal species, or outbreak vs. non-outbreak) to identify genes available in or linked with certain strains (e.g. identification of genes specific for broiler necrotic enteritis outbreak strains). Filip Van Immerseel presented some examples of the use of the technique is different bacterial species, under which Clostridium difficile. Anouk Lanckriet described the development of a novel transposon mutagenesis approach. She started with a description of the technique in general and then argumented why the currently available transposon mutagenesis approaches for C. perfringens are not ideal. Indeed, until now mostly Tn916 was used, and this transposon inserts multiple times in the genome, what is not ideal in screening for virulence genes using phenotypical tests. The novel developed technique uses a phage Mu transposon and this leads to single insertions in the genome. A mutant bank of about 3300 mutants has currently already being generated. Finally a brief description was given on the use of the Clostron technique that was used in the laboratories of Nigel Minton and Julian Rood to make mutants in specific genes. To end, Fillip Van Immerseel stated that the use of comparative phylogenomics, and transposon mutagenesis combined with a good phenotypical screening method, can lead to the identification of virulence genes for necrotic enteritis in broilers. These genes can then be knocked out using the Clostron technique to validate the role of these genes in broiler necrotic enteritis.

Charlotte Valat and **Henrik Christensen** presented the work done from the European project (FP6) POULTRYFLORGUT. Poultry carry human enteric pathogens, such as Campylobacter and Salmonella, in their intestinal tracts, often without exhibiting clinical signs. Scientific and agro-food experts have recently noted increased prevalence of these and less common contaminants in flocks, possibly stemming from regulatory and other changes affecting feeding, raising techniques or processing and distribution. However, data are scarce on the effects of these changes, and knowledge

of the ecology of poultry intestinal flora is very poor, due to a lack of accurate methodology. The three-year POULTRYFLORGUT project aims at providing a strong factual basis for the optimization of the hygienic quality of the poultry products meant for human consumption, and addressing the economic assessment of changes in poultry practice and effect of organizational arrangements in the poultry food chain. It will focus on the control of the intestinal flora of the broilers and laying hens, including the food-borne pathogens. The approach to study and reduce microbial risks associated with consumption of poultry products (eggs, meat) concerns the different steps of poultry products production from farm to fork. Developing methodologies using novel molecular techniques will allow the global and integrative study of the intestinal flora of poultry: PCR-SSCP (Polymerase Chain Reaction – Single Strand Conformation Polymorphism), DGGE (Denaturing Gradient Gel Electrophoresis), FISH (Hybridization in situ par fluorescence), and T-RFLP (Restriction Fragment Length Polymorphism). It will also help to define and validate intestinal health or pathological microbiological criteria in poultry, to study the interaction between the intestinal flora and food-borne pathogens and, consequently, to improve the characterization of the digestive disorders. Both speakers presented some results on the use of the different techniques to quantify the composition of the poultry gut microbiota.

Richard Ducatelle overviewed the currently important problem of dysbacteriosis in broilers. This is a less defined problem that is clearly emerging in the EU broiler industry simultaneously with the ban of growth promoting antibiotics. It is a poorly described condition of the gut and is either or not a synonym for conditions such as ‘wet litter’, ‘non-specific bacterial enteritis’, ‘small intestinal bacterial overgrowth’, ‘malabsorption’, and many more. The common clinical denominator is increased water content of faeces and reduced digestibility of feed with indigested residues visible in the faeces. There are economical and welfare concerns resulting from dysbacteriosis. In many cases, but not all, dysbacteriosis is linked to increased feed conversion, decreased body weight and poor performance in general. It is generally believed that, dysbacteriosis is a condition in which the interaction between the gut microbiota and the host is impaired, such that the gut health is not optimal. It is suggested that the altered composition of the gut microbiota induces changes in the gut wall, including morphological changes (villus length decreases, crypt depth increases, epithelial cell damage,) and inflammatory reactions (infiltration of immune cells in the wall). This is influenced by nutrition. The combination of a suboptimal microbiota combined with effects on the gut wall would then most likely interfere with digestive processes, eventually leading to poor performance, and induce enteritis. Unpublished results of Richard Ducatelle’s laboratory illustrate that a wheat/rye (high in non-starch polysaccharides) based diet without enzymes and antimicrobial growth promoters induces massive T-cell infiltration in the gut wall, decreases the length of villi, induces villus fusion and apoptosis in the crypts and tips of the villi, and many more. This coincides with decreased performance. The situation can be reversed by adding antimicrobial growth promoters to the feed. In contrast, corn based (low in non-starch polysaccharides) diets do not result in the above mentioned problems. The gut microbiota composition in the studied groups was different, and certain microbiota groups were only present in the gut of the animals that received the wheat/rye diet, as detected by t-RFLP. The role of Clostridium in this syndrome is hitherto unclear. These data clearly show the interaction between nutrition, microbiota and the host response in dysbacteriosis. Until now, a clear definition of dysbacteriosis is missing, and it is intriguing how an undefined and under-investigated syndrome can produce such economical problems in the broiler industry.

3. Assessment of the results, contribution to the future direction of the field

The presentations and the scientific discussions at the ESF workshop led to knowledge about the C. perfringens induced diseases, since combining the human and veterinary medical knowledge on different but related (same agent) diseases led to interesting discussion on the pathogenesis of the diseases. It became clear that there are still considerable deficiencies however in the understanding of the mechanisms of virulence that lead to clinical manifestation of disease (necrotic enteritis) in poultry and the epidemiologic factors involved in the carriage of cpe positive C. perfringens strains and their transfer to humans through the food chain. It also became clear from the discussions that cpe positive C. perfringens is an important and yet underestimated cause of food poisoning in humans for which poultry meat possibly constitutes and important, yet incompletely understood source of infection.

The participants came to the conclusion that, by bringing together the expertise from the human medical and the veterinary field, progress could be made in understanding and preventing these problems. Therefore, a common structure for a FP7 proposal was supported by all participants and the major output of the ESF workshop is the current workout of a proposal by the participants. This means that research on the ESF workshop topic can be funded if successful, but even in case of a non-successful proposal research groups will certainly seek interaction with each other seen the relevant complementarities between some laboratories. Thus the results of the ESF workshop can have a large impact on research activities on the topic of C. perfringens induced diseases in animals, especially since the topic has been highly under-investigated. Furthermore, the FP7 proposal will also contain a workpackage on control strategies (vaccines, probiotics, diagnostics,) to prevent C. perfringens induced diseases in poultry, and thus this can have an impact on the development of control products by European companies.

4. Final programme

Tuesday 23 October 2007

08:35	*Welcome and introduction* **Filip Van Immerseel (Ghent, BE)**
08:45	**Presentation of the European Science Foundation (ESF)** **Martin Röllinghoff** (Standing Committee for the European Medical Research Councils)
09:00	**Group presentations**
10:00	*Coffee Break*
10:30	***C. perfringens* food poisoning: an important issue?** **Simon Hardy (Oslo, NO) and Miia Lindström (Helsinki, FI)**
11:15	***C. perfringens* toxins and virulence factors** **Richard Titball (Exeter, UK)**
12:00	*Lunch*
13:45	**Necrotic enteritis in poultry: an overview** **Magne Kaldhusdal (Oslo, NO)**
14:30	**Use molecular biology in *C. perfringens* research** **Filip Van Immerseel and Anouk Lanckriet (Ghent, BE)**
15:15	*Coffee Break*
15:45	**Dysbacteriosis and intestinal health problems in poultry** **Richard Ducatelle (Ghent, BE)**
16:30	**Framework Programme 7 structure and how it works** **Saskia Vanden Broeck (Ghent, BE)**
17:15	**Concluding discussion**
19:30	*Dinner*

Wednesday 24 October 2007

08:30	Structure and contents of European collaborative action: setting up a detailed proposal **Filip Van Immerseel (Ghent, BE)**
09:00-12:00	**Discussion on structure of project proposal: controlling necrotic enteritis based on mechanistic insights in pathogenesis of the disease**

5. Statistical information on participants

Nationalities:

Belgium	4
France	1
Germany	1
United Kingdom	3
Denmark	3
Sweden	1
Finland	2
Norway	5
Swizerland	1

Gender participation: Male 15, Female 6

6. Final list of participants

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