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Bibliography

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VIRULENCE ATTRIBUTES OF GASTROINTESTINAL *ESCHERICHIA COLI* ISOLATES

Analysis of type 1 fimbriae expression in verotoxigenic *Escherichia coli*: a comparison between serotypes O157 and O26

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Microbiology, 2001, Vol. 147, No. 1, pp. 145–152.

Previous research has shown that verotoxin-producing *Escherichia coli* (VTEC) O157 strains appear unable to express type 1 fimbriae although other serotypes such as O26 and O118 can. This study has investigated the molecular basis of this difference. The study confirmed the presence of a 16 bp deletion within the regulatory region of fimA (fim switch) in 63 VTEC O157 strains but not in other VTEC serotypes tested. The fim switch was shown to be detectable only in the phase off orientation in VTEC O157, but detection of the switch in the phase on orientation correlated with the degree of mannose-sensitive yeast agglutination in VTEC O26. Repair of the 16 bp deletion in the VTEC O157 fim switch region restored phase-variable expression of fimA in a permissive background. Non-O157 VTEC, especially O26 and O118, can be pathogenic in cattle; the role of type 1 fimbriae in this and colonization is discussed.

The AIDA autotransporter system is associated with F18 and stx2e in *Escherichia coli* isolates from pigs diagnosed with edema disease and postweaning diarrhea

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Clinical and Diagnostic Laboratory Immunology, 2001, Vol. 8, No. 1, pp. 143–149.

Pathogenic *Escherichia coli* strains are known to cause oedema disease (OD) and postweaning diarrhoea (PWD) in piglets. Although the exact mechanisms of pathogenicity that lead to ED-PWD remain to be elucidated, *E. coli*-borne Shiga-like toxin and adhesion-mediating virulence factors such as F18 adhesin or F4 fimbriae are believed to play a central role in OD-PWD. In light of these observations we investigated whether another *E. coli* adhesin, the plasmid-encoded AIDA (adhesin involved in diffuse adherence) might also be present in OD-PWD-causing *E. coli* isolates. For rapid screening for the AIDA system in large numbers of isolates, a multiplex PCR method along with a duplex Western blot procedure was developed. When screening 104 strains obtained from pigs with or without OD-PWD, we observed a high prevalence of the AIDA operon in porcine *E. coli* isolates, with over 25% of all strains being AIDA positive, and we could demonstrate a significant association of the intact AIDA gene (orfB) with OD-PWD, while defects in orfB were associated with the absence of disease. Although our data hint toward a contribution of AIDA to OD-PWD, further studies will be necessary since the presence of the AIDA genes was also associated with the presence of the Shiga-like toxin and F18 adhesin genes, two reported virulence factors for OD-PWD.

afa-8 gene cluster is carried by a pathogenicity island inserted into the tRNAPhe of human and bovine pathogenic *Escherichia coli* isolates

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Infection and Immunity, 2001, Vol. 69, No. 2, pp. 937–948.

We recently described a new afimbrial adhesin, AfaE-VIII, produced by animal strains associated with diarrhoea and septicaemia and by human isolates associated with extraintestinal infections. The afa-8 operon, encoding AfaE-VIII adhesin, from the human blood isolate *E. coli* AL862 was carried by a 61-kb genomic region

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with characteristics typical of a pathogenicity island (PAI), including a size larger than 10 kb, the presence of an integrase-encoding gene, the insertion into a tRNA locus (pheR), and the presence of a small direct repeat at each extremity. Moreover, the G+C content of the afa-8 operon (46.4%) was lower than that of the *E. coli* K-12/MG1655 chromosome (50.8%). Within this PAI, designated PAI IAL862, we identified open reading frames able to code for products similar to proteins involved in sugar utilization. Four probes spanning these sequences hybridized with 74.3% of pathogenic afa-8-positive *E. coli* strains isolated from humans and animals, 25% of human pathogenic afa-8-negative *E. coli* strains, and only 8% of fecal strains (P=0.05), indicating that these sequences are strongly associated with the afa-8 operon and that this genetic association may define a PAI widely distributed among human and animal afa-8-positive strains. One of the distinctive features of this study was that *E. coli* AL862 also carried another afa-8-containing PAI (PAI IIAL862), which appeared to be similar in size and genetic organization to PAI IAL862 and was inserted into the pheV gene. We investigated the insertion sites of afa-8-containing PAI in human and bovine pathogenic *E. coli* strains and found that this PAI preferentially inserted into the pheV gene. The GenBank accession numbers for the sequences reported herein are AF072900, AF286670 and AF286671.

Necrotoxigenic *Escherichia coli* type-2 invade and cause diarrhoea during experimental infection in colostrum-restricted newborn calves

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Veterinary Microbiology, 2001, Vol. 81, No. 4, pp. 315–329.

There exists experimental evidence that, necrotoxigenic *Escherichia coli* (NTEC) strains producing the cytotoxic necrotising factor 1 cause intestinal and extra-intestinal disease in piglets. On the other hand, no experimental model has been developed with NTEC strains producing the cytotoxic necrotising factor 2. A total of 14 colostrum-restricted calves were orally challenged with 2 strains isolated from the faeces of a diarrhoeic calf (B20a) or from the heart blood of a septicaemic calf (1404). All calves had diarrhoea that lasted until 8 of them were destroyed. In those calves, diarrhoea was correlated with the faecal excretion of the challenge strains. At necropsy, vascular congestion of the intestinal mucosa, hypertrophy of the mesenteric lymph nodes (MLN) and some congestion of the lungs were observed. Bacteriology confirmed the colonization of the intestine by the challenge strains, which were also recovered from the heart blood, lungs and liver. Histological sections confirmed enterocolitis, lymphadenitis and limited bronchopneumonia. In the intestinal tissue sections, bacteria testing positive in an *in situ* DNA hybridization assay with a CNF2 probe were observed. Those results were confirmed by immunohistochemistry with a polyclonal anti-O78 and monoclonal anti-F17b antisera. Three of the 5 control calves receiving either saline or a CNF-F17a strain (25KH09) had no clinical signs or lesions. The other 2 presented profuse liquid diarrhoea but those calves were positive for the presence of K99+ *E. coli*. In this model, both NTEC2 strains were able to colonize the intestine, cause long-lasting diarrhoea and invade the blood stream with localization in various internal organs in colostrum-restricted conventional newborn calves.

Virulence characterization of strain *Escherichia coli* – BK99

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Ciencia Rural, 2001, Vol. 31, No. 3, pp. 455–459.

In order to evaluate the pathogenicity and antimicrobial resistance pattern of BK99 *E. coli* strain, several tests were assessed: slide agglutination for detection of the fimbriae F5, STa production, haemolytic and colicin activity, pathogenicity assessment using piglet inoculation and antimicrobial resistance to drugs. The strain BK99 showed the following profile: F1+, F5+, STa+, Col V+, Hly-, STR, KAR, NOR, TTR and SFR. It produced clinical disease and death of infected piglets. Moreover, it was possible to recover the BK99 strain from diarrhoeic faeces and from the gut contents of the piglets, with high rate of recovery of colonies expressing fimbriae F5+. The present results suggest that the *E. coli* BK99 strain could produce virulence factors and experimentally reproduce neonatal colibacillosis in pigs.

Virulence factors of *Escherichia coli* isolated from diarrhoeic piglets

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Medycyna Weterynaryjna, 2001, Vol. 57, No. 7, pp. 503–506.

The isolated strains of *E. coli* from diarrhoeic pigs in northeast Poland were investigated for the presence of fimbriae with the following antigenic specificity: F4, F5,

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F6, F17, F18 and F41. Out of 204 strains studied only 4 possessed fimbriae F41, 2 F17 and 2 F4 strains. None of the genetic element coding for LT, STa, STb enterotoxins and shiga variant toxin was detected in any of the studied strains. More than 90% of the *E. coli* strains were sensitive to gentamicin, excenel, enrofloxacin, amoxicillin, lincospectin, flumequine and apramycin. The range of strains that were sensitive to neomycin, nitrofurantoin, colistin and chloramphenicol were 87.25, 75.5, 75.48 and 65.6%, respectively. Only 53.92, 51 and 50% of the strains were sensitive to coliprim, streptomycin and ampicillin. Tetracycline showed the least antimicrobial activity in the present study. Only 8.8% of the strains were susceptible to the above antibiotic.

Virulence factors in *Escherichia coli* isolated from the blood of bacteremic neonatal calves

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Veterinary Microbiology, 2001, Vol. 78, No. 3, pp. 241–249.

Twenty-five *Escherichia coli* isolates from the blood of critically ill bacteraemic calves sampled in two separate studies (1991 and 1993) on a calf-rearing farm housing over 15000 calves, in the San Joaquin Valley, California were studied. Isolates were characterized for O serogroups and for pathotypes as determined by the presence of specific virulence factors including heat-labile enterotoxin (LT), heat-stable enterotoxins a and b (STa, STb), verotoxins 1 and 2 (VT1, VT2), cytotoxic necrotizing factor (CNF), aerobactin, intimin Eae and P, F17 and CS31A fimbrial adhesins, and resistance to bactericidal effects of serum. These isolates constituted a heterogeneous group. However, isolates were mostly aerobactin positive and often resistant to the bactericidal effects of serum. Isolates of pathotypes O78 (n=6), O119:CS31a (n=3), and P positive but O non-typable (n=3) were associated with a high mortality rate. The remaining isolates belonged to diverse pathotypes, often possessing the adhesins P, F17, CS31A and Eae but belonging to O serogroups other than O78 and O119, and were less frequently associated with mortality. Although no virulence factor common to all isolates was identified, the capacity to use iron by the presence of aerobactin that is important to the capture of iron was a predominant factor. Moreover, certain pathotypes appear to be associated with primary colisepticaemia whereas other pathotypes may cause a bacteraemia without necessarily leading to septicaemia.

Identification of eae genes in *Escherichia coli* strains isolated from pigs with postweaning diarrhoea

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Veterinary Record, 2001, Vol. 148, No. 8, pp. 241–243.

The prevalence of eae genes in *E. coli* isolated from pigs with postweaning diarrhoea, as determined by PCR analysis, is described. 224 *E. coli* strains were selected from 372 strains isolated from weaned pigs. Six (2.7%) *E. coli* strains were positive for the eae gene as determined by the presence of the 840 bp-amplified product obtained with primers Int-Fc and Int-Rc. The remaining 218 (97.3%) did not generate a PCR amplicon of 840 bp or any other size. All eae-positive isolates were negative for fimbrial antigens F4, F5, F6, F17 and F41, and none of these strains possessed the fedA gene of F18 fimbriae. None of the *E. coli* strains with the eae genetic marker harboured enterotoxin genes LT1, ST1 and ST11, or shiga toxin 1 and 2, including the stx2e variant genes. The presence of the eae gene makes the strains examined similar to human enteropathogenic *E. coli* and therefore pigs, like cattle, should be considered as a reservoir of attaching and effacing *E. coli* bacteria which are potentially pathogenic for humans.

Prevalence of eae and shiga toxin genes among *Escherichia coli* strains isolated from healthy calves

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Journal of Veterinary Medicine. Series B, 2001, Vol. 48, No. 1, pp. 67–72.

Strains of *Escherichia coli* (n=390), isolated from 132 healthy 4- to 8-week-old calves, were tested by polymerase chain reaction (PCR) for the eae (intimin) gene and shiga toxin genes (stx1 and stx2). All strains were also analysed for F5, F17 and F41 fimbriae and for the heat-labile (LT) and heat-stable (STI and STII) genetic markers. Overall, the eae gene was detected in 84 (21.5 %) of the strains tested. Only 21 (5.4 %) isolates were positive for stx1 (18 strains) or stx2 (three strains); nine of the stx1-positive isolates also possessed the eae gene. A high percentage (29.2 %) of the isolates tested expressed F17 but no enterotoxin genes were detected. None of the eae- or stx-positive strains belonged to the O157 serogroup.

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Characterisation and clonal relationships of Shiga-toxigenic *Escherichia coli* (STEC) isolated from Australian dairy cattle

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Veterinary Microbiology, 2001, Vol. 79, No. 4, pp. 323–335.

A total of 136 Shiga toxin-producing *Escherichia coli* (STEC) isolated during a longitudinal survey of three Australian dairy farms [date not given] were examined to determine their virulence factors, serotype and genomic relationships. This study aimed to assess the potential of these STEC to cause disease in humans and to analyse the on-farm ecology of STEC. Virulence factors (stx, eae, ehxA) were used as determinants of potential to be enterohaemorrhagic *E. coli* (EHEC) and were examined using polymerase chain reaction (PCR). Among the cattle groups tested, calves, both before and during weaning, shed the most putative EHEC and were the main source of serotypes commonly associated with human disease. *E. coli* O157:H7 and *E. coli* O26:H11 represented 9.4 and 7.8% of cattle STEC isolates, respectively, with other putative EHEC serotypes reported for the first time from cattle. Based on serotype and virulence factors, 20% of STEC were putative EHEC. Pulsed-field gel electrophoresis (PFGE) was used to compare the genomic profiles of STEC from dairy farms. Isolates common to cattle and the farm environment were identified. Multiple strains of STEC with high clonal turnover were detected in the faeces of cattle, and isolates appeared to be specific to individual farms. To fully assess the pre-slaughter EHEC risk factors on-farm, examination of STEC virulence is as important as determination of STEC prevalence.

SURFACE PROTEINS OF *ANAPLASMA MARGINALE*

Highly conserved regions of the immunodominant major surface protein 2 of the genogroup II ehrlichial pathogen *Anaplasma marginale* are rich in naturally derived CD4+ T lymphocyte epitopes that elicit strong recall responses

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Journal of Immunology, 2001, Vol. 166, No. 2, pp. 1114–1124.

Genogroup II *Ehrlichia*, including the agent of human granulocytic ehrlichiosis, *Ehrlichia phagocytophila*, and the bovine pathogen *Anaplasma marginale*, express a markedly immunodominant outer membrane protein designated major surface protein 2 (MSP2). MSP2 is encoded by a multigene family, resulting in the expression of variant B cell epitopes. MSP2 variants are sequentially expressed in the repeated cycles of rickettsemia that characterize persistent *A. marginale* infection and control of each rickettsaemic cycle is associated with development of a variant-specific IgG response. Importantly, these persistent rickettsaemic cycles are controlled at levels 100–1000 times lower than those responsible for clinical disease during acute infection. Control of rickettsaemia during persistence could result from an anamnestic Th lymphocyte response to conserved regions of MSP2 that enhances the primary Ab response against newly emergent variants. Comparison of MSP2 variants reveals conserved N and C termini flanking the central, surface-exposed hypervariable region that represents the variant B lymphocyte epitopes. We demonstrate MSP2-specific CD4+ T lymphocyte recognition of epitopes common to several strains of *A. marginale* and the related pathogen *A. ovis*. Furthermore, T lymphocyte lines from three individuals identified six to nine overlapping peptides representing a minimum of four to seven dominant or subdominant epitopes in these conserved N and C termini. Immunodominant peptides induced high levels of IFN-gamma, a cytokine associated with protection against *Ehrlichia* and needed for rapid generation of variant-specific IgG2. The presented data support the potential importance of a strong Th lymphocyte response to invariant MSP2 epitopes in controlling rickettsaemia during persistent infection to subclinical levels.

Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells

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International Journal for Parasitology, 2001, Vol. 31, No. 2, pp. 145–153.

Anaplasma marginale is a tick-borne ehrlichial pathogen of cattle for which six major surface proteins (MSPs) have been described. The MSP1 complex, a heterodimer composed of MSP1a and MSP1b, was shown to induce a protective immune response in cattle and both proteins have been identified as putative adhesins for bovine erythrocytes. In this study the role of MSP1a and MSP1b as adhesins for bovine erythrocytes and tick cells was defined. Msp1alpha and msp1beta1 genes from the Oklahoma isolate *of A. marginale* were cloned and

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expressed in *Escherichia coli* K-12 under the control of endogenous and tac promoters for both low and high level protein expression. Expression of the recombinant polypeptides was confirmed and localized on the surface of transformed *E. coli*. The adhesion properties of MSP1a and MSP1b were determined by allowing recombinant *E. coli* expressing these surface polypeptides to react with bovine erythrocytes, *Dermacentor variabilis* gut cells and cultured tick cells derived from embryonic *Ixodes scapularis*. Adhesion of the recombinant *E. coli* to the three cell types was determined using recovery adhesion and microtitre haemagglutination assays, and by light and electron microscopy. MSP1a was shown by all methods tested to be an adhesin for bovine erythrocytes and both native and cultured tick cells. In contrast, recombinant *E. coli* expressing MSP1b adhered only to bovine erythrocytes and not to tick cells. When low expression vectors were used, single *E. coli* expressing MSP1a was seen adhering to individual tick cells while reaction of tick cells with the *E. coli*/MSP1a/high expression vector resulted in adhesion of multiple bacteria per cell. With electron microscopy, fusion of *E. coli* cell membranes expressing MSP1a or MSP1b with erythrocyte membranes was observed, as well as fusion of tick cell membranes with *E. coli* membranes expressing MSP1a. These studies demonstrated differential adhesion for MSP1a and MSP1b for which MSP1a is an *A. marginale* adhesin for both bovine erythrocytes and tick cells while MSP1b is an adhesin only for bovine erythrocytes. The role of the MSP1 complex, therefore, appears to vary among vertebrate and invertebrate hosts.

Identification of antigenic differences that discriminate between cattle vaccinated with *Anaplasma centrale* and cattle naturally infected with *Anaplasma marginale*

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International Journal for Parasitology, 2001, Vol. 31, No. 2, pp. 179–186.

Monoclonal antibodies were raised against the vaccine strain of *Anaplasma centrale* used in Australia. A monoclonal antibody that reacted with an 80 kDa antigen was used to develop an *A. centrale*-specific fluorescent antibody test that will be useful for confirming species identity in patent infections. Another monoclonal antibody that reacted with a 116 kDa antigen was used to develop an *A. centrale*-specific competitive inhibition enzyme-linked immunosorbent assay (ELISA) for the serological identification of vaccinated cattle. The sensitivity of the ELISA was 100% in cattle experimentally infected with *A. centrale*, 97.1% in a vaccinated beef herd and 98.3% in a vaccinated dairy herd. The specificity of the ELISA was 98.6% in non-vaccinated cattle outside the *A. marginale*-endemic area, 97.9% in non-vaccinated cattle within the *A. marginale*-endemic area and 100% in cattle experimentally infected with *A. marginale*. The ELISA detected antibodies to *A. centrale* in cattle up to 9 years after vaccination with no apparent decrease in sensitivity. The assay has proved extremely valuable in Australia for investigating reported failures of multivalent live vaccines used to protect cattle against anaplasmosis and babesiosis, and should be similarly useful elsewhere in the world where these types of vaccines are used, e.g. Israel and South America.

Analysis of the 16S rRNA gene sequence of *Anaplasma centrale* and its phylogenetic relatedness to other ehrlichiae

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Clinical and Diagnostic Laboratory Immunology, 2001, Vol. 8, No. 2, pp. 241–244.

The nucleotide sequence of the *Anaplasma centrale* 16S rRNA gene was determined and compared with the sequences of ehrlichial bacteria. The sequence of *A. centrale* was closely related to *Anaplasma marginale* by both level-of-similarity (98.08% identical) and distance analysis. A species-specific PCR was developed based upon the alignment data. The PCR can detect *A. centrale* DNA extracted from 10 infected bovine red blood cells in a reaction mixture. *A. centrale* DNA was amplified in the reaction, but not other related ehrlichial species. The sequence of the partial 16S rRNA gene of *A. centrale* has been deposited in the GenBank data library under the accession number AF283007.

Use of repetitive DNA elements to define genetic relationships among *Anaplasma marginale* isolates

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FEMS Microbiology Letters, 2001, Vol. 197, No. 2, pp.139–143.

A study was conducted to investigate the effectiveness of repetitive extragenic palindromic (REP)-polymerase chain reaction (PCR) and enterobacterial repetitive intergenic consensus (ERIC)-PCR in differentiating *A. marginale* strains from different regions. *A. marginale*

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isolates were obtained from cattle in different regions of Brazil (Mato Grosso do Sul, Minas Gerais, Parana, Pernambuco, Rio Grande do Sul, Sao Paulo), in Florida, USA, and an *A. centrale* strain from Uruguay. REP-PCR and ERIC-PCR revealed similar patterns among *A. marginale* isolates obtained from the same region, and a lower degree of similarity between *A. marginale* and *A. centrale*. It is concluded that both methods can differentiate *A. marginale* strains.

Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae)

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Veterinary Parasitology, 2001, Vol. 97, No. 1, pp. 65–76.

A. marginale is a tickborne ehrlichial pathogen of cattle that causes the disease anaplasmosis. 6 major surface proteins (MSPs) have been identified on *A. marginale* from cattle ticks, of which three, MSP1a, MSP4 and MSP5, are from single genes and do not vary within isolates. The other three, MSP1b, MSP2 and MSP3, are from multigene families and may vary antigenically in persistently infected cattle. Several geographic isolates have been identified in the USA, which differ in morphology, protein sequence and antigenic properties. An identifying characteristic of *A. marginale* isolates is the molecular weight of MSP1a, which varies in size among isolates due to different numbers of tandemly repeated 28–29 amino acid peptides. For these studies, genes coding for *A. marginale* MSP1a and MSP4, msp1alpha and msp4, respectively, from nine North American isolates were sequenced for phylogenetic analysis. The phylogenetic analysis strongly supports the existence of a southeastern clade of *A. marginale* comprised of Virginia and Florida isolates. Analysis of 16S rDNA fragment sequences from the *A. marginale* tick vector, *Dermacentor variabilis*, from various areas of the USA was used to evaluate possible vector-parasite co-evolution. Our phylogenetic analysis supports identity between the most parsimonious tree from the *A. marginale* MSP gene data and the tree that reflected the western and eastern clades of *D. variabilis*. These phylogenetic analyses provide information that may be important to consider when developing control strategies for anaplasmosis in the USA.

Phylogenetic relationships of *Anaplasma marginale* and *'Ehrlichia platys'* to other *Ehrlichia* species determined by GroEL amino acid sequences

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International Journal of Systematic and Evolutionary Microbiology, 2001, Vol. 51, No. 3, pp. 1143–1146.

The heat-shock protein (GroEL) genes of *Anaplasma marginale*, *Ehrlichia muris* and *'Ehrlichia platys'* were sequenced and compared with the GroEL of other species of *Ehrlichia*. The GroEL amino acid sequences of *A. marginale* and *'E. platys'* were most similar to the GroEL sequence of *E. phagocytophila*, with which they formed one group with 6–10% divergence. The *E. muris* GroEL was most closely related to the GroEL of two unclassified strains (HF-565 and Anan), then to *E. chaffeensis*, *E. canis*, *E. ewingii and Cowdria ruminantium*, forming a second distinct group (0.3–8.6% divergence). The GroELs of *E. risticii* and *E. sennetsu* were very similar to one another (only 2% divergence), forming the third group. The first two groups were relatively closely related (17–20% divergence), while the third group was only distantly related to the first two groups (62–73% divergence).

AFRICAN SWINE FEVER VIRUS IN *ORNITHODOROS*

Transovarial transmission of African swine fever virus in the argasid tick *Ornithodorus moubata*

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Medical and Veterinary Entomology, 2001, Vol. 15, No. 2, pp. 140–146.

To determine filial infection prevalence of experimentally infected colony *Ornithodorus moubata* ticks for African swine fever virus (ASFV), 3 groups of ticks were used: uninfected controls; orally infected with the VIC T90/1 isolate; and orally infected with the LIV 13/33 isolate of ASFV. Filial infection prevalences were not constant but were highly variable between egg batches from different ticks and between successive egg batches from the same tick. Filial infection prevalences ranged from 1.8% to 31.8% for ticks infected with the VICT90/1 isolate and from 1.2% to 35.5% for ticks infected with the LIV 13/33 isolate. A similar pattern was noted after the third feed. Immunohistochemisty showed that virus replicates in the developing larval cells and not in the yolk sac cells or within the outer layers of the eggs. The results show that ASFV can replicate to a high titre ($10^{5.1}\log_{10}HAD_{50}$) within the larval cells of the developing egg.

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African swine fever virus structural protein pE120R is essential for virus transport from assembly sites to plasma membrane but not for infectivity

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Journal of Virology, 2001, Vol. 75, No. 15, pp. 6758–6768.

Immunoelectron microscopy revealed that protein pE120R localizes at the surface of the intracellular virions. Consistent with this, coimmunoprecipitation assay showed that protein pE120R binds to the major capsid protein p72. Moreover, it was found that, in cells infected with an ASFV recombinant that inducibly expresses protein p72, the incorporation of pE120R into the virus particle is dependent on p72 expression. Protein pE120R was also studied using an ASFV recombinant in which E120R gene expression is regulated by the *Escherichia coli* lac repressor-operator system. In the absence of inducer, pE120R expression was reduced about 100-fold compared to that obtained with the parental virus or the recombinant virus grown under permissive conditions. One-step virus growth curves showed that, under conditions that repress pE120R expression, the titer of intracellular progeny was similar to the total virus yield obtained under permissive conditions, whereas the extracellular virus yield was about 100-fold lower than in control infections. Immunofluorescence and electron microscopy demonstrated that, under restrictive conditions, intracellular mature virions are properly assembled but remain confined to the replication areas. Altogether, these results indicate that pE120R is necessary for virus dissemination but not for virus infectivity. The data also suggest that protein pE120R might be involved in the microtubule-mediated transport of ASFV particles from the viral factories to the plasma membrane.

Effects of infection of the tick *Ornithodorus moubata* with African swine fever virus

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Medical and Veterinary Entomology, 2000, Vol. 14, No. 4, pp. 355–360.

The effects of infection with African swine fever virus (ASFV) on adult and nymphal *Ornithodorus moubata* ticks were examined in 3 groups of ticks: an uninfected control group; one group infected with the VIC T90/1 isolate of ASFV; and another group infected with the LIV 13/33 isolate of ASFV. Infection with ASFV did not affect the oviposition rates of infected ticks when compared with uninfected ticks. There was no difference between infected and uninfected ticks in progeny hatching rates and first nymphal stage feeding rates. Feeding rates of infected adult ticks were also unaffected. However, a significant increase in mortality rates was observed amongst the adult ticks that fed on an infective bloodmeal compared to ticks fed on an unifected bloodmeal.

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African swine fever virus IAP homologue inhibits caspase activation and promotes cell survival in mammalian cells

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Journal of Virology, 2001, Vol. 75, No. 6, pp. 2535–2543.

African swine fever virus (ASFV) A224L is a member of the inhibitor of apoptosis protein (IAP) family. We have investigated the antiapoptotic function of the viral IAP both in stably transfected cells and in ASFV-infected cells. A224L was able to substantially inhibit caspase activity and cell death induced by treatment with tumor necrosis factor alpha and cycloheximide or staurosporine when overexpressed in Vero cells by gene transfection. We have also observed that ASFV infection induces caspase activation and apoptosis in Vero cells. Furthermore, using a deletion mutant of ASFV lacking the A224L gene, we have shown that the viral IAP modulates the proteolytic processing of the effector cell death protease caspase-3 and the apoptosis which are induced in the infected cells. Our findings indicate that A224L interacts with the proteolytic fragment of caspase-3 and inhibits the activity of this protease during ASFV infection. These observations could indicate a conserved mechanism of action for ASFV IAP and other IAP family members to suppress apoptosis.

MYCOBACTERIUM PARATUBERCULOSIS AND THE BOVINE IMMUNE SYSTEM

An evaluation of a modified interferon-gamma assay for the detection of paratuberculosis in dairy herds

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Veterinary Immunology and Immunopathology, 2001, Vol. 79, No. 1–2, pp. 69–81.

Whole blood samples were obtained from multiple dairy herds in Pennsylvania and in Wisconsin which were

previously determined to be infected with *Mycobacterium paratuberculosis* (MpS) (Johne's disease) by faecal culture. Blood samples were shipped overnight to the National Animal Disease Centre (NADC) in Ames, IA for processing and interferon-gamma (IFN-gamma) analysis. Blood samples were incubated alone (non-stimulated) or with concanavalin A (ConA), a T-cell mitogen used as a positive control in the assay, for 18 h. In addition, samples were incubated with *M. avium* purified protein derivative (AvPPD), *M. bovis* purified protein derivative (BoPPD), or a whole cell sonicate of *M. paratuberculosis* for 18 h to elicit antigen-specific IFN-gamma production. After incubation, plasma was harvested and analyzed for IFN-gamma by ELISA. Values for IFN-gamma for non-stimulated blood samples (background) were consistently low for animals in all herds evaluated. In contrast, ConA stimulation of blood samples evoked a significant secretion of IFN-gamma regardless of infection status or faecal culture results for individual cows, indicating that immune cells were still viable after overnight shipment and capable of responding to stimulation. Antigen-specific IFN-gamma results were positively correlated with infection status as determined by previous faecal shedding and/or current faecal shedding of *M. paratuberculosis*. Accuracy of the IFN-gamma assay for correctly predicting infection status of individual cows in the herds with low levels of infection ranged from 50 to 75% when used as a single test. Combined use of the IFN-gamma test and a commercial ELISA antibody test accurately predicted infection status of 73% of cows from a dairy herd with a high level of *M. paratuberculosis* infection and 90% from a well-characterized group of dairy cows at the NADC. These results indicate that the antigen-specific IFN-gamma assay is a very sensitive diagnostic tool for detection of subclinical paratuberculosis in cattle and may be useful on an individual animal basis to remove infected animals from the herd.

Paratuberculosis in Danish dairy herds: development and testing of improved methods for the diagnosis of paratuberculosis

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International Dairy Federation Symposium on on-farm control and diagnosis of paratuberculosis, The Atomium, Brussels, Belgium, 27 January, 2001.
Bulletin of the International Dairy Federation, 2001, No. 364, pp. 60–64.

Two hundred and fifty-two cattle selected from dairy herds infected with *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) and 119 cattle selected from dairy herds presumably free of paratuberculosis were tested in a prospective cohort study. Preliminary results from the evaluation of a test for cellular immunity (IFN-gamma test) to infection with *M. a. paratuberculosis* showed a marked difference in the frequency of test-positives in the 2 cohorts. 113 (45%) animals tested positive from the infected herds and 3 (3%) animals tested positive from the presumably free herds. Consistently test-positive animals were from 9–12 months of age and older, indicating a possibility of unveiling infected animals at an early stage of infection when other tests for paratuberculosis are negative. Four culture-positive cows developing clinical paratuberculosis changed from test-positive to test-negative in the IFN-gamma test. We assume that a change from test-positive to test-negative in IFN-gamma test may predict onset of clinical paratuberculosis. However, further results from the prospective study are necessary to strengthen these assumptions.

Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections

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Journal of Veterinary Medicine, Series B, 2001, Vol. 48, No. 3, pp. 185–195.

After immunization of 4 calves with a live modified *Mycobacterium paratuberculosis* vaccine the course of the humoral and cell mediated immune reactions was studied during a 2-year clinical investigation. Furthermore, the possibility of shedding of the vaccine strain and the influence of the vaccination on the tuberculin skin test was determined. In addition to standard procedures recently developed diagnostic methods (antibody enzyme linked immunosorbent assay, interferon-gamma test, polymerase chain reaction) were used. A cell mediated immune reaction, reflected in an increased, specifically induced, interferon-gamma production developed much earlier (1–2 weeks post-immunization) than humoral immunity (8–16 weeks post-gamma immunization). While the increase in antibody titres was transient, declining to extremely low levels 48–60 weeks post-immunization, cell mediated immunity remained detectable until the end of the investigation. Spread of the vaccine strain into the body and shedding were never detected during the whole course of the study except for one colon site in one calf. As late as 2 years after vaccine application positive or doubtful skin reactions against *M. bovis* purified protein

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derivative were measured, reflecting possible interference of the immunization with the diagnosis of bovine tuberculosis. At the end of the investigation, a positive cell mediated immune reaction was also detected in the control animal although clinical, pathological and bacteriological examinations gave no indication for a mycobacterial infection.

Fibronectin attachment protein homologue mediates fibronectin binding by *Mycobacterium avium* subsp. *paratuberculosis*

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Infection and Immunity, 2001, Vol. 69, No. 4, pp. 2075–2082.

Attachment of *Mycobacterium avium* subsp. *paratuberculosis* to host tissue and penetration of mucosal surfaces are pivotal events in the pathogenesis of Johne's disease. Fibronectin (FN) binding is required for attachment and internalization of several mycobacteria by epithelial cells *in vitro*. The objective of this study was to further characterize the FN binding activity of *M. avium* subsp. *paratuberculosis*. *M. avium* subsp. *paratuberculosis* strains 5781 and 6594 were recovered from faeces of adult beef cattle with clinical paratuberculosis. Although the bacteria bound FN poorly at pH above 7, brief acid pretreatment greatly enhanced FN binding within the pH range (3 to 10) studied. A 4.6-kbp fragment from an *M. avium* subsp. *paratuberculosis* genomic library was found to contain a 1107-bp open reading frame that shows very high nucleotide sequence identity with that of the FN attachment protein (FAP) gene of *M. avium* subsp. *avium*. Pretreatment of FN with an FN-binding peptide from *M. avium* subsp. *avium* FAP abolished FN binding, indicating that *M. avium* subsp. *paratuberculosis* binds FN in a FAP-dependent manner. Pretreatment of *M. avium* subsp. *paratuberculosis* with anti-FAP immunoglobulin G did not abrogate FN binding; blocking occurred only when anti-FAP was added together with FN. FAP was detected by immunofluorescence only in lipid-extracted *M. avium* subsp. *paratuberculosis*. Western blotting and immunoelectron microscopy revealed that FAP is located near the interior of the cell envelope of *M. avium* subsp. *paratuberculosis*. The results indicate that a FAP homologue mediates the attachment of FN to *M. avium* subsp. *paratuberculosis*. Further, given the subcellular location of FAP, it is considered that this protein operates at the terminus of a coordinated FN binding system in the cell envelope of *M. avium* subsp. *paratuberculosis*. The nucleotide sequences of FAP-P has been assigned the GenBank accession number AY007557.

Diagnostic techniques for paratuberculosis

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Bulletin of the International Dairy Federation, 2001, No. 362, pp. 5–17.

This report reviews the currently available diagnostic techniques applied for detection *of M. paratuberculosis* infection in dairy cattle. Available techniques are based on detection of the etiologic agent or detection of the immune response of the host. Traditional culture is still widely used, but alternative methods, such as radiometric culture media, have proven superior to traditional culture in analytical sensitivity and speed of detection. IS900 based polymerase chain reaction (PCR) may become an alternative if problems of PCR inhibition are efficiently overcome. The absorbed indirect enzyme-linked immunosorbent assay-technique is the preferred method for detection of antibodies to *M. paratuberculosis*. However, antibody detection can only be applied on cattle no younger than 15 months of age. Detection of cell-mediated immunity, by interferon-gamma enzyme immunoassay, may become preferable for early diagnosis of paratuberculosis if problems of unspecific antigens and uncertainty in interpretation are overcome. A combination of immunological tests for screening purposes and herd-level information, and agent detection-based techniques for diagnosis confirmation are preferable. The application of more than one test and quantitative interpretation of test results provide valuable tools for diagnosis of paratuberculosis.

Use of Hoechst 33342 staining to detect apoptotic changes in bovine mononuclear phagocytes infected with *Mycobacterium avium* subsp. *paratuberculosis*

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Clinical and Diagnostic Laboratory Immunology, 2001, Vol. 8, No. 2, pp. 460–464.

Mycobacterium avium subsp. *paratuberculosis* is an intracellular pathogen of macrophages that causes a chronic enteritis (Johne's disease) in ruminants. The purpose of this study was to determine whether *M. avium* subsp. *paratuberculosis* infection causes apoptosis in bovine monocytes. Using Hoechst 33342 staining, we observed increased numbers of apoptotic monocytes within 6 h of infection with *M. avium* subsp. *paratuberculosis*, and these numbers increased further at 24 and 48 h. This effect appeared to require viable bacilli, because monocytes infected with heat-killed *M. avium*

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subsp. *paratuberculosis* did not exhibit a significant increase in apoptosis. Preincubation of monocytes with bovine growth hormone prior to infection with *M. avium* subsp. *paratuberculosis* did not significantly alter the number of apoptotic cells.

Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection

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Infection and Immunity, 2001, Vol. 69, No. 3, pp. 1492–1498.

Bovine paratuberculosis is caused by infection of young calves with *M. avium* subsp. *paratuberculosis*. In some of the chronically infected cows the long asymptomatic stage (2 to 4 years) is followed by a rapid progression to a clinical stage due to protein-losing enteropathy, which will ultimately be fatal. The current dogma is that in early stages of disease the cell-mediated responses predominate, whereas in the clinical stage of the disease the humoral responses prevail, possibly signaling a switch in immune reactivity related to disease progression. We developed IgM-, IgA-, and IgG1- and IgG2-isotype-specific ELISA for *M. avium* subsp. *paratuberculosis*-derived antigens (heat shock proteins of 70 kDa (Hsp70) and 65 kDa (Hsp65), lipoarabinomannan (LAM), and *M. avium* subsp. *paratuberculosis* purified protein derivative PPD (PPDP)). The serological responses of cows (n=176) in different stages of paratuberculosis were used to evaluated the putative shift in immune responsiveness. In the clinical stage, the PPDP-specific IgG1 responses were increased compared to those in the asymptomatic stage. However, total IgG1 and IgG2 and the Hsp70-, Hsp65-, and LAM-specific isotype responses were decreased in the clinical stage compared to those in the asymptomatic stage of disease. Thus, the classical pattern was found only for PPDP antigens and the IgG1 isotype. For other antigens and isotypes and the total IgG levels, the response pattern was different and indicates that there is no uniform association with increased antibody responses during the progression from the asymptomatic stage to the clinical stage of bovine paratuberculosis. The complete nucleotide sequence of *M. avium* subsp. *paratuberculosis* Hsp70 is available in the GenBank database under the accession number AF254578.

Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds

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American Journal of Veterinary Research, 2001, Vol. 62, No. 2, pp. 270–274.

A study was conducted to determine whether vaccination with a killed vaccine prevents faecal shedding of *M. avium* subsp. *paratuberculosis* in cattle, to compare effectiveness of a culture and cull programme in vaccinated (n=25) and nonvaccinated (n=29) herds and to compare paratuberculosis-related preventive management in vaccinated (n=2) and nonvaccinated (n=2) herds. 58 commercial dairy herds in the Netherlands were studied. Differences were not detected among the 25 herds that were vaccinated; culture results were positive for *M. avium* subsp. *paratuberculosis* in 4.4% of herds. In 29 herds that had not been vaccinated, culture results were positive in 6.7%. The percentage of positive results on culture decreased from 10.9% and 5.7% to 3.5% and 0%, respectively, in the 2 vaccinated herds. In the 2 non-vaccinated herds, percentages decreased from 6.1% and 16.5% to 0% and 2.3%, respectively. Management practices were different between herds that were vaccinated and herds that were not; owners of herds that were not vaccinated followed more preventive management procedures and practiced less feeding of raw milk to calves. It is concluded that vaccination of calves with a killed vaccine does not prevent transmission of *M. avium* subsp. *paratuberculosis*; therefore, hygienic practices remain essential in herd management.

Regulation of expression of major histocompatibility antigens by bovine macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* or *Mycobacterium avium* subsp. *avium*

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Infection and Immunity, 2001, Vol. 69, No. 2, pp. 1002–1008.

M. avium subsp. *paratuberculosis* and *M. avium* subsp. *avium* are antigenically and genetically very similar organisms; however, they differ markedly in their virulence for cattle. We evaluated the capacity of bovine macrophages infected with *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* to express major histocompatibility

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complex (MHC) class I and class II antigens on their surface and to interact with primed autologous lymphocytes. Infection of bovine macrophages with *M. avium* subsp. *paratuberculosis* promoted the downregulation of MHC class I and class II molecules on the macrophage surface within 24 and 12 h, respectively. Alternatively, MHC class II expression by *M. avium* subsp. *avium*-infected macrophages was not detected until 24 h after infection, and the magnitude of the decrease was smaller. Decreased MHC class I expression by *M. avium* subsp. *avium*-infected macrophages was not detected. Unlike *M. avium* subsp. *paratuberculosis*-infected macrophages, *M. avium* subsp. *avium*-infected macrophages upregulated MHC class I and class II expression after activation by gamma interferon or tumour necrosis factor alpha. Further, *M. avium* subsp. *avium*-infected macrophages were lysed by primed autologous lymphocytes, whereas M. *avium* subsp. *paratuberculosis*-infected macrophages were not. Overall, the results support the hypothesis that the difference in the virulence of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* for cattle is dependent on a difference in the capacity of the organisms to suppress mycobacterial antigen presentation to T lymphocytes.

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