Offered Talks and Poster Section

The conference was structured around invited keynote lectures supported by offered papers in related areas from delegates. These offered papers were allocated to talk or poster presentations and submitted abstracts collated for publication in two sections:

1) Talks (prefaced by the letter T) - Speakers are denoted by an asterisk.
2) Posters (prefaced by the letter P).

T1. Taxonomy of the *Pasteurellaceae:* A Difficult Problem Even When the Phylogeny is Known.

F.E. Dewhirst*, B.J. Paster, G.J. Fraser and I.Olsen, Department of Molecular Genetics, Forsyth Dental Center, Boston, MA 02115, USA and Department of Microbiology, Dental Faculty, University of Oslo, Oslo, Norway.

We have determined full 16S rRNA sequences for over 90 strains representing 80 species in the family *Pasteurellaceae*. Since our most recent report on the phylogeny of this group (Zbl. Bakt. 279:35-44, 1993), we have obtained sequences for 16 additional strains: Bisgaard taxa 10, 15, 16, 17, 18, and 20, *Pasteurella haemolytica* CCUG 28148, "A/P-like" CCUG 28030, H. *influenzae-murium* CCUG 6515, P. *haemolytica-* like CCUG 28018, *Haemophilus*-like CCUG 19808, *H. paragallinarum*-like CCUG 18783, P. *multocida* ss *septica* CCUG 17977, and three strains of *A. actinomycetemcomitans*. Sequences were determined by direct Sanger sequencing of purified 16S rRNA. Sequences were aligned and a homology matrix was determined by using only those positions at which 90% of strains possessed sequence information. A phylogenetic tree was constructed using the neighbour joining method. The new strains are all members of the family *Pasteurellaceae* and fall within clusters described previously. If the 16S rRNA based analysis accurately reflects the phylogeny of the *Pasteurellaceae,* our results present a dilemma for the taxonomy of the family. Branching of clusters within the family are deep (bush like) and complex. It would seem that the family needs to be divided to over 20 genera, or reduced to a single genera.

T2. Deoxyribonucleic Acid Relatedness Among Porcine V Factor-Dependent *Pasteurellaceae.*

K. Mϕller*, National Veterinary Laboratory, Bülowsvej 27, DK-1790, Copenhagen; M. Kilian, Institute of Medical Microbiology, University of Aarhus, Denmark; F. Grimont and P.A.D. Grimont, Service des Enterobacteries, Institut Pasteur, F-75724 Paris Cedex 15, France.

Deoxyribonucleic acid relatedness studies (S1 nuclease method) of selected V factor-dependent *Pasteurellacea* from the porcine respiratory tract showed that taxon Minor group and the taxa previously designated C, D/E, and F form distinct groups only distantly related to each other (1 to 26% homology), and to other selected members of the family *Pasteurellaceae* (3 to 39% homology). Three homology groups were identified in taxon Minor group and two in taxa D/E and F indicating some heterogeneity within the individual taxa. The study confirms that the taxa Minor group, C, D/E, and F warrant specific recognition. However, the exact genus affiliation can not be concluded on the basis of available data.

T3. Population Genetics of Non-Capsulate *Haemophilus influenzae.*

K.J. Forbes* and T.H. Pennington, Medical Microbiology, Aberdeen University, Aberdeen, U.K.

Haemophilus influenzae is an important human pathogen with the preponderance of invasive disease isolates being capsulate, usually type b, and non-invasive disease isolates typically being non-capsulate. Several studies have demonstrated that the genetic structure of these two groups are completely different. Linkage disequilibrium of genetic markers is found amongst the capsulate strains with extensive genetic similarity being shared between members of each clone. This is in stark contrast to non-capsulate strains where genetic similarities are minimal and groups of indistinguishable strains (clones) are often small in size and indeed frequently contain only single representatives. This genetic heterogeneity of non-capsulate strains has been analysed using genotypic methods: genomic DNA fingerprinting, Southern hybridisation, and gene RFLP's and sequencing. Each of these methods can be used to establish genetic relationships between strains but the conclusions

that can be drawn from these are strongly influenced by the method used. These comparative analyses can be used to test the hypothesis that if the predominant mechanism of genomic evolution in non-capsulate strains is solely vertical transmission of genetic material, then strain phylogenies should all be identical irrespective of the method. If this is not the case, then horizontal transmission of genetic material must have disrupted the original clonal framework of the strains.

T4. Use of Biogrouping and DNA Fingerprinting to Monitor Transmission of *Pasteurella* Between Bighorn and Domestic Sheep.

A.C.S. Ward,* M.D. Jaworski and D.L.Hunter, University of Idaho, Caine Veterinary Teaching and Research Center, Caldwell, Idaho USA.

Deaths resulting from respiratory infections associated with *Pasteurella* species occur in both domestic and wild sheep populations. Exposure of animals to strains of *Pasteurella* new to their population may result in colonization of nasal mucosa, tonsillar crypts, and in some instances disease. Tests were conducted on over 300 *Pasteurella* isolates from bighorn and domestic sheep which had been in close proximity to monitor transmission. Biochemical tests (biogrouping) provided a basis for initial screening of the isolates. *Pasteurella* isolates having the same biogroup were subjected to DNA fingerprinting as a final step to determine if transmission had occurred between sheep groups. Transmission from domestic to bighorn sheep was conclusively demonstrated in contact pen exposure with disease being evident in both sheep species. Organisms with identical fingerprints were also detected in one range situation without any evidence of disease in either sheep populations. The route of transmission was not demonstrable in the latter case.

T5. Characterization of *Actinobacillus seminis*.

J.C. Low[1], I. Bromley[1], W. Donachie[2], D. Somerville[1], [1]SACVS Edinburgh, Bush Estate, Penicuik, Midlothian, [2]Moredun Research Institute, Edinburgh.

The identification of Gram-negative pleomorphic bacilli isolated from ovine semen is difficult as many of the bacterial species associated with ram infertility are biochemically inactive and published descriptions are often ambiguous and misleading. In this study, isolates of *Actinobacillus seminis* from clinical specimens, semen and vaginal swabs and other members of the *Pasteurellaceae* family have been compared on the basis of cultural characteristics, biochemical reactions, APIZYM profiles and by examining their outer membrane protein profiles in SDS-PAGE. This study was funded by SOAFD.

T6. Protective Capacity of the Antigenic 40-Kilodalton LppB Lipoprotein of *Haemophilus somnus* Against Experimental Infection in Cattle.

C.R. Rioux*[1],R.J. Harland,[1] M. Theisen[1], N.A. Rawlyk[1] and A.A. Potter[1&2], Veterinary Infectious Disease Organization[1] and Canadian Bacterial Diseases Network,[2] Saskatoon, Saskatchewan, Canada.

We have previously reported the cloning and characterization of the gene *lppB* coding for a 40-kDa outer membrane lipoprotein of *H. somnus*. *Escherichia coli* transformants producing LppB adsorbed Congo red, a property associated with virulence in pathogenic bacteria, and reacted strongly with bovine hyperimmune serum raised against *H. somnus*. The deduced amino acid sequence of LppB shows similarities to that of new lipoprotein D (NlpD) of *E. coli*. Recombinant LppB was found at low levels in *E. coli* as seroreactive polypeptides, in the 25- to 40-kDa range, which could be partially purified by aggregate preparation. To evaluate the protective capacity of LppB against experimental *H. somnus* challenge, cattle were vaccinated twice with recombinant LppB prior to intravenous infection with virulent *H. somnus*. Compared to placebo animals, cattle vaccinated with LppB showed less severe clinical signs such as fever, weight loss, and

lameness. The survival rates were 0 and 88% for the placebo and vaccinated calves, respectively. LppB elicited a strong, specific antibody response in cattle. Therefore, LppB is a potential antigen for use in vaccines to prevent haemophilosis.

T7. Protection of Ruminants with *Pasteurella haemolytica* A1 Capsular Polysaccharide (CP) Vaccines Containing MDP or MDP-GDP.

K. Brogden,* B. DeBey, F. Audibert, H. Lehmkuhl and L. Chedid. USDA-ARS-NADC Ames, IA; Univ. California, Tulare, CA; and VACSYN S. A. Paris, France.

To improve CP immunogenicity, vaccines were prepared containing 1.0 mg CP with and without MDP (range 0.2 - 1.0 mg) or MDP-GDP (range 0.1 - 1.0 mg). After immunisation, vaccines with MDP or MDP-GDP induced significantly higher IgM, IgG1, and IgG2 titres. After challenge, lambs vaccinated with CP+0.05 mg MDP or CP+MDP-GDP had significantly less pulmonary consolidation and concentrations of *P. haemolytica* in lesions compared to CP vaccinated or nonvaccinated lambs. After challenge, calves vaccinated with CP containing 0.2 mg MDP, 0.5 mg MDP, or 1.0 mg MDP-GDP also had significantly less pulmonary consolidation and concentrations of *P. haemolytica* in lung lesions compared to CP vaccinated or nonvaccinated calves. Vaccines containing CP+0.5 mg MDP and CP+1.0 mg MDP-GDP induced bactericidal antibodies by 7 days and were more efficacious than two commercial vaccines.

T8. Induction of RTX Neutralizing Antibody by Exposure of Pigs to a Unique Strain of *Actinobacillus suis* and Resultant Protection Against *Actinobacillus pleuropneumoniae.*

B. Fenwick, Department of Pathology and Microbiology, Kansas State University.

Eight *Actinobacillus pleuropneumoniae* (App) free pigs from a newly derived high health status herd were inoculated intranasally with a live strain of *A. suis*. Very mild clinical signs developed approximately 8 hours after the inoculations and soon resolved. Within three weeks significant App hemolysin neutralising titers developed, yet all pigs remained negative to App by complement fixation assay and ELISA. Eight additional age matched 'control' pigs from the same herd were mixed with the *A. suis* exposed pigs and both groups challenged intranasally with the type strain of App serotype 1. Two days later all pigs were killed and post-mortem examinations conducted. Prior exposure to *A. suis* and the resultant induction of cross neutralising titers provided significant ($P<0.04$) protection against App disease. These findings have implications in the understanding of the factors that may influence the occurrence of disease by those organisms that produce related RTX proteins as well as suggesting novel methods by which to provide protection.

T9. Seroepidemiology of Undifferentiated Fever in Feedlot Calves.

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A seroepidemiological study was conducted in western Canada to investigate the relationships between *Pasteurella haemolytica*, *Haemophilus somnus*, undifferentiated fever, and mortality in 1,219 feedlot calves. Blood samples were obtained from each animal upon arrival at the feedlot and again at approximately 33 days of the feeding period. In addition, interim samples were obtained from animals which required treatment for undifferentiated fever (Fever) and a corresponding number of healthy control animals (Control). The overall, haemophilosis, and BRD or haemophilosis mortality rates were significantly ($p<0.05$) higher in the Fever group than in the Control group. The Fever animals had significantly ($p<0.05$) lower *Pasteurella haemolytica* anti-leukotoxin (PHAL) titers at arrival than the Control animals. In addition, the factorial change in PHAL titers of the Fever animals from arrival to the convalescent sampling (4.7X) was significantly ($p<0.05$) higher than the factorial change in PHAL titers over the same interval for the

control animals (3.5X). There were no significant ($p \geq 0.05$) differences in the *Haemophilus somnus* (HS) arrival titers, interim titres, or the factorial changes in titers from arrival to the interim sampling between the Fever and Control animals. However, the convalescent HS titres of the Fever animals were significantly ($p<0.05$) lower than the Control animals. The HS arrival titers of animals that died of BRD were significantly ($p<0.05$) lower than the Control animals. Moreover, the factorial change in HS titers of the Fever animals from arrival to the convalescent sampling (2.2X) was significantly ($p<0.05$) lower than the factorial change in HS titers over the same interval for the Control animals (3.2X). The HS arrival titers of animals that died of BRD were significantly ($p<0.05$) lower than the HS arrival titers of animals which lived. In summary, the results of this study show that both *Pasteurella haemolytica* and *Hemophilus somnus* are significant pathoges of the early feeding period. Moreover, animals which develop undifferentiated fever and/or die during the feeding period are less likely to respond serologically to one or both of these pathogens. Additional studies are necessary to investigate these relationships further.

T10. Large-Scale Use of PCR for the Detection of Toxigenic *Pasteurella multocida* in Nasal and Tonsillar Swabs of Pigs.

E. Kamp[1]*, G. Bokken[1], T. Vermeulen[1], M. de Jong[2], H. Buys[1], F. Reek and M. Smits[1], [1]Institute for Animal Science and Health, P.O. Box 65, 8200 AB, Lelystad, [2]Animal Health Service Centre, P.O. Box 9, 7400 AA Deventer, The Netherlands.

We developed a PCR for the detection of toxigenic *Pasteurella multocida* by using two primer sets in the gene that encodes the dermonecrotic toxin. Target DNA in clinical samples was isolated with GuSCN and diatom earth as described by Boom *et al.* (J. Clin. Microbiol. (1990) 28: 495). To enable large scale use of the PCR we adapted this method to a microtiter plate format. In this way we could process 96 clinical samples simultaneously. To test the sensitivity of the PCR we examined nasal or tonsillar swabs from 345 pigs of 7 herds known to be infected with the toxigenic *P. multocida* by conventional bacteriological methods and by PCR. Toxigenic *P. multocida* were isolated from 23 samples, forty-two samples were found positive in PCR. All samples positive for toxigenic *P. multocida* were also positive in PCR. To test the specificity of the PCR we examined swabs of 211 pigs of 5 herds which have a certificate to be free from toxigenic *P. multocida*. Toxigenic *P. multocida* were not isolated from these samples. Two samples from pigs of one herd were positive in PCR. We concluded that the PCR is more sensitive than bacteriological examination and that the PCR has a high sensitivity.

T11. Field Evaluation of *Pasteurella haemolytica* Immunogen in Lambs.

F. Morales A.; L.Jaramillo M.; J. Tortora P. and F.Trigo T.*
CENID-MICROBIOLOGIA/FMVZ/UNAM Cd. Universitaria, Mexico D.F. 04510

The purpose of this work was to evaluate the efficacy and inocuity of a live *Pasteurella haemolytica* (PH) immunogen in sheep in the field. The study was conducted in five farms located in the Ajusco area, near Mexico City. Ninety six lambs were subcutaneously immunized with a live culture of PH. Eighty one lambs were sham inoculated and remained as the control group. Before and after immunization anti-capsule and anti-cytotoxin antibodies to PH were evaluated. Clinico pathological follow up was also conducted with the lambs until weaning. Anti-capsule and anticytotoxin antibody titres showed seroconversion to the immunization ($p < 0.05$). Five animals developed pneumonia, 4 in the control group (4.9%) and only one (1%) in the vaccinated group. Sera samples were taken for serological analysis when the animals presented clinical pneumonia and 15 days later. Results revealed that pneumonic animals were seronegative to anti-cytotoxin antibodies and that 15 days later presented a titre higher than in the vaccinated animals. These results suggest that the infection was caused by PH and that its cytotoxin played an important role in the disease. It can also be considered that the protection

induced by the PH immunization was capable of reducing pneumonia in lambs. Lastly, the inocuity of the immunogen was demonstrated by the absence of post-vaccination reactions.

T12. Synergistic Effect of a P2-Homologous Outer Membrane Protein and ApxI Toxin in Protection Against *Actinobacillus pleuropneumoniae.*

J.F. van den Bosch*, A.N.B. Pubben, F.G.A. van Vugt, and R.P.A.M. Segers. Intervet International, P.O.Box 31, NL-5830 AA Boxmeer, The Netherlands.

A 42kDa outer membrane protein (OMP), expressed by all *A. pleuropneumoniae* (App) serotypes, was found to be homologous to the P2-OMP of *H. influenzae* type b. The strongly cytotoxic and haemolytic RTX toxin ApxI (formerly HlyI) is expressed by App serotypes 1, 5a, 5b, 9, 10 and 11. Efficacy experiments in pigs revealed that vaccines containing either OMP or ApxI did protect against mortality but not against the development of lung lesions after challenge with App serotype 1 or 5a, whereas vaccines containing both OMP and ApxI induced full protection. Experiments in mice showed a synergistic effect of OMP and ApxI antigens with regard to protection against App serotype 1 challenge. It was concluded that App subunit vaccines should contain the common P2-homologous OMP in addition to Apx toxins.

T13. A Novel *Pasteurella haemolytica* A1 Vaccine Killed by Ultraviolet Light was Efficacious in Goats Against a Transthoracic Challenge.

C.W. Purdy*, USDA-ARS, Bovine Respiratory Disease Unit, Bushland, TX; D. C. Straus, Texas Tech University, HSC, Lubbock, TX, U.S.A.

A 10 hour old pool of virulent *Pasteurella haemolytica* A1 (PhA1) containing 10^{10} CFU/ml was killed by ultraviolet light (315 nm) exposure for 1 hour. The PhA1 bacterin was tested repeatedly for viability: none was found. On days 0 and 21, 25 goats each received lml of PhA1 (10^{10}CFU/ml) subcutaneously (SC) with various carrier systems which determined the bacterin treatment groups: agar beads (AG, n=6); polyacrylamide beads (PA, n=7); saline suspension (SA, n=6); and oil adjuvant (OA, n=6). Positive controls (PC, n=9) were injected transthoracicly into the left lung with 1 ml of 7.25 X 10^5 live PhA1 in PA (1st vaccination) and 4.4 X 10^6 live PhA1 in PA (2nd vaccination). Negative controls were injected SC with only PA on days O and 21. All goats were challenged in the right lung with 2.07 X 10^8 live PhA1on day 35 and necropsied 4 days later. Vaccine efficacy was demonstrated by smaller ($P < 0.0001$) (cm^3) consolidated pneumonic tissue lesions in the vaccinated groups at the challenge site: NC, 160; PC, 1; PA,6; AG, 7; SA, 8; OA, 2.

T14. Comparison of DNA from Organisms Classified with the 3rd Taxon of *Pasteurella haemolytica* or *P. haemolytica, sensu stricto.*

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Organisms which could not be classified with *P. haemolytica* biotypes A or T were originally classified as a 3rd taxon of *P. haemolytica* by Frederiksen. Previous investigations have linked some members of this group with *P. haemolytica* biovar 1 (*P. haemolytica* sensu stricto). The results of the present study, which included ribotyping of 32 and 28 strains respectively of 3rd taxon and biovar 1, demonstrated 37 ribotypes after digestion with HindIII. Computation using Taxan4 showed the existence of 6 major clusters within the 3rd taxon. With the exception of one strain all strains of biovar 1 clustered together. Using different coefficients of similarity or clustering methods did not change the overall clustering pattern. Within the 3rd taxon a correlation was demonstrated between the observed clusters and fermentation of L(+) arabinose and decarboxylation of ornithine. Three strains representing the 3rd taxon, which according to previously published

DNA:DNA hybridization represent separate species, were allocated to 3 different clusters in the present study. However, strain P 733 of 3rd taxon which according to DNA:DNA hybridizations showed 85% similarity with the type strain of *P. haemolytica* did not cluster with biovar 1. Further investigations are needed before final conclusions can be drawn as to the taxonomical position of the 3rd taxon of *P. haemolytica*.

T15. *In vivo* Association of *Actinobacillus pleuropneumoniae* with the Respiratory Epithelium of Pigs.

P. Dom*, F. Haesebrouck, R. Ducatelle and G. Charlier[1], [1]Laboratory of Veterinary Bacteriology, University of Gent and NIDO, Brussels, Belgium.

The ability of *Actinobacillus pleuropneumoniae* serotype 2, 5, 9 and 10 strains to associate *in vivo* with the epithelium of the porcine respiratory tract was investigated after intranasal inoculation of hysterectomy-derived and colostrum-deprived pigs. At 90 minutes post-inoculation multiple focal early inflammatory pulmonary lesions were observed in which histologically different, more or less concentric zones could be distinguished. In the centre of these pneumonic areas bacteria were associated with infiltrated cells and exudate. In the surrounding zones, bacteria were intimately associated with the epithelium of the alveoli and the cilia of the terminal bronchioles, as observed by light- and electron microscopy. Bacteria were only sporadically associated with the epithelium of the upper respiratory tract. In view of these findings, we propose the hypothesis that adherence of *A. pleuropneumoniae* to lower respiratory epithelial cells, possibly leading to a local high concentration of RTX toxins at the surface of eukaryotic cells, constitutes an important initial step in pathogenesis. (This study was supported by IWONL, Brussels, Belgium).

T16. Virulence of Different Serovars of *Haemophilus parasuis* for Cesarean-Derived, Colostrum-Deprived Pigs.

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Strains representing *H. parasuis* serovars 2, 4, 5, 12, 13, 14, and a nontypeable strain, were evaluated for virulence using an intratracheal inoculation model in CDCD pigs. Inoculation of only 100 CFUs of some strains resulted in systemic infection and death within a few days post-challenge. Based on the number of CFUs required to produce death, all strains representing serovars 2, 5, 12, 13, and the nontypeable strain, were highly virulent, while two strains representing serovar 4 were of moderate virulence. Two serovar 14 strains, which differed in outer membrane protein (OMP) profile, were also examined. One serovar 14 strain caused death in all pigs inoculated with as few as 3×10^3 CFU, while the other strain caused death in only 1 of 3 pigs inoculated with as many as 1×10^8 CFU. These data concur with previous reports indicating that both serovar and OMP profile may be predictors of virulence for *H. parasuis*.

T17. Ciliostasis and Colonization of Toxigenic *Pasteurella multocida*.

J.P. Nielson[1,2]* and S. Rosendall[1], [1]University of Guelph, Ontario, Canada, [2]National Veterinary Laboratory, Copenhagen, Denmark.

The relation between ciliostasis and predisposing factors to nasal colonization with toxigenic *Pasteurella multocida* was studied. Ciliostasis was induced within 4 hours in cultured explants of nasal mucosa by acetic acid (0.01%), hydrogen peroxide (0.05%), and *Bordetella bronchiseptica* (5×10^8 CFU/ml), while PMT was ciliostatic at a concentration of 50 µg/ml only. Toxigenic *P. multocida* in concentrations from 10^8 CFU/ml to 10^{10} CFU/ml did not affect ciliary beating. Unilateral intranasal installation of 1% acetic acid or 10 % hydrogen peroxide, caused a unilateral impairment of the mucociliary transport but intraperitoneal injection of 300 ng PMT/ kg body weight did not. Pigs treated unilaterally

intranasally for two days with 5 % hydrogen peroxide developed unilateral short-lived colonization with toxigenic *P. multocida,* and a significant unilateral turbinate atrophy.

These findings indicate that ciliary arrest might be a mechanism common to predisposing factors for nasal infection with toxigenic *P. multocida.*

T18. Determination of the Hyaluronic Acid Presence in the Capsular Material of *Pasteurella Multocida* Types A and D Isolated from Porcine Lungs.

J.A. Gutierrez-Pabello[1], J.E. Smith[2] and F. Suarez-Guemes[1*], [1]Departamento de Microbiologia e Immunologia, FMVZ, UNAM Mexico D.F. 04510, [2]University of Surrey, Guildford, Surrey, UK.

A total of 20 strains of *Pasteurella multocida* was isolated from porcine lungs. Seven smooth colony strains belonged to serotype D, whereas 12 highly mucoid and one smooth strains were classified as type A by the indirect haemagglutination test. A salt gradient paper chromatography was used for the detection of hyaluronic acid. All *P. multocida* strains tested, developed a blue spot at the same level as the purified hyaluronic acid did. Serotype A strains showed a more distinct and bigger spot than serotype D cultures. Findings suggest that the capsular material of serotype D strains may contain hyaluronic acid as serotype A does, or may have a different substance that migrates to the same level.

T19. *Actinobacillus pleuropneumoniae* Encodes a Periplasmic Copper Zinc Superoxide Dismutase.

P.R. Langford* and J.S. Kroll, Molecular Infectious Diseases Group, Department of Paediatrics, St Mary's Hospital Medical School, London, W2 1PG.

Bacterial superoxide dismutases (SODs) are metallo-enzymes which form important cytosolic defences against oxygen toxicity. We have recently discovered that *A. pleuropneumoniae* has both a conventional MnSOD (SodA) and a second enzyme, CuZnSOD (SodC) - a rare bacterial example of this protein. The deduced amino acid sequence contains an N terminal domain characteristic of a signal peptide, suggesting that the enzyme is exported. Attempts to isolate periplasmic contents of *A. pleuropneumoniae* were unsuccessful and we therefore used an indirect strategy to examine CuZnSOD location. We expressed a fusion of *5'-sodC* to a promoterless gene encoding leader-peptide-deficient TEM1-β-lactamase in *E. coli.* As β-lactamase activity in the fusion is only detectable if the protein is exported to the periplasm, this allows identification of leader peptides. Clones growing on ampicillin-containing medium were found to have in-frame fusions downstream of the putative leader peptide. A clone with an in-frame fusion within this region did not have β-lactamase activity. These results strongly suggest that *A. pleuropneumoniae* CuZnSOD is periplasmic. Superoxide can cross the bacterial outer membrane but not the cytoplasmic membrane, suggesting that CuZnSOD may protect against an exogenous source of superoxide, produced for example during host defence activity.

This work was supported by the Biotechnology and Biological Sciences Reseach Council.

T20. *Pasteurella multocida* Toxin as a Mitogen.

A.J. Lax*, M.G. Smyth, P.B. Mullan and P.N. Ward. Institute for Animal Health, Compton, Newbury, Berks, RG16 0NN, U K.

We have previously shown that the *Pasteurella multocida* toxin (PMT) is a potent mitogen for cultured Swiss 3T3 fibroblast cells where it stimulates the initiation of DNA synthesis and an increase in cell number in quiescent cells. Its sequence shares a motif found in several toxins which ADP ribosylate a target[2] , but mutations within this motif do not affect its activity[3]. PMT has limited homology to the *Escherichia coli* toxin CNF1[4], but its

significance is unknown. We will report here on the sensitivity of PMT to a variety of proteases, and the effects of denaturants on the toxin. The effects of site directed mutagenesis on toxic activity will also be described. We have shown that PMT is also a potent mitogen for primary derived osteoblasts and chondrocytes.

Rozengurt *et al.* (1990) Proc Nat Acad Sci USA 87, 123 - 127: 2. Lax *et al.*(1990) FEBS Letters 277, 59 - 64: 3. Ward *et al.* (1994). FEBS Letters 342, 81 - 84: 4. Falbo *et al.* (1993). Infect. Immun. 61, 4909 - 4914.

T21. Species Specificity of *Actinobacillus actinomycetemcomitans (Aa)* Leukotoxin (Ltx)

E.T. Lally and I.R. Kieba, University of Pennsylvania, Philadelphia, PA, USA 19104-6002.

Aa Ltx belongs to the RTX (repeats in toxin) family of bacterial cytolysins. While extensive amino acid homology exists amongst the various RTX, the cellular and species specificities remain unique for individual toxins. For example, *Aa* Ltx will only kill human monomyelocytes while the *P. haemolytica (Ph)* leukotoxin *(lkt)* kills bovine lymphoid cells. The present studies were undertaken to determine the domain of Ltx that is responsible for species specificity. PCR-generated fragments of the *lktA* gene from *Ph* were spliced into naturally occurring restriction sites of the *ltxA* gene. After expression in tandem with the *ltxC* gene from the Pλ promoter, cells were sonicated and the ability of the respective lysates to kill either HL-60 (human) or BL3 (bovine) cells was assessed by trypan blue exclusion. Over 75% of the *ltxA* gene product can be replaced with *lktA* with no effect on its ability to kill the human cells. The critical area required for the chimeric toxins to recognize human target cells is a 246 aa fragment (residues 694-940) that contains the GGXGXDXUX glycine-rich repeats. Initial attempts to produce mutants with splice sites within this region resulted in loss of toxicity. Recently, the three-dimensional structure of a related protein with glycine-rich repeats has been solved. Utilization of atomic coordinates from these data should permit construction of a model of the repeat region of the *A. actinomycetemcomitans* leukotoxin and subsequent development of toxin chimeras that further define the fine structure of species specificity of RTX.

T22. Identification and Structural Characterisation of an Iron- Regulated Haemopexin Receptor in *Haemophilus influenzae* Type B.

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Haemophilus influenzae type b (Hib) uses haem as a source of essential porphyrin and iron and is capable of acquiring haem bound to the serum haem-binding protein haemopexin. We therefore examined the mechanism by which this pathogen acquired haem from haemopexin. Haemopexin binding to whole cells of Hib after growth in an iron-restricted medium was a specific, time-dependent, saturable process. From competitive binding assays with ^{125}I labelled haem-haemopexin, the affinity (K_2) was estimated to be 0.3 μM with approximately 3600 receptors per cell. Haemopexin affinity chromatography of solubilised membranes yielded three proteins of 57, 38 and 29 kDa. Trypsinisation of whole cells resulted in the loss of haemopexin-binding and correlated with the degradation of only the 57 kDa protein indicating that this protein, which can be renatured to bind haemopexin after SDS-PAGE and Western blotting, is the major surface exposed component of the haemopexin receptor. N-terminal amino-acid sequence analysis indicated that the 38 kDa protein was in fact the outer membrane porin protein P2. These data establish the existence of a haemopexin receptor and indicate a possible role for the P2 porin in the acquisition of haem from haemopexin.

T23. Cloning of a DNA Sequence Specific to Type B Isolates of *Pasteurella multocida* that Cause Haemorrhagic Septicaemia (HS).

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Haemorrhagic septicaemia (HS) is a peracute disease of cattle and buffalo that is caused by specific serotypes of the bacterium *Pasteurella multocida*. During the last fifty years, five main serotyping systems have been developed to classify the *P. multocida* species. Despite the attempts at classification, there has been no system developed to date that will definitively identify HS-causing strains of *P. multocida*. A modified subtractive hybridisation method has allowed the isolation of a DNA sequence that specifically hybridises to type B isolates that cause HS. This technique involves competitive hybridisation of *Sau* 3A l-digested DNA from the isolate 0131 (Izatnager 25) and biotin labelled sonicated DNA from 0130 (Izatnager 52). Double stranded digested DNA was enriched by paramagnetic separation and subsequently cloned into a plasmid vector. Recombinant clones were screened against a range of *P.multocida* isolates by slot blot hybridisation. Analysis by this method has identified a candidate clone for identification of Carter type B HS-causing isolates of *P.multocida*.

T24. *Haemophilus felis* as a Cause of Disease in the Cat.

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21 isolates of *Haemophilus* sp were obtained from cats and six isolates were characterised biochemically and found to resemble *H. felis*. The organisms were isolated from healthy SPF cats and from cases of disease such as pleurisy, conjunctivitis, rhinitis, pharyngitis, pneumonia, bronchitis and from the vagina in infertility.Six isolates were obtained in pure culture suggesting that the organism might be a cause of disease. Further evidence for this supposition was provided by the demonstration by immunoblotting of antibody to an isolate obtained from an SPF cat with respiratory disease following successful treatment of the disease.

T25. Cloning of a DNA Region Putatively Involved in the Encapsulation of *Actinobacillus pleuropneumoniae.*

C.K. Ward and T.J. Inzana*, Virginia Tech, Blacksburg, VA.

The capsular polysaccharide (CP) is a required virulence factor of *Actinobacillus pleuropneumoniae* (Ap). Our aim is to clone and sequence the serotype 5 capsule *sfc* genes to better define the role of capsule in virulence and immunoprotection. A 5.3 kb *xbal* fragment of strain J45 chromosomal DNA that hybridized with two DNA probes specific for the *Haemophilus influenzae* type b (Hib) Region 1 (capsule export) genes was cloned and restriction mapped. Portions of this cloned Ap DNA hybridized with genomic DNA from Ap serotypes 1, 2, 7, 9, indicating conservation among serotypes. Chromosomal walking was used to clone an adjacent 5.8 kb region of Ap DNA that would presumably encode structural genes required for CP biosynthesis. A portion of this adjacent DNA hybridized with DNA from other Ap serotype 5 strains, but not with DNA from Ap serotypes 2, 7, or 9, indicating serotype-specificity of this region. In addition, our cloned export genes could complement and partially restore the mutated *kps* export genes of *E. coli* Kl. These results indicate that the Ap capsule belongs to the conserved group II family of capsules in Gram-negative bacteria.

T26. Cloning and Characterization of a Bacteriophage Lysozyme Gene from *Haemophilus somnus*.

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Haemophilus somnus is an opportunistic pathogen associated with several bovine disease syndromes. The ability to scavenge the essential nutrient iron from the host is well established for bacterial pathogens. An *in vitro* plate bioassay was used to determine that *H. somnus* was capable of using bovine transferrin, haemoglobin, or haemin as a sole source of iron. A genomic cosmid library was screened for clones able to bind haemin (Hmb+). An Hmb+ clone was isolated and the phenotype was localized to a 1.6 kb fragment. DNA sequencing and Tn*phoA* mutagenesis indicated that an open reading frame *(hmb)* coding for a secreted 21.4 kDa polypeptide was responsible for the Hmb+ phenotype. Anti-Hmb antiserum detected a recombinant 22,000 M_r protein in Western blots. The predicted amino acid sequence of Hmb was homologous to the primary sequence of lysozymes (murein hydrolase; EC 3.2.1.17) from several bacteriophages. Furthermore, *hmb* could complement amber mutations in the "lysozyme" genes of bacteriophages lambda (*R* gene) and P2 (*K* gene), enabling these mutant bacteriophages to lyse a non-permissive *E. coli* host. Whole cell lysates of *E. coli* expressing Hmb could lyse EDTA-killed *E. coli* cells. These data suggest that *hmb* encodes a lysozyme of bacteriophage origin. The presence of bacteriophages in *H. somnus* is now being investigated.

T27. Construction and Analysis of a *Pasteurella haemolytica* Mutant Unable to Synthesize Three Membrane Lipoproteins.

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A single genetic locus from *Pasteurella haemolytica* serotype A1, consisting of three tandemly arranged genes encoding 28-30 kDa membrane lipoproteins, was replaced with a mutated locus which carries the β-lactamase-encoding gene from a 4.2 kbp *P. haemolytica* plasmid. The inactivated locus was introduced into *P. haemolytica* by electroporation of a plasmid which carries the mutated locus but is incapable of replicating in *P. haemolytica*. Southern and western blot analyses indicate that the wild-type locus was replaced by the mutated locus through a double crossover recombination event and that the three membrane lipoproteins were no longer produced by the mutant strain. The effects of this mutation on the growth of *P. haemolytica in vitro* and on the production of other *P. haemolytica* membrane proteins, capsule, and leukotoxin were examined. These methods for mutagenesis should be useful in constructing mutant loci which can be used to analyze the roles for various *P. haemolytica* proteins in the pathogenesis of bovine pneumonic pasteurellosis.

T28. Preliminary Characterization of the Latent Haemolysin Induced in *Escherichia coli* by the *Actinobacillus pleuropneumoniae* HLYX Protein.

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The *hlyX* gene of the swine pathogen *A. pleuropneumoniae* encodes a protein which is very similar to the global regulatory protein, FNR of *E. coli* and like FNR, can regulate the expression of the *frdA*, *ndhII*, and *nar* genes. In addition, HlyX is able to induce the synthesis of a latent haemolysin. Haemolysin production is highest during late log phase and can be detected only in cells grown under anoxic conditions. Non haemolytic mutants of *E. coli*/pT51 *(hlyX+)* were created using Tn*phoA*/Tn*phoA'* elements carried on lambda phage. From P1 transduction studies, all of these mutations appeared to map to the same

region. The sequence of the regions flanking one of the transposon insertions was determined, but no homology could be detected with any known *E. coli* gene. Haemolytic recombinants were also isolated in *E. coli (hlyX-)* carrying a wild type *E. coli* mini Mu library. These cells grew poorly and were only weakly haemolytic. When *hlyX was* introduced, growth rates and haemolytic activity increased.

T29. Plasmid Mediated NAD-Independent *Haemophilus Paragallinarum* and Their Use in Poultry Vaccine Manufacture.

R.R. Bragg*, L. Coetzee and J.A. Verschoor, University of Pretoria, South Africa.

Since 1990, NAD-independent isolates of *H. paragallinarum* have been isolated from chickens showing typical symptoms of infectious coryza in South Africa. These NAD-independent isolates were tested with a panel of three locally produced monoclonal antibodies (Mab) against *H. paragallinarum.* Mab patterns obtained for the NAD independent isolates were similar to those obtained with the normal, NAD dependent strains. Plasmid mediated NAD independence has been reported in *H. Parainfluenzae.* In the light of this work, it was decided to attempt plasmid transformation, with the NAD-independent isolates as plasmid donors and well characterized *H. paragallinarum* isolates as plasmid recipients. This was successfully carried out. Strain 0083, which is widely used in the manufacture of poultry vaccines against infectious coryza, was successfully transformed into a NAD-independent strain. It was established, using chicken raised antibodies against strain 0083 that the hemagglutinin of the transformed isolates was not affected during transformation. It is thus theoretically possible to use transformed NAD-independent strains of *H. paragallinarum* for the manufacture of poultry vaccines.

T30. Global Distribution of DNA Sequences Associated with Site Specific Integration of Large Antibiotic Resistance Plasmids in *Haemophilus influenzae.*

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A large conjugative *Haemophilus influenzae* resistance plasmid has been shown to excise from a specific location in the tRNA leucine gene of a *H. influenzae* strain isolated in the U.K. (strain 1056). The geographical extent of this phenomenon is unknown. Antibiotic resistant isolates of *H. influenzae* from North America, South America, Greece, Asia and the U.K. were analyzed by PCR and DNA sequencing to identify similarity between these disparate strains and strain 1056. PCR primers were designed to amplify the *att* P site of free plasmid and the *att* L and *att R* sites (junction fragments) of integrated plasmid. Strains from each geographical location amplified product with *att* P, *att* L and *att* R primers. PCR products of *att* P and *att* R from strains representative of each geographical location were sequenced and found to have high homology. This data shows that plasmid from a world-wide distribution share common sequences associated with recombination and excision of plasmid. These plasmids all integrate at the same site in the tRNA leucine gene of *H. influenzae,* thus suggesting a common mode of site specific recombination.

T31. Development of *Actinobacillus pleuropneumoniae* as a Bacterial Vaccine Vector.

C.T. Prideaux*, L. Pearce, C.L. Wright and A.L.M. Hodgson, CSIRO Division of Animal Health, PMBI, Parkville, Australia, 3052.

Actinobacillus pleuropneumoniae (APP) is the causative agent of porcine pleuropneumonia, a severe respiratory disease of pigs. We are currently developing APP for use as a bacterial vaccine vector system capable of delivering and expressing foreign genes in pigs. The establishment of such a system requires the identification of a number of key elements such as promoters to regulate foreign gene expression, secretory signals to allow export of foreign proteins from the bacteria, and a broad host range plasmid shuttle system suitable for the introduction, and stable expression of foreign genes in APP. Currently we are evaluating two plasmids for use in vector construction, pIG1 and

pIG3, obtained from Australian pig isolates of *Pasteurella multocida* and APP respectively. These two plasmids have been evaluated for their ability to transform APP isolates, their rate of loss from APP during passage without the presence of selectable pressure, and the ability of foreign genes inserted onto these plasmids to be authentically expressed utilising various promoters.

T32. Construction and Characterization of a *Pasteurella- Actinobacllus-Escherichia coli* Shuttle Vector.

C.L.Wright[1,2]*, R.A. Strugnell[2] and A.L.M. Hodgson[1], [1]CSIRO Division of Animal Health, Parkville, Victoria 3052, Australia, [2]Department of Microbiology, Melbourne University, Parkville, Victoria 3052, Australia.

The Gram-negative organism, serotype D *Pasteurella multocida* (Pm), is a causative agent of swine atrophic rhinitis (AR), a chronic disease of young pigs that results in the atrophy of nasal turbinates, facial deformities and reduced growth rates. A plasmid vector system for Pm will be required to enable the development of a new, live recombinant AR vaccine. A 5.4 kb streptomycin and sulphonamide resistance plasmid (pIG1) was isolated from an Australian strain of Pm and was not only found to replicate in *E.coli*, but also *Actinobacillus pleuropneumoniae* and *Pasteurella haemolytica*. Nucleotide sequence analysis of pIG1 revealed that approximately half of the plasmid consisted of the drug resistance genes and the remainder encoded replication and putative mobilisation functions. A series of vectors have been constructed that contain the replication region from pIG1, various antibiotic resistance genes and a multiple cloning site. These plasmids should facilitate the genetic manipulation of Pm and other members of the HAP group.

T33. Outer Membrane Protein and Lipopolysaccharide Analyses as Taxonomic and Epidemiological Tools for *Pasteurella haemolytica.*

R.L. Davies, Department of Microbiology, University of Glasgow, Scotland, U.K.

The outer membrane protein (OMP) and lipopolysaccharide (LPS) profiles of over 200 isolates of *P. haemolytica* were examined by SDS-PAGE and Western-blotting analysis. The isolates analyzed included representatives of all the recognized A and T serotypes as well as various untypeable isolates, and originated from both Europe and North America. Analysis of OMP and LPS profiles using these techniques was capable of differentiating between isolates having the same serotype, of demonstrating similarities between isolates of different serotypes, and of demonstrating both similarities and significant differences between untypable and typable isolates. The usefulness and applications of these techniques in taxonomic and epidemiological studies of *P. haemolytica* will be discussed in further detail.

T34. Invasive-Disease-Causing and Nasopharyngeal Isolates of *Haemophilus influenzae* Type B are Different.

N.I. Leaves*, M.. Barbour, J.Z. Jordens, T.E.A. Peto and D.W.M. Crook, PHLS Haemophilus Reference Laboratory, Oxford, U.K.

Haemophilus influenzae type b (Hib) is a commensal of the human upper respiratory tract, from where carriage can result in invasive disease. As part of an investigation into the possible bacterial factors associated with the progression from carriage to invasive disease, fifty-six naso-pharyngeal isolates of Hib (NP-Hib) from healthy subjects were compared with fifty-nine invasive isolates of Hib (I-Hib) from the same region and time period. The isolates were characterised using outer membrane proteins (OMPs), ribotyping and capsular genotyping. They were also examined for the presence of large plasmids associated with ampicillin resistance using whole plasmid probing and a PCR-based technique. The I-Hib population structure, when assayed using ribotyping and OMPs, was similar to that described previously. However, the NP-Hib population possessed a smaller

proportion of the major clone (P=0.06) and a more varied selection of the more unusual subtypes; there was no difference in capsular genotype distribution. Plasmid DNA was found in a significantly higher proportion of NP-Hib than I-Hib (P = 0.006) and was not always associated with antibiotic resistance. These results suggest that the population structure of I-Hib is different from NP-Hib and that I-Hib is a subset of NP-Hib. Further, the preponderance of plasmid in NP-Hib suggests that strains containing plasmid are less likely to cause invasive Hib disease.

T35. Characterisation of Porcine Isolates of *Pasteurella multocida*, Including the use of Multilocus Enzyme Electrophoresis (MEE).

M.P. White[1]*, G. Chew[2], and D.J. Hampson[2], [1]University of Sydney, NSW, Australia, [2]Murdoch University School of Veterinary Studies, WA, Australia.

P. multocida is associated with 2 important clinical syndromes in pigs: pneumonia and atrophic rhinitis. A total of 115 predominantly porcine respiratory tract isolates which had been presumptively identified as *P. multocida* was subjected to capsular typing, somatic serotyping, toxigenicity testing, biochemical activity profiling, subspeciation and MEE. Whole cell protein profiles and outer membrane protein profiles on SDS-PAGE were produced from 25 isolates. Several significant correlations were observed between the MEE type of isolates and their capsule type, somatic serotype, biochemical activity and subspecies classification.

(This work was funded by the Australian Pig Research and Development Corporation).

T36. Multivariate Analysis of Fatty Acid Contents of Outer Membrane Vesicles from *Actinobacillus*, *Haemophilus* and *Pasteurella* spp.

I. Olsen*, V. Myhrvold and I. Brondz, Department of Oral Biology, University of Oslo, Oslo, Norway.

Outer membrane vesicles are released from Gram-negative cell walls during their growth. The vesicles may either penetrate tissue locally or are spread with the blood to more peripheral sites. Vesicles are probably important virulence factors due to their contents of enzymes and toxins. The present study used gas chromatography to analyse the fatty acid contents of isolated outer membrane vesicles in spp. of *Actinobacillus*, *Haemophilus* and *Pasteurella*. Both reference and clinical strains were used. Data were treated with multivariate analysis which distinguished between *Actinobacillus actinomycetemcomitans* (class 1) and *Haemophilus influenzae* (class 2) (F-test, 95% confidence limits). *Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, and *Pasteurella multocida* were distinct from both these classes, except for *H. aphrophilus* ATCC 33389^T which fell in class 1 (border line). C14-3OH was a major fatty acid in all species while C10-3OH, C12-3OH, and C16-3OH were minor components. *H. aphrophilus* exhibited simpler chromatograms than *A. actinomycetemcomitans*: it did not contain C10-3OH or C16-3OH fatty acids. The abundance of 3OH fatty acids suggested that outer membrane vesicles from the examined species are rich in lipopolysaccharide.

T37. Heterogeneity of *Actinobacillus actinomycetemcomitans* Demonstrated with Multivariate Analysis of Fatty Acid Data from Outer Membrane Vesicles.

I. Brondz*, V. Myhrvold and I. Olsen, Department of Oral Biology, University of Oslo, Oslo, Norway.

We have previously used a variety of chemical characters and multivariate chemosystematics to demonstrate that *Actinobacillus actinomycetemcomitans* is a heterogeneous species (I. Brondz and I. Olsen, Oral Microbiol Immunol 1993;8:129-133). In the present study, the fatty acid contents of isolated outer membrane vesicles were used to see if this heterogeneity could be confirmed. A difference in the fatty

acid contents of outer membrane vesicles from *A. actinomycetemcomitans* strains may be of importance to the variation in their virulence. Fatty acid data from *A. actinomycetemcomitans*, which were provided after gas chromatographic analysis, were treated with SIMCA analysis and compared with those of similar data from vesicles isolated from *H. aphrophilus, H. paraphrophilus, H. influenzae* and *P. multocida*. *A. actinomycetemcomitans* could be divided into two strain groups which were distinguished at 95% confidence limits (F-test). One group contained ATCC 33384^T, ATCC 29522, SUNY 366, HK 435, and Q 1247; the other comprised ATCC 29524, ATCC 29523, FDC 2112, FDC 511, and SUNY 489. This assignment of strains into groups supported our previous findings. Both groups were distinct from *H. aphrophilus, H. paraphrophilus, H. influenzae,* and *P. multocida*. Presence of OH acids suggested that vesicles of the examined species are abundant with lipopolysaccharide.

T38. Effects of Antibiotic on Host TNFα and PGE_2 Responses in Sheep after Subcutaneous Infection with *Pasteurella haemolytica* Biotype T Serotype 15.

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Pasteurella haemolytica biotype T produces systemic disease in lambs similar in speed and clinical signs to endotoxic shock. Endotoxic shock comprises a cascade of reactions involving endotoxin and inflammatory mediators, particularly TNFα and PGE_2. Preliminary results in 3 lambs showed that antibiotic (oxytetracycline) may inhibit the release of TNFα and help prolong survival. In the present work sixteen specific pathogen-free lambs aged 10 weeks were dosed subcutaneously with 3 x 10^8cfu *P. haemolytica* T15. Seven lambs served as controls, 9 were treated with oxytetracycline intramuscularly 2-3 hours post-infection (p.i.). Blood samples taken before and at intervals after infection were analysed for bacteria, TNFα and PGE_2 and selected metabolites. Bacteraemia, detected at 2 hours p.i. in all control and 7 treated lambs, was controlled by antibiotic. One lamb died immediately after treatment. All lambs became febrile (rectal temperature ≥ 40.5°C) by 2.5 ± 0.2 hours p.i. and clinical and biochemical signs for lambs which died were typical of endotoxic shock. Mean (± SEM) age at death was 8 ± 3 hours (n=7) and 20 ± 4 hours (n=7) for control and treated lambs, respectively. PGE_2 concentrations increased in 14 lambs (peak concentrations ranging from 16-307pg/ml plasma) but significant TNFα responses (peak range 4-55ng/ml plasma) occurred only in control lambs.

T39. Inflammatory Cytokines and Lung Injury in Bovine Pneumonic Pasteurellosis.

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We hypothesise that acute lung injury seen in bovine pneumonic pasteurellosis is caused by interactions between various cellular constituents in the lung and inflammatory mediators, stemming from the initial interaction of *Pasteurella haemolytica* LPS and leukotoxin with alveolar macrophages (AMs). A large body of experimental data suggests that AM-derived tumor necrosis factor α (TNFα) and interleukin-1β(IL-1β) indeed are responsible in the pathogenesis of lung injury seen in animal models of sepsis-related human adult respiratory distress syndrome (ARDS). These findings are especially relevant because the pathogenesis of lung injury seen in bovine pneumonic pasteurellosis resembles human ARDS lung injury. In this study, we examined the kinetics of gene expression and release of TNFα and IL-1β by bovine AMs stimulated with LPS or purified leukotoxin (LPS-free) obtained from *P. haemolytica* Al. Results from Northern blot analysis indicate that there was a time-dependent expression of TNFα and IL-1β mRNA by AMs stimulated with LPS. Peak TNFα and IL-1β mRNA expression was seen at 1 hr, declined significantly by 16 hr and was undetectable at 24 hr after stimulation with 1 μg/ml of LPS. Production of bioactive

TNFα peaked at 4 hr while bioactive IL-1β production peaked at 24 hr. There was a dose dependent increase in concentration of bioactive TNFα and IL-1β secreted by bovine AMs stimulated in the presence of 0.1-10 μg/ml of LPS. The expression of cytokines mRNA and secretion by LPS-stimulated AMs were inhibited by polymyxin B. AMs stimulated with purifed leukotoxin at subcytolytic concentrations also expressed TNFα and IL-1β mRNA with peak steady state mRNA levels observed with 1 leukotoxin unit/ml. The kinetics of mRNA expression observed with leukotoxin-stimulated AMs differed from that of LPS-stimulated AMs and was not inhibited by polymyxin B. Using *in situ* hybridization, we have demonstrated TNFα and IL-1β expression associated with pneumonic lung lesions from *P. haemolytica* induced experimental pneumonic pasteurellosis. These data suggest that AM-derived inflammatory cytokines may contribute to the pathogenesis of lung injury in bovine pneumonic pasteurellosis.

T40. Passive Immune Cross-Protection in Mice with Antisera to Haemorrhagic Septicaemia and Fowl Cholera Strains of *Pasteurella multocida.*

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Mice were passively immunised with antisera made against *P. multocida* that cause haemorrhagic septicaemia (HS; serotypes B:l, B:2, B:3,4, B:4, E:2) and fowl cholera (FC; serotypes A:3, A:5). They were then challenged with homologous and heterologous serotypes. All serogroup B antisera protected against all serogroup B strains, regardless of somatic serotype. Reciprocal cross-protection did not occur among serogroup A strains. Some antisera produced cross-protection which did not correlate to specific somatic or capsule antigens. Cross-protection also occurred between certain strains that cause HS and those that cause FC. This cross-protection was not related to antigens that react in the capsule indirect haemagglutination test, H proteins, or a and b soluble antigens. Immunoblots of the various HS and FC serotypes that reacted with cross-protective antisera showed at least 20 bands of common mobility. The most intense common reactions occurred with antigens in the 59-87 kDa range.

T41. Immune Responses of Calves to Different *Pasteurella haemolytica* A1 Vaccines.

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This study was designed to evaluate the immune responses of three experimental streptomycin dependent (Sm^D) live *Pasteurella haemolytica-Pasteurella multocida* (BioCor, Omaha, NE) vaccines grown in different media (A, B, and C), two commercial bacterin/toxoid vaccines available in the United States, and two experimental subunit vaccines derived from *P. haemolytica* 12296, one consisting of leukotoxin (LKT), capsular polysaccharides (CP), and outer membrane proteins, and the other containing iron regulated outer membrane proteins (IROMPs) and CP. Four ELISAs were optimized to evaluate the ability of the vaccines to elicit antibody responses against the LKT, CP, surface antigens (SA), and IROMPs of *P. haemolytica* A1. The ideal cut-off for each ELISA was determined using a receiver operating characteristic curve (ROC) on sera from 30 field vaccinated and 30 prevaccinated cattle. At these cut-off points the specificity and sensitivity of the ELISAs against SA, IROMPs, LKT, and CP were 86% and 90%, 86% and 100%, 72% and 100%, and 88% and 100% respectively. Sm^D vaccine trial was performed in conventionally raised calves. Sm^D vaccinates showed significant increases in antibody levels against CP and SA, while no increases were detected against LKT and IROMPs. Analysis of magnitude of antibody responses in Sm^D vaccinates indicated a better efficacy of the vaccine produced in media C. Studies evaluating serologic responses to LKT, SA, CP, and IROMPs generated by commercial and subunit vaccines are underway. Preliminary results

with commercial vaccines indicate poor antibody responses against LKT. In addition, challenge studies will be used to determine the vaccine efficacy in terms of lung lesion and clinical scores and their correlation to the magnitude of antibody responses to LKT, CP, SA, and IROMPs.

T42. Nad (V-Factor) - Independent and Typical *Haemophilus paragallinarum* Infection in Commercial Chickens: a Five Year Field Study.

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An unusual bacterium causing respiratory disease in chickens emerged in South Africa in February 1989. The disease clinically resembled infectious coryza but the organism differed from typical *Haemophilus paragallinarum* especially in that it did not require V-factor for growth. The bacterium has been termed an NAD-independent *H. paragallinarum*. A study of avian Haemophili isolated from diseased chickens in Natal over the past five years showed three types to be present; typical *H. paragallinarum*, NAD-independent *H. paragallinarum* and *H. avium* (now transferred to the genus *Pasteurella*). Before the end of 1989 the NAD-independent *H. paragallinarum* had become the predominant isolate and thereafter was isolated from commercial chickens in other regions of South Africa. The disease affected all strains of chickens in an overall age range of 14 days to 66 weeks and was responsible for upper respiratory disease of layers and upper and lower respiratory disease in broilers. It was commonly isolated from diseased adult birds previously vaccinated against typical *H. paragallinarum*. Broilers were most commonly infected from 3 weeks of age and layers within the placement to peak production period.

T43. Antigens Involved in Cross-Protection Between *Actinobacillus pleuropneumoniae* Biotypes-Serotypes in Pigs.

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Four groups of 5 gnotobiotic pigs were intranasally inoculated with an *Actinobacillus pleuropneumoniae* biotype 1-serotype 2 (*App* 1-2) strain (producing RTX toxins ApxII and ApxIII), an *App* 1-10 strain (producing ApxI), an *App* 2-2 strain (producing ApxII) or saline (controls), respectively. Six weeks later, all pigs were exposed to *App* 1-2 endobronchial challenge. Severe clinical signs were only observed in all control pigs, one pig immunized with *App* 1-10 and two pigs immunized with *App* 2-2. These pigs died within 36 hours post inoculation. In the other pigs, clinical signs were mild or absent, and none of these pigs died. At the time of challenge neutralizing antibodies against ApxI only, Apx II only and both Apx II and III were present in sera of pigs immunized with *App* 1-10, *App* 2-2 and *App* 1-2, respectively. Opsonizing antibodies against *App* 1-2 were only detected in sera of 8 of the 12 pigs that survived. SDS-PAGE and Western Blot analysis revealed that supernatants of 12-hour-old cultures of the *App* strains contained 3 common antigens. These results indicate that immune mechanisms other than Apx neutralizing antibodies were involved in cross-protection of pigs immunized against *App* 1-10 and challenged with *App* 1-2. (This study was supported by IWONL, Brussels, Belgium).

T44. Lesions of *Actinobacillus suis* Infection in Swine.

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Gross, histological and microbiological findings from a retrospective study of 36 confirmed cases of *Actinobacillus suis* infection in swine necropsied since 1988 will be presented and discussed. In the majority of cases, *A. suis* was isolated alone; in the remainder usually with one other of a variety of pathogens, including *Streptococcus suis* and *Pasteurella* species, with one case of concurrent cytomegalovirus infection. Age of affected animals ranged from 4 days to young adult, with the majority of cases occurring from 2 to 16 weeks. The multiple manifestations of the disease include septicaemia; polyserositis; arthritis and periarthritis; endocarditis; embolic pneumonia, hepatitis and nephritis. Pleuro-pneumonia has been seen also, and with increasing frequency in the last two years. An association with minimal disease herd status has been noted. Pathogenesis will be discussed. Is this an emerging disease?

T45. Efficacy And Safety Of A *Pasteurella haemolytica* A1 Bacterin Toxoid (One Shot™) Under Experimental Conditions.

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A *P. haemolytica* Bacterin Toxoid vaccine (One Shot™) has been developed for the protection of cattle against pneumonic pasteurellosis. Beef calves challenged at two weeks post-vaccination showed an 81% (subcutaneous [SC] vaccination) to 94% (intramuscular [IM] vaccination) reduction in lung lesions compared to controls. One SC dose offered little protection to animals challenged with *P. haemolytica* at 3 days post-vaccination, but provided a 52% reduction in lung involvement, compared to controls, when challenged at 7 days post-vaccination. An 87% reduction in lung lesions, compared to controls, occurred when animals maintained under field conditions were challenged at 4.25 months post-SC vaccination. *P. haemolytica* was consistently re-isolated from lung lesions. Serological status of animals did not necessarily correlate with protection. Vaccination of cattle at twice the recommended antigen level or with five vaccinations over a four month period caused no significant systemic reactions. A significant rise in body temperature occurred at 8 hours post-vaccination, but returned to normal levels by 24 hours post-vaccination. Localized swellings at the injection site in some animals were encountered by either route of administration, but decreased in size over time. The vaccine is considered safe and efficacious by either route of administration.

P1. The Efficacy of a *Pasteurella haemolytica* A1 Vaccine Given as a Single Dose Against Experimental Pneumonic Challenge In Cattle.

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The efficacy of single dose administration of a commercially available *Pasteurella haemolytica* A1 culture supernatant vaccine (PRESPONSE) was tested in an experimental challenge model in cattle. One dose of vaccine was as effective in protecting cattle from development of pneumonia as was administration of the recommended 2 doses. Also, leukotoxin neutralizing and bacterial agglutinating titres were similar in both groups on the day of challenge and in both cases, the titres were correlated with protection. It is hypothesized that calves are naturally primed to *P. haemolytica* through commensal colonization of the upper respiratory tract, and therefore, respond in an anamnestic nature to delivery of a single dose of the vaccine. Both serologic and protection results presented here support this theory of natural priming.

P2. Experimental Evaluation of *Pasteurella haemolytica* Immunogen in Lambs.

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This study was conducted to evaluate the protective effect of live *Pasteurella haemolytica* (PH) and its leukotoxin on lambs upon challenge. Forty lambs were used and randomly distributed in four groups. Group one was the control group, receiving subcutaneously only growth media. Group two was subcutaneously immunized with a live culture of PH Al. Group three was intradermically immunized with a live culture of PH. Group four received PH leukotoxin, which was subcutaneously administered with complete Freund's adjuvant. All animals were exposed with PI-3 virus and then challenged with a field strain of PH Al, and subsequently slaughtered to evaluate pulmonary lesions. Control lambs presented fever after challenge. Anticapsule and anti-leukotoxin antibody titres were higher in all immunized groups compared with the control group ($p< 0.05$). Pulmonary lesions were more severe in the control group (<0.05), but there was not significant difference among the immunized groups. These results show that the protection conferred with live PH vaccine or its leukotoxin is satisfactory to protect lambs against pulmonary lesions produced experimentally by PH challenge. Furthermore, inocuity of the immunogens used in the present study due to the absence of post-vacunal reactions, was demonstrated.

P3. Vaccination of Lambs Against Pneumonic Pasteurellosis in Experimental Conditions.

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The aim was to evaluate the effects of vaccination with different antigens of *Pasteurella haemolytica* (PH), on the control of pneumonic pasteurellosis in lambs. Forty two lambs were divided into six groups, with seven animals each. Five groups were inoculated subcutaneously with different immunogens of PH: group A, was vaccinated with 0.5 ml of live PH harvested in the log phase, at concentration of $1x10^9$ CFU/ml; group B, was the control group; group C, with a commercial bacterin; group D, 2 ml of leukotoxin; group E, 2ml of surface antigens extract (SAE) plus leukotoxin; group F, SAE plus leukotoxin plus adjuvant. On day 35, all lambs were exposed to PI-3 virus; on day 42 were transthoracically challenged with 2 ml of a suspension of live PH (1×10^9 CFU/ml). All lambs were euthanized seven days after challenge. Sera samples were obtained on days 0,7,14,21,35,42. Each serum was tested for antibodies to PH by indirect hemagglutination test, and with the simple visual assay for determination of leukotoxin neutralizing antibody titres. Mean antibody titres were compared using the variance analysis, and the Tukey test, which showed a significant difference ($P < 0.05$) in those lambs immunized with leukotoxin,

live PH, and commercial bacterin. Pneumonic lesions in lambs from groups A, C, and D were smaller than in groups B, E, and F. Vaccination of lambs with live PH and leukotoxin enhanced lung resistance as shown by reduction in lesions and increase in antibody titers.

P4. Serotypes of *Pasteurella haemolytica* and *Pasteurella multocida* Isolated From Pneumonic Domestic Ruminants in Mexico.

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This study was conducted to isolate and determine the capsular and somatic serotypes of *Pasteurella multocida* (PM) and the capsular serotypes of *Pasteurella haemolytica* (PH) from bovine, ovine and caprine lungs with inflammatory lesions. A total of 232 lungs with inflammatory lesions were collected and these yielded 117 isolates of Pasteurella sp. Forty two strains were identified as PH and 75 as PM. All PH were biotype A. The serotypes, which were determined by indirect haemagglutination, were as follows: in sheep there were 28 strains (39% were A8, 32% A2, 11% A1, 7% A6, 4% A5, and 7% untyped); in cattle there were 12 strains (58% were A1, 17% were A2 and A6, and 8% untyped); in goats there were 2 strains (A5 and A8). For PM, 60% were capsular type A and 27% capsular type D, according with the acriflavine and hyaluronidase techniques. Somatic serotypes were determined by gel immunodiffusion and represented 72% in sheep for serotype 3; 9% for serotype 15; 4% for serotype 7; 2% for serotypes 4,10,12, and 14; and 7% untyped. In cattle 77% were serotype 3, 8% serotype 4, 4% serotypes 7 and 12, and 8% were untyped; whereas in goats, with 3 strains one was serotype 3, one serotype 15 and one untyped. The distribution of serotypes is compared and contrasted with other related studies.

P5. Comparative Evaluation of Two Commercial Atrophic Rhinitis Vaccines

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A dual-adjuvant atrophic rhinitis (AR)/pneumonia vaccine (Ambico B*P*M) containing *Bordetella bronchiseptica, Pasteurella multocida* type A, *Pasteurella multocida* type D (PmD) toxoid whole cells and *Mycoplasma hyopneumoniae* was compared to a commercial oil-adjuvanted AR vaccine containing *B. bronchiseptica* and PmD toxoid in a passive immunity pig model. Groups of four pregnant gilts were vaccinated according to label directions with either of the products. Four additional gilts remained unvaccinated as controls. The litters were not vaccinated. Reproduction of AR was achieved by infecting half of each litter at 4 days post-farrow with 10^9 cfu *Bordetella bronchiseptica* and at 8 days post-farrow with 10^9 cfu toxigenic PmD. The litters were euthanized six weeks post-infection. The B*P*M vaccine reduced nasal turbinate atrophy by 85% and the oil-adjuvanted product reduced turbinate atrophy by 74% in comparison to controls. The oil-adjuvanted product induced greater serum and colostrum antibody titres vs. *B. bronchiseptica* and PmD toxin, but this product did not confer greater protection to pigs nursing vaccinated gilts. It is possible that other immunogens from PmD are also effective in protecting pigs in addition to the PmD-toxoid.

P6. Modulation of Local Defence Mechanisms by Aerogenous Immunizations Against Porcine *Pasteurella multocida* Pneumonias.

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In investigations into pathogenesis and immunoprophylaxis of porcine *Pasteurella multocida* (*P.m.*)-induced pneumonias, only aerogenous immunizations with *P.m.* bacterins via aerosol caused an increase in the pulmonary clearance associated with a decrease in the severity of experimentally induced *P.m.*-pneumonias. In addition, specific IgA antibodies in the bronchoalveolar lavage fluid were elevated, whereas specific IgG antibodies showed no

reaction. To determine cellular effector mechanisms involved in the defence of *P.m.* infections, the phagocytosis of fluoresceinated *P.m.*-whole cells by alveolar macrophages (AM) in comparison to blood monocytes (BM) was measured using flow cytometry. Furthermore, the accessory function of AM and BM was examined by detecting their ability to stimulate the proliferation of blood T-cells. In aerogenously immunized pigs, the percentage of blood monocytes phagocytosing *P.m.* bacteria (%P) was significantly higher than in control animals, associated with an increased mean fluorescence intensity (MFI). AM of immunized pigs showed also an increased %P, but a decreased MFI, possibly reflecting a faster degradation of ingested bacteria. The accessory function of AM but not BM was enhanced after aerogenous immunization. These data indicate an important role of specific local IgA antibodies and activated AM in the defence of *P.m.* pneumonias in pigs.

P7. Capsule Depolymerization of *Pasteurella multocida.*

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Capsules of serogroups A, D and F *P. multocida* were depolymerized by treating with chondroitinase AC. After treatment, antigens were absorbed onto red blood cells and tested in indirect haemagglutination (IHA) tests with anti-capsule sera. Treatment of serogroup D and F strains resulted in increased IHA titres. Whereas, IHA titres to serogroup A were diminished. Enzyme treated bacteria were agglutinable in antisera, but non-treated bacteria were not. Chondroitinase AC treatment of capsulated *P.multocida* enhanced phagocytosis by swine neutrophils. However, the degree of phagocytosis was less than that of naturally-occurring, non-capsulated variants derived from parent capsulated strains.

P8. Immune Response of Colostrum Fed Dairy Calves to Capsular Polysaccharide of *Pasteurella haemolytica* A1.

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The ability of young colostrum fed dairy calves to respond to the capsular polysaccharide of *Pasteurella haemolytic* A1 was investigated. Calves were vaccinated, at 2 and 4 weeks of age with a culture supernatant vaccine (Presponse, Langford, Inc.); blood samples were collected at weekly intervals. Antibodies binding the capsular polysaccharide were determined using isotype specific (IgG1, IgG2, and IgM) enzyme immunoassays. A fourfold increase in IgM antibodies was evident in 18% of the calves after one dose of vaccine, with an additional 23% seroconverting after the second dose. Increases in IgG1 and IgG2 antibody titres of this magnitude were present in 9% and 23% of the calves respectively, after the second dose of vaccine. There was a negative correlation between pre-vaccination IgG1 antibody titre and IgG1 IgG2 and IgM responses to vaccination (r= -0.78, -0.19, and -0.75, respectively). Thus vaccination can induce an active immune response to capsular polysaccharide in calves with low passive (IgG1) antibody titres.

P9. Epidemiology of Human Infections by *Pasteurella, Actinobacillus* and Related Species (or Group) in France.

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A retrospective study of *Pasteurella, Actinobacillus* and related species infections was performed by the *Pasteurella* and Related Bacteria Working Group from 1992 to 1993. Among the 379 cases recorded, wound infections (bites, scratches and punctures) were the most common forms of pasteurellosis (68.6%). In human infections unrelated to

animal bites, respiratory tract diseases and septicemia were the predominant infections with respectively 17 and 9%. Next in importance were urogenital (2%), central nervous system (1.3%), abdominal (0.5%), and various (1.6%) infections. The infections were principally caused by *P. multocida* (61.4%), *P. canis* (9.6%), *P. stomatis* (4%), *P. dagmatis* (2.3%), and related species (EF-4, *Neisseria weaveri, Weeksella zoohelcum* and *Capnocytophaga canimorsus:* 16.4%). In a few cases, *Pasteurella (pneumotropica, bettyae* and SP group: 3%), and *Actinobacillus (ureae* and *lignieresii:* 2.3%) were isolated. The majority of animal wound infections were treated with β-lactamins (penicillins) or cyclines; with other forms, β-lactamins (penicillins and cephalosporins) were more likely.

P10. *In vitro* Activity of Selected Antimicrobial Agents Against HAP Organisms Isolated from BRD, SRD, and ORD.

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The minimum inhibitory concentrations (MIC) for eleven antimicrobial agents commonly used in veterinary therapy were determined with 1047 strains of HAP organisms isolated from bovine (BRD), swine (SRD), and ovine (ORD) respiratory disease. MIC determinations were conducted in accordance with NCCLS guidelines. Ceftiofur was the most active compound with an MIC_{90} of ≤0.06 μg/ml for all isolates tested. Enrofloxacin was also very active with MIC_{90} of ≤0.06 μg/ml for the BRD and SRD strains and 0.13 μg/ml for the ORD strains. In comparison, ampicillin was less active with an MIC_{90} of 32.0 μg/ml for *P. haemolytica* and 16.0 μg/ml for *A. pleuropneumoniae.* Similarly, tetracycline was much less active with MIC_{90} ranging from 16.0 to 32.0 μg/ml for BRD and SRD isolates. Sulfamethazine demonstrated poor activity against the isolates tested with MIC_{90} of ≥256.0 μg/ml for the BRD and SRD strains tested.

P11. Serious Infection of the Forefinger Due to *Pasteurella* "Sp" Group Following a Guinea Pig Bite.

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Case report: a previously healthy 31-year-old woman was bitten on April 23rd, 1993, at the left forefinger by her guinea pig. After cleansing of the wound, she was empirically treated with oxacillin and anti-inflammatory. Two days later, she was admitted to the hospital because a cellulitis of the hand: the forefinger looked inflammatory with purulent discharge, lymphangytis of the forearm and axillary adenopathy. After surgical treatment, she received pristinamycin for 5 days then cloxacillin. On May 4th, because a severe arthritis of the interphalangeal joint with necrosis of the tendon, antibiotherapy was changed for amoxycillin-clavulanate. On May 5th, the gravity of the sepsis necessitated an amputation at the second phalanx of the forefinger, and the patient improved with amoxicillin-clavulanate for 10 days. Bacterial culture of the joint obtained on May 4th evidenced *Pasteurella* "SP" group susceptible *in vivo* to amoxycillin-clavulanate. *Pasteurella* "SP" group is a rare organism usually isolated from guinea pig but seldom from humans, likely because it is not widely spread and probably because of its low virulence: actually it is almost always found in patients with predisposing conditions. This report also shows it can be responsible for very severe infection even in individuals without any particular risk factor.

P12. Bacteriophage Genes From *Haemophilus somnus*: Homology to Genes From Bacteriophages P2 and Hp1.

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Haemophilus somnus is a bovine pathogen and etiological agent of "hemophilosis". While investigating iron uptake in *H. somnus,* a 7.8 kb fragment of genomic DNA coding for a hemin-binding (Hmb+) phenotype was cloned. DNA sequencing (7808 bp) indicated a series of unidirectional open reading frames (orfs). Database analysis of *H. somnus* predicted polypeptides showed homology to proteins in other bacteriophages. The greatest homology occurred between 7 non-consecutive *H. somnus* polypeptides and the DNA packaging, capsid, and tail proteins of bacteriophage P2. Interrupting the P2 homology were 4 polypeptides that were similar to the predicted lysis proteins of the *H. influenzae* temperate bacteriophage HP1. A prominent feature was the identical gene order within the regions of homology. The *H. somnus* phage genes are organized like the P2 genes, however, HP1 lysis genes have replaced the P2 lysis genes. This type of recombination between bacteriophages is consistent with Botstein's modular evolution theory. The highly conserved gene order in bacteriophages, particularly in the area known as the "lysis cassette", increases the likelihood of successful recombination between distantly related bacteriophages. Polyclonal antiserum against *H. somnus* orf2 (P2 major capsid protein homologue) will help determine whether this DNA is part of a functional bacteriophage.

P13. Molecular Investigation of the Role of ApxI and ApxII in the Virulence of *Actinobacillus pleuropneumoniae* Serotype 5.

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The *apxI* gene locus of a non-haemolytic mutant of *Actinobacillus pleuropneumoniae* (Ap) serotype 5 contained a major deletion that spanned most of *apxICABD*. Use of monoclonal antibodies confirmed that ApxI was not synthesized, and that ApxII was synthesized but not exported. The *apxICABD* genes and *apxIBD* genes were cloned into a broad host-range vector and electroporated into the mutant to obtain strains JF1064 and JF1060, respectively. JF1064 exported ApxI and ApxII, and produced haemolytic activity exceeding that of parent strain J45; JF1060 produced only weak haemolytic activity. In mouse virulence studies JF1064 was significantly more virulent than J45, while JF1060 was significantly less virulent than J45 or JF1064, but more virulent than the nonhaemolytic strain. JF1064 and J45 were similar in the capability to cause pleuropneumonia in pigs. JF1060 maintained the ability to induce pleuropneumonia in pigs, although higher doses were required to induce similar lesions. This study shows a direct correlation between Apx toxin production and virulence in Ap.

P14. Characterization of a Restriction Endonuclease From *Pasteurella haemolytica* Serotype A1 and Construction of a Gene-Replacement *Aroa* Mutant.

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A new restriction endonuclease, *PhaI,* was isolated from *P. haemolytica* serotype 1, strain NADC D60, obtained from pneumonic bovine lung. *PhaI* recognizes the 5 base non-palindromic sequences 5'-GCATC-3' and 5'-GATGC-3'. Cleavage occurs 5 bases 3' from the former recognition site and 9 bases 5' from the latter. A gene encoding for a methyltransferase which protects against *PhaI* cleavage was cloned from *P. haemolytica* into *E. coli*. Whereas unmethylated plasmid DNA containing a *P. haemolytica* origin of replication was unable to transform *P. haemolytica* when introduced by electroporation, the

same plasmid DNA obtained from *E. coli* which contained cloned *PhaI* methyltransferase could do so. The *aroA* gene of *P. haemolytica* serotype A1 was cloned and sequenced. A *P. haemolytica* ampicillin-resistance fragment was cloned into the unique *NdeI* site of *aroA*. A hybrid plasmid was constructed by joining the *aroA* replacement plasmid with the 4.2 kb *P. haemolytica* plasmid which encodes streptomycin resistance. Following *PhaI* methylation, the hybrid plasmid was introduced into P. *haemolytica* by electroporation. Allelic exchange between the replacement plasmid and chromosome of P. *haemolytica* gave rise to an ampicillin-resistant mutant which was unable to grow on medium deficient in tryptophan. Although transformation efficiency with methylated hybrid plasmid was $< 10^3/\mu g$, the hybrid was capable of unstable replication in *P. haemolytica,* so this system may be suitable for construction of additional gene-replacement mutants

P15. Restriction Barriers in *Pasteurella multocida* Type D Strains From Pigs.

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A system for transfer of foreign DNA into type D *P. multocida* is being developed. A natural streptomycin/sulphonamide resistance plasmid, pPM1, which could be transferred into *Escherichia coli* DH5 alpha was used. It was found that this plasmid could not be transferred between certain *P. multocida* strains or between *E. coli* and some *P. multocida* strains by electroporation (1). This evidence suggests that there are several restriction barriers present in these *P. multocida* strains. To overcome the restriction barrier(s) a toxigenic strain LFB3 was mutated using ethyl-methanesulphonate (2). The mutation resulted in a strain which could take up pPM1. Plasmid pPM1 isolated from this strain could be transferred to wild type LFB3.

(1) Conditions for transformation of *Pasteurella multocida* by electroporation. Jablonski L., Sriranganathan N., Boyle S.M., and Carter G.R. 1992. Microbial Pathogenesis 12 63-68.

(2) Experiments in Molecular Genetics. J.H. Miller. 1973. Cold Spring Harbor.

P16. A Native Plasmid Of *Pasteurella haemolytica* Serotype A1: DNA Sequence Analysis and Investigation of its Potential as a Vector.

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The complete nucleotide sequence was determined for a 4.3 kilobase pair plasmid, pAB2, isolated from a bovine strain of *Pasteurella haemolytica* serotype A1 . The plasmid encodes a Rob-1 type beta-lactamase and contains a region with significant DNA homology to elements of the mobilization (mob) region of the *E. coli*, ColE1. pAB2 can be transferred to *P. haemolytica* by transformation and electroporation and can also be mobilized for conjugative transfer using *E. coli* helper functions. An insertion mutant of pAB2 (pTC2/81) carrying a copy of Tn5 was transferred to *E. coli* K12 by conjugation. This could be transferred to *E. coli* HB101, but not to *P. haemolytica* serotypes A1 or A2 by transformation . However, a derivative of pTC2/81 containing only a fragment of Tn5 was able to transform *P. haemolytica*. Two further constructs containing the pAB2 presumptive origin of replication and the ampicillin resistance gene and cloned kanamycin resistance gene or a fragment of the *P. haemolytica* A1 leukotoxin A gene, were similarly able to transform *E. coli* but not *P. haemolytica*. This has demonstrated that the pAB2 is capable of acting as an *E. coli* / *P. haemolytica* shuttle vector. However, the nature of the cloned DNA sequences are important for transformation and stable plasmid replication. This suggests the presence of a restriction system or DNA modification system in *P. haemolytica*. To overcome this attempts have been made to use chemical mutagenesis to select mutants which are susceptible to transformation.

P17. Characterization Of *Actinobacillus Pleuropneumoniae Strains* By Ribosomal Intergenic Sequences.

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Actinobacillus pleuropneumoniae is the aetiologic agent of contagious porcine pleuropneumoniae. The disease is widespread among pigs, and results in mortality, chronic lung lesions, and pleurisy. The aim of this study, was to characterize the species further and determine the intraspecies relationship, based on the ribosomal intergenic regions. Eight strains, each representing 1 of the 8 serotypes found in Denmark, 13 international reference strains and 3 strains of the closely related species *Actinobacillus lignieressi,* were included in the study. The intergenic regions were amplified by PCR, using primers matching the conserved regions in the surrounding ribosomal genes. This resulted in amplification of 2 intergenic regions of approximately 500 and 600-650 bases, from all the strains. The intergenic regions were sequenced with the Taquence cycle sequencing kit. From sequence similarity, a phylogenetic tree was constructed using the Neighbor-Joining method, and analyzed by bootstrapping. The intergenic sequences of these strains allowed a clear discrimination between the 2 closely related species, *Actinobacillus pleuropneumoniae* and *Actinobacillus lignieressi.*

P18. The *Haemophilus Influenzae Sxy-1* Mutation is in a Newly-identified Gene Essential for Competence.

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DNA is abundant in mucosal environments and may be a significant source of nucleotides for those mucosal pathogens able to take it up. In *Haemophilus influenzae*, competence for DNA uptake is increased 100-fold to 1000-fold by the sxy-1 mutation, which we have mapped to an ORF adjacent to the rec-1 locus. Insertional inactivation of sxy entirely prevents both DNA uptake and transformation, indicating that it encodes a function essential for competence. In contrast, a multicopy plasmid containing the wildtype sxy gene confers constitutive competence on wildtype cells, suggesting that Sxy may be a positive regulator of competence whose activity is increased by the sxy-1 mutation. However, sxy-1 is a point mutation causing only a Val-Ile substitution in the Sxy protein. Three other point mutations in sxy cause similar levels of hypercompetence but do not change the specified protein at all. However, each of these four point mutations is predicted to destabilize a region of potential secondary structure at the 5' end of the sxy mRNA. In wildtype cells formation of this stem may depend on the rate of transcription, thus permitting translation of sxy mRNA and induction of competence only when nucleotide pools are depleted.

P19. Evaluation of a PCR Detection System of *Actinobacillus pleuropneumoniae* in Mixed Cultures from the Respiratory Tract of Pigs.

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A PCR for detection of *A. pleuropneumoniae* was evaluated for its suitability as a diagnostic tool. All of the 102 tested Danish isolates of *A. pleuropneumoniae* reacted in the PCR and a 985-bp product was amplified. Mixed cultures from 70 lungs with clinical signs of pleuropneumonia were tested. The results of the PCR assay were compared with those of culture, and all isolation positive lung cultures tested positive in the PCR. Approximately one third of the isolation negative lungs were, however, positive in the PCR test, suggesting a superior sensitivity of the PCR test to that of culture. Cultured samples from tonsils of 101 pigs from 9 different herds were examined by PCR for the presence of the

985-bp target sequence and 65% reacted positive compared to 23% positive by culture. A significantly higher detection level was achieved, when the tonsils were precultured on selective media as compared to non-selective media. The results show that a combination of culture and PCR is a valuable method for the identification of *A. pleuropneumoniae* in pigs.

P20. Localization of an Epitope Involved in Neutralizing the Leukotoxin from an A1 Serotype of *Pasteurella haemolytica.*

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Fragments of the Lkt A gene from A1 serotype of *P. haemolytica* were generated by restriction digests or by PCR. These were cloned into a plasmid vector for expression as a fusion protein with β-galactosidase. The resulting fusion proteins were characterized by immunoblotting for their reaction with a monoclonal antibody which has a high toxin neutralizing titre. The epitope was localized to a 20 AA region located at the C terminus of the LktA molecule and the epitope of a non-neutralizing antibody was located a further 20 AA downstream. Although the minimal recombinant peptides were not antigenic, a protein encompassing a 200 AA region of the C terminus was immunogenic and evoked production of neutralizing antibodies in SPF lambs.

Neither of the monoclonals used reacted with T10 serotype LktA on blots or neutralized T10 toxin. Comparison of the deduced AA sequence in the region of these epitopes showed considerable sequence divergence between A1 and T10 toxins.

P21. Serological Profile of a Porcine Herd Using a Hemolysin Neutralization Assay to Detect *Actinobacillus pleuropneumoniae* Antibodies.

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The purpose of this study was to determine the serological status of a pig herd in relation to *Actinobacillus pleuropneumoniae* infection. Two gilts, four sows and their litters were bled six times at 1, 5, 9, 13, 19, and 25 weeks of age. One hundred per cent of the females, 68% of the piglets from the gilts, and 93% of the piglets from the sows were classified as positive on week one, by the hemolysin neutralization assay. By week 5 none of the piglets showed positive titres, they remained seronegative until week 13. Seroconversion arose when the animals were moved to the finishing pen. When the study was finished, at week 25, 20% of the sampled population was positive, and 26% was classified as suspicious. Clinical disease did not appear during the study, and pleuropneumonic lesions were not found at the slaughter house. According to these results, herd immunity is a key in the infected farms to diminish the clinical disease.

P22. The Occurrence of *Pasteurella haemolytica* Serotypes Amongst Cattle, Sheep and Goats in Southern Africa.

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Pasteurella haemolytica is a frequent cause of disease amongst cattle and sheep in southern Africa, causing millions of rands in losses. In cattle it is usually seen amongst animals in feedlots, whilst it occurs in sheep under intensive as well as extensive conditions. Over an eight year period from September 1986 to March 1994, a total of 497 specimens were received from sheep and goats for the isolation of *Pasteurella* organisms, from seven different geographical areas in southern Africa. In all these regions *P. haemolytica* serotype 6 was the most frequent, followed closely by types 9 and 15. One hundred and sixty eight isolates could not be typed (33.8 %). Four disease syndromes were associated with these serotypes, pneumonia being the largest with 51.3 % cases, blue udder in ewes (28.14 %),

septicaemia in 8.66 % and other miscellaneous causes 11.9 %. From cattle, 96 specimens were submitted for the isolation and typing of *Pasteurella* organisms, 41 % belonged to *P. haemolytica* type 1 and originated from pneumonia specimens whilst 25 % were not typeable. Serotype 1 was isolated from 56.71 % of all cases presented for examination, from the whole region.

P23. Serological and Molecular Characterisation of V-Factor (NAD)- Independent *Haemophilus paragallinarum.*

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Fifteen isolates of V-factor (NAD)-independent strains of *H.paragallinarum* isolated from poultry in South Africa were characterised by biochemical typing, serotyping, restriction endonuclease analysis (REA) and ribotyping. The isolates were deliberately selected to be as diverse as possible. All the isolates were shown to be typical of the species in biochemical properties and all were serovar A. REA was performed with three enzymes. All the isolates gave identical REA profiles. Ribotyping was performed using a probe which consisted of the plasmid pUC19 into which the 16S rRNA operon of *H. paragallinarum* had been inserted. All the isolates gave the same ribotyping profile. Our results strongly suggest that these NAD-independent isolates are clonal in nature.

P24. A Co-agglutination Test for Serotyping *Pasteurella haemolytica.*

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A co-agglutination test was adopted for detection of *Pasteurella haemolytica* type specific antigens in lung lesions even if no viable bacteria could be cultured The co-agglutinating reagents were prepared by coating protein-A producing *Staphylococcus aureus* cells with hyperimmune sera raised against *P. haemolytica* type strains. Homologous reactions of bacterial suspension, saline extract and boiled saline extract antigens were good, but some one-way cross-reactions appeared. Ninety-three per cent of 64 field strains examined could be serotyped using the coagglutination test. The coagglutination test detected *P. haemolytica* type specific antigens in the lung specimens of 4 calves, 5 sheep which had succumbed due to naturally occurring pneumonia, 20 calves experimentally infected with *P. haemolytica* Al, and in 36% of the lung specimens of slaughtered field sheep with chronic lung lesions. This test is recommended as an additional method for fast and reliable serotyping of *P. haemolytica.*

P25. A Soluble 70 kilodalton Protein Present in Cells of *Pasteurella haemolytica* A2

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A monoclonal antibody (Mab) was raised against whole live A2 *P. haemolytica* cells in mice primed with outer membrane proteins of iron-deficient A2 cells. It recognises a cytoplasmic / periplasmic 70kDa protein found in A2 iron- deficient cells as did serum from either a convalescent sheep or an animal vaccinated with a sodium salicylate extract of A2 iron-deficient cells. The Mab recognises the antigen in A2 cells grown in either nutrient broth culture or $\alpha\alpha'$dipyridyl-treated culture broths, but not in cells of several other serotypes eg A1, 6, 7, 9, T3, 4, 10, 15 whether iron-replete or iron-deficient. The soluble protein was purified by affinity chromatography on a column of mouse IgG - Sepharose and after SDS/PAGE and transfer to PVDF had an N-terminal sequence of 18 amino acids which was similar to that of the DnaK heat shock "chaperone" protein of *E. coli.* However,

the protein did not seem to possess the "consensus" sequence belonging to the general family of heat shock proteins. Its location in the cell could be modified by the presence of iron in the growth medium. Thus the protein could be differentiated from the other immunologically active iron- regulated OMPs of *P. haemolytica* A2.

P26. Rapid Preparation of Competent Cells by Filtration.

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Collection and washing of cells by centrifugation is a time consuming and inconvenient component of many microbiological protocols. We have found that filtration onto disposable membrane filters can efficiently replace the centrifugation steps in many procedures. To demonstrate the utility of filtration, we have substituted it for centrifugation in standard competence protocols for *Escherichia coli, Haemophilus influenzae*, and *Saccharomyces cerevisiae.* With all three protocols filtered cells showed transformation efficiencies comparable to or higher than those of cells prepared by centrifugation. Furthermore, the filtered cells were competent sooner, both because filtration is faster than centrifugation and, in the case of *E. coli*, because competence was highest immediately after filtration. The procedure can be easily adapted to collect cells for many other purposes, and should be especially useful in metabolic studies and in preparing cells for electroporation, as it facilitates both washing and resuspension of cells.

P27. Variations of the Chromosomal Structure Between Serotypes 1-12 and Biovars 1 and 2 of *Actinobacillus pleuropneumoniae.*

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Entire chromosomal DNA of all 12 *Actinobacillus pleuropneumoniae* serotype reference strains and of field strains from some selected serotypes and also from biovar 2 strains has been analyzed using infrequently-cutting restriction enzymes and field inversion gel electrophoresis (FIGE). The size of the chromosome of the *A. pleuropneumoniae* type and serotype 1 reference strain 4074^T was calculated to be 1850 +/- 100 kilobasepairs (kbp). The other serotype reference strains have a very similar chromosome size. The restriction fragment patterns obtained from the different serotype reference strains show that their chromosomal structures differ. However, the serologically related serotypes 1, 9,11, serotypes 5a and 5b and also serotypes 6 and 8 have very similar patterns. Filed strains with the same serotype show the same patterns, even when they originated from different geographical areas. Analysis of PCR-amplified 16S rRNA genes with frequently-cutting restriction enzymes does not distinguish between serotypes and biovars.

P28. Clinical Isolates of *Actinobacillus actinomycetemcomitans* From Three European Cities Carry Highly Related Prophages.

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Actinobacillus actinomycetemcomitans is a putative periodontal pathogen. Others had suggested that bacteriophages of *A. actinomycetemcomitans* might be associated with disease activity. We characterized five lysogenic *A. actinomycetemcomitans* strains isolated from different patients from three geographic locations (Basel, Freiburg i. Br., Amsterdam). They belonged to two genotypes as revealed by RFLP analysis. One of the strains showed a DNA restriction modification system. The temperate bacteriophages induced from these hosts were related: The phages have the same virion structure. The phage DNA genomes were investigated by restriction fragment pattern analysis and Southern hybridizations. All five phages had a genome size of about 43 kb and showed

identical or similar restriction fragment patterns. Hybridization experiments indicated extensive DNA homologies. However, the phages formed three immunity groups. These results may suggest, but do not prove, a common genetic trait providing a selective advantage to lysogenic *A. actinomycetemcomitans*.

P29. High-Molecular-Mass LPS are Involved in *Actinobacillus pleuropneumoniae* Adherence to Porcine Respiratory Tract Cells.

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Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia. We have identified the lipopolysaccharides (LPS) as the major adhesin of *A. pleuropneumoniae*. Using immunoelectron microscopy and flow cytometry, we showed in the present study that LPS were well exposed at the surface of this encapsulated micro-organism. Inhibition of adherence of *A. pleuropneumoniae* with extracted LPS was performed on porcine trachea frozen sections. Acid hydrolysis of LPS revealed that the active component of LPS was not lipid A but the polysaccharides. LPS from *A. pleuropneumoniae* serotypes 1 and 2 were separated by chromatography on Sephacryl S-300 SF according to their molecular mass. The adherence inhibitory activity was found in the high-molecular-mass fractions. These fractions contained KDO and neutral sugars, and were recognized by a monoclonal antibody directed against *A. pleuropneumonia-* O-antigen but not recognized by a monoclonal antibody against capsular antigen.

P30. Serotyping of *Actinobacillus pleuropneumoniae* Serotype 5 Strains Using a Monoclonal Antibody Against Lipopolysaccharides O-Chain in a Latex Agglutination Test.

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A latex agglutination test has been developed for serotyping *Actinobacillus pleuropneumoniae* serotype 5 strains. Latex particles were sensitized with a murine monoclonal antibody recognizing an epitope on the serotype 5 LPS-O chain as shown by SDS-PAGE and Western blotting. A total of 144 *A. pleuropneumoniae* and 14 common bacterial species associated with swine were tested by mixing 50µl of latex reagent with the same volume of a dense suspension (MacFarland #5) of bacterial cells grown for 18 h on PPLO agar plates. All *A. pleuropneumoniae* strains had been previously serotyped using a standard procedure. The latex agglutination test was rapid (few minutes), easy to perform and overall a good correlation was found with the standard techniques. In addition, the sensitized latex particles were stable for at least two months.

P31. Purification and Characterisation of Bovine Lipopolysaccharide Binding Protein.

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Lipopolysaccharide binding protein (LBP) is a recently described acute phase protein which is implicated in modulating the host responses to lipopolysaccharide (LPS) from Gram-negative bacteria. LBP-like activity has been identified in bovine serum and in this study LBP was purified from acute phase serum using ion exchange chromatography over Bio-Rex 70 and Econo-Pac Q. On SDS-PAGE, bovine LBP demonstrated a single band with a molecular radius of 58 kDa. Functionally, bovine LBP resembled LBPs from other species in that it enhanced binding of LPS to monocytes and increased the sensitivity of monocytes

to LPS by at least a 100-fold. However, immunoglobulins to rabbit LBP or recombinant human LBP did not cross- react with bovine LBP. Studies on LBP activity during the acute phase response demonstrated a four-fold increase 36 hours after a single intratracheal inoculation of *Pasteurella haemolytica* Al. Isolation of LBP will allow further investigations on LBP-mediated responses to LPS in cattle.

P32. Identification of a Conserved, Phase Variable Epitope in the Lipooligosaccharide of *Haemophilus somnus*.

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Haemophilus somnus (Hs) is a multisystemic, pathogenic bacterium of bovines. As with *Haemophilus influenzae* (Hi) and *Neisseria gonorrhoeae* (Ng), the lipooligosaccharide (LOS) of disease isolates of Hs undergoes phenotypic phase variation *in vivo* and *in vitro*, whereas preputial isolates do not. Monoclonal antibodies (MAb) to the LOS of Hi, Hi biogroup *aegyptius*, and Ng were examined for reactivity with the LOS of Hs strain 738 and LOSs from 13 phase variants of strain 738. Hs LOS bands of M_r 4200 to 4300 reacted with 1 MAb to Ng LOS, 3 MAb to Hi biogroup *aegyptius* and 1 MAb to Hi LOS. Hs LOS bands <4000 M_r did not react with any MAb. All phase variants of strain 738 tested reacted with the same MAb as strain 738. Hs preputial isolate IP produced a single LOS band of M_r 3300, did not react with any MAb, and did not undergo LOS phase variation. A $(CAAT)_5$ probe reacted with Hs 738 and Hi DNA of 4-5 kb, but did not react with Hs DNA from non-disease isolate IP. Thus, at least 1 phase variable epitope seems to be conserved in Hs disease isolates, and a common mechanism for LOS phase variation may occur in mucosal Gram-negative bacteria.

P33. The Influence of *Haemophilus somnus* Lipooligosaccharide on Acrosomal Status of Rabbit Sperms.

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The ejaculated rabbit sperms were incubated with different doses of *H.somnus* lipooligosaccharide (LOS).The number of viable cells, their motility and acrosomal status were examined at different time intervals and capacitation conditions. The percentage of sperms with partially or completely damaged acrosomes was dependent on the LOS concentration and on the time of incubation. The viability and motility of rabbit spermatozoa were not affected during the incubation in comparison to control samples. It seems that endotoxin of *H.somnus* may induce the acrosomal reaction of rabbit sperms. This phenomenon may contribute to poor reproductive performances of infected females.

P34. Comparison of Bovine and Ovine Isolates of *Haemophilus somnus.*

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Recent studies indicate that *Haemophilus agni* and *Histophilus ovis* from sheep and *Haemophilus somnus* from cattle are biochemically and culturally very similar. These organisms produce varied and frequently severe infections in the two animal species. Isolates from different types of infections in the two animal species were evaluated to detect differences at the biochemical, molecular, and antigenic levels. Six different biogroups were detected by an expanded panel of biochemical tests. Restriction enzyme analysis (REA) revealed marked differences between bovine and ovine isolates, between bovine disease and preputial isolates and even among ovine carrier isolates. All isolates had

the major 78, 60, and 40kDa antigens on Western blots. However, minor differences were detectable in the low molecular weight antigens. Although marked differences were detected in biochemical tests and in REA profiles, similarities of antigens of ovine and bovine isolates suggest that vaccines containing these major antigens may provide protection against disease in both sheep and cattle.

P35. Development of Disrupted Whole-cell *Actinobacillus pleuropneumoniae* (App) Vaccines.

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We previously reported the development of an effective App whole cell vaccine utilizing growth conditions that optimize antigen production, disruption/ inactivation of cells by sonication to improve antigen presentation, and a light oil adjuvant (Emulsigen™). In the present study, we evaluated other adjuvants and disruption/inactivation methods to improve commercial feasibility. In preliminary experiments, based on serum antibody responses, we found light oil adjuvants(Emulsigen and Imugen™) were superior to vegetable oil and aluminum hydroxide. Heat inactivation (HI) or disruption via a "French press" (FP) were similar to sonication, and the optimal organism concentration was approximately 10^9 per dose. The following vaccines (Imugen adjuvant) were evaluated against homologous experimental challenge with serotype 5: A-- HI/$10^{9.5}$, B-- FP/$10^{9.5}$, C-- FP/$10^{8.9}$, CON-- control. The percent pneumonia and mortality by vaccine were: A-- 10.6, 1/7; B-- 4.4, 0/7; C-- 12.1, 1/7; and CON-- 37.5, 7/7. The modifications did not alter the efficacy of our original sonicated-whole-cell vaccine.

P36. Specificity and Sensitivity of an ELISA for *Actinobacillus pleuropneumoniae* (App).

M.H. Mulks* and B.J. Thacker, Michigan State University, East Lansing, Michigan, USA.

We previously reported the development of an ELISA for measuring App antibodies using an outer membrane antigen prepared by sucrose density gradient centrifugation. This ELISA has been useful for evaluating serum immune responses within controlled experiments. However under field conditions, pigs will often develop low to moderate antibody levels even though they appear to be App-free. Recently, reference sera from pigs experimentally infected with gram-negative bacteria were made available. The sera were tested blind and a key was provided later by the reference laboratory. The number of positive sera/total tested, by organism for each coating antigen serotype (1 and 5, respectively) was: App serotype 1-- 23/24, 20/24; App serotype 5-- 28/28, 26/28; App serotype 7-- 11/22, 19/22; *Actinobacillus suis*-- 14/16, 15/16; *Haemophilus parasuis*-- 0/16, 10/16; *Pasteurella multocida*-- 2/18, 3/18; and *E. coli*-- 0/10, 1/10. Immunoblot analysis revealed several obvious cross-reactive proteins, and suggested that capsular polysaccharide and lipopolysaccharide also cross-react. Development of App specific serotests can be hindered by cross-reactive antigens from other Gram-negative bacteria.

P37. Cloning And Expression Of *Actinobacillus pleuropneumoniae* (App) Riboflavin Synthesis Genes.

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Riboflavin synthesis genes from APP serotype 5 were cloned and expressed in E. *coli DHS5*-α. APP serotype 5 chromosomal DNA was isolated and restricted with *HindIII*. Fragments ranging from 4 to 7 kb in length were ligated into the cloning vector pUC 19. Transformation of *E. coli* with the ligation mixture yielded one clone with a 5.0 kb insert that produced a water-soluble extracellular yellow compound. The absorption spectra of the compound were similar to that of riboflavin with absorption peaks at 444.5 and 373 nm. Shifts in absorption due to acidification and reduction with sodium dithionate also

compared well with riboflavin. Mass spectroscopy with positive and negative ion fast atom bombardment confirmed that the extracellular product was riboflavin (Vitamin B-2). Riboflavin is produced at 40 μg/mL in a 24 hour culture grown in minimal salts media with N-Z amine. Production is not dependent on IPTG induction indicating presence of the endogenous APP promoter. Partial double-strand sequencing data indicates the presence of at least two genes with good homology at the amino acid level to *rib* A and *rib* H from *Bacillus subtilis* . It is possible that an entire operon with homology to the *Bacillus rib* GBAHT operon may be present in APP. Further sequencing will determine whether homologues to other *rib* genes exist in APP.

P38. Evaluation of a Polyclonal Blocking ELISA Detecting Antibodies to *Actinobacillus pleuropneumoniae* Serotype 2.

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A polyclonal blocking ELISA detecting antibodies to *A.pleuropneumoniae* serotype 2 was compared to the conventionally used Complement-Fixation (CF) assay in three different samples: 1) test samples from SPF-breeding and multiplying herds. 2) consecutive serum samples from SPF sows. 3) aerosol-inoculated SPF pigs. 1) The ELISA was shown to have a higher herd-sensitivity, but a lower herd-specificity than the CF-assay. 2) The SPF sows were serological negative in three consecutive samplings, but sows with blocking percentages in the 'cut-off' area, tends to remain there in all three samplings. 3) However, some of the aerosol-inoculated pigs showed specific serological reactions in the 'cut-off' area of the blocking percentages and thereby excluded the possibility of adjusting the 'cut-off' level in order to solve the herd specificity problem. Serological reactions in the 'cut-off' area of this ELISA need to be verified by other signs of *A.pleuropneumoniae* serotype 2 infections in herd health surveillance programs.

P39. Screening of *Actlnobacillus pleuropneumoniae* Field Isolates on Expression of ApxI, ApxII, ApxIII Toxins and 42kDa-OMP.

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A total of 135 *A. pleuropneumoniae* (App) strains, approx. 10 representatives of each of the presently known 13 serotypes, was isolated from diseased pigs in various countries all over the world. The strains were tested for expression of the RTX toxins ApxI, ApxII and ApxIII, and for expression of a common 42kDa P2-homologous outer membrane protein (OMP), by Western blotting using specific monoclonal (toxins) and polyclonal (OMP) antibodies. The vast majority of 96% of the strains had the same antigen expression profile as the corresponding App serotype reference strain. Combined with efficacy testing in pigs against various App serotypes, these results provide strong evidence that an App subunit vaccine containing ApxI, ApxII, ApxIII and OMP antigens will induce protection against all encountered App strains and serotypes.

P40. An *Actinobacillus pleuropneumoniae* Subunit Vaccine Inducing Protection in Pigs Against all Serotypes.

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At present 13 different *A. pleuropneumoniae* (App) serotypes are known, which all can cause disease in fattener pigs. The different App serotypes express one or two of three different RTX toxins (ApxI-III), resulting in four different toxin profiles among

serotypes. In addition, all serotypes express a P2-homologous 42kDa outer membrane protein (OMP). A subunit vaccine containing purified ApxI, ApxII, ApxIII and OMP in an aqueous adjuvant was tested for efficacy in SPF pigs against challenge with various App serotypes. The vaccine induced excellent protection against challenge with App serotypes 1, 2, 5a, 7, 9 and 10. Since these serotypes are representatives of all four different toxin profiles seen among the 13 App serotypes, it is concluded that the vaccine will be protective against all presently known App serotypes.

P41. Role of Apx Toxins of *Actinobacillus pleuropneumoniae* in *vitro*.

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The Apx toxins of *Actinobacillus pleuropneumoniae* are thought to be important virulence factors. To study the role of Apxl and Apxll, we constructed knock-out mutants of strain S 4047 (serotype 1). Phenotypes of the mutants were ApxI-/ApxII+, ApxI+/ApxII-, and ApxI-/ApxII-. The effects of the mutants on porcine PMNs were studied in a chemiluminescence assay and in a chemotaxis assay. Wild type and the ApxI-/ApxII+ and ApxI+/ApxII- mutants first activated and then killed the PMNs. This was not observed for the ApxI-/ApxII- mutant. Wild type and the ApxI+/ApxII- mutant clearly attracted PMNs but in the vicinity of the bacteria the directed migration was arrested. This was not observed for the ApxI-/ApxII+ and the ApxI-/ApxII- mutants. Our results with these mutants agree with those obtained with affinity purified toxins alone.

P42. Production and Characterization of Monoclonal Antibodies Against the 42 kDa Outer Membrane Protein of *Pasteurella haemolytica* A1.

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Pasteurella haemolytica serotype A1 is the organism most frequently associated with a fibrinous pleuropneumonia of feedlot beef cattle in the United States. *P. haemolytica* A1 produces major outer membrane proteins (OMPs) of approximately 30 kDa and 42 kDa. In this study, we purified the 42 kDa OMP after preparative SDS-polyacrylamide gel electrophoresis of *P. haemolytica* A1 outer membranes. The gel-purified protein was used to immunize mice for the production of monoclonal antibodies (MAbs). Hybridomas were subsequently screened by ELISA against *P. haemolytica* A1 cell envelopes. Seven individual hybridomas, which produced MAbs that recognized the 42 kDa OMP, were isolated. Western blot analyses revealed that all seven MAbs recognize a protein of similar molecular weight (42 kDa) in *P. haemolytica* strains of serotypes A1, A2, A6, and A9 as well as in untypeable strains. Immunoelectron microscopy and whole cell ELISA assays suggest that some of the MAbs are directed against surface-exposed regions of the 42 kDa protein.

P43. *Pasteurella haemolytica* Leukotoxin and its Interaction With Target Cells.

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P. haemolytica leukotoxin (LKT) is one of the RTX family of toxins and is thought to be one of the major virulence factors in pneumonic pasteurellosis in cattle and sheep. Because of its apparent specificity for ruminant leukocytes, it may be a prime determinant of the host range of *P. haemolytica*. A large number of *P. haemolytica* isolates, including representatives of all serotypes and untypables, has been examined for LKT production in supernates of log phase cultures. There was some variation in the amount and molecular weight of LKT produced by different strains, as judged by SDS-PAGE and immunoblotting

with monoclonal antibodies. There were also marked differences in leukotoxic activity as measured by a chemiluminescence assay with bovine and ovine neutrophils. Some strains produced apparently normal amounts of LKT protein but which had low toxicity. Data will be presented on the target cell specificity of these LKT preparations in terms of their cell-binding, leukotoxic and haemolytic activities for different mammalian species. The properties of the native LKT from *P. haemolytica* will be compared with those of recombinant LKT from *E. coli.*

P44. Evaluation of a *Pasteurella haemolytica* Recombinant Sialoglycoprotease Fusion Protein (Rgcp-F) Vaccine.

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Recombinant sialoglycoprotease fusion protein (rGcp-F) was used in a vaccination and challenge trial in cattle. Calves received two doses of one of the following vaccines: saline plus adjuvant, Presponse (culture supernate vaccine), rGcp-F alone with adjuvant, Presponse enriched with rGcp-F, Presponse enriched with rLkt (recombinant leukotoxin), Presponse enriched with rGcp-F and rLkt. Following first vaccination with rGcp-F most calves seroconverted as shown by Western Blot. As expected, vaccination with Presponse was approximately 70% efficacious. Enrichment with both rGcp-F and rLKT enhanced protection, but enrichment with either alone resulted in improvement that was not significantly better than Presponse alone. This failure of rLKT alone to enhance Presponse, which was expected from previous experiments, may reflect the unusually severe challenge in this particular trial (all controls died within 5 days). Given this, it was surprising to discover that rGcp-F alone provided protection that was significantly better than saline (no vaccine) and not statistically less than Presponse. Protective efficacy approximated 40%. This is the first demonstration of protection using a single *P. haemolytica* antigen.

P45. The Distribution of the Sialoglycoprotease Gene and Activity Among *Pasteurella haemolytica* Serotypes 1 to 16.

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An examination of the distribution of the sialoglycoprotease (Gcp) gene and activity among *Pasteurella haemolytica* serotypes 1 to 16 was recently completed; along with other selected Pasteurellacae, including several clinical isolates of *P. haemolytica* serotype 1. Southern blot hybridization analysis and Gcp activity assay indicated that virtually all recognized *P. haemolytica* A biotypes contained the *gcp* gene and proteolytic activity. The exception was serotype 11 which revealed an alternative genetic organization and exhibited no proteolytic activity [Abdullah *et al;* Biochem. Soc. Trans. 18: 901-903]. Furthermore, all recognized T biotypes were negative for both gene and activity. This may have relevance in the differential pathogenicity and host range of A and T biotypes.

P46. Cloning and Molecular Characterization of the *GalE* Gene of *Pasteurella haemolytica* A1.

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The enzyme UDP-galactose-4-epimerase (GalE) catalyzes the epimerization of UDP-galactose to UDP-glucose. This enzyme has been shown to play an important role in the biosynthesis of lipopolysaccharide (LPS) in a variety of Gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium, Neisseria meningitidis,* and *Haemophilus influenzae.* The LPS of P. *haemolytica* Al has been implicated as a virulence factor in the organism' s pathogenesis. To investigate the biosynthesis of P. *haemolytica* Al LPS at the

molecular level, the *galE* gene was cloned on a 5.5 kbp plasmid insert. This recombinant plasmid could complement and restore the synthesis of the entire O-antigen in a *S. typhimurium galE* mutant. Several proteins are expressed from the *galE* complementing DNA, including a protein of approximately 37 kDa which is similar in size to other GalE proteins. A 2.3 kbp sub-clone that encodes the 37 kDa protein is homologous to the *H. influenzae* type b *galE* gene.

P47. The Evaluation of Usefulness of Selected Polyclonal Antibodies for Immunocytochemical Diagnosis of *Haemophilus somnus*.

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The results of bacteriological examination in diagnosis of *Haemophilus somnus* infection are often difficult to interpret because of high cultivation requirements for this micro-organism and the overgrowth with saprophytic flora. We decided to work out the immunohistochemical method for detection of *H.somnus in situ*, based on using the monovalent sera against selected components of outer membrane protein (OMP) complex. The OMP of *H.somnus* was isolated according to Kania *et al.*(1992). After the separation of the OMP in SDS-PAGE, the major components: 67, 40 and 22 kDa were cut out and used for immunization of rabbit and goat, according to Boulard and Lecroisey (1982). The developed immune sera were controlled with ELISA and immunoblotting against H.somnus and other related bacteria whole antigens. Rabbit sera against 67 and 22 kDa OMP showed wide cross reactivity with *Pasteurella multocida, P.haemolytica* and Escherichia coli. Only goat immune serum against 40kDa OMP showed strong reactivity with *H.somnus* and very weak cross-reactivity with *P.multocida*. IgG class (both IgGl and IgG2 isotype) was isolated from immune serum and negatively absorbed with supernatant of *P.multocida* sonicate coupled to sepharose 4B. This antibody was successfully used in immunocytochemical detection of *H.somnus*.

P48. Protective Efficacy of a *Pasteurella haemolytica* A1 Iron-Regulated Bacterin Vaccine in Calves.

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The protective efficacy of an iron-regulated bacterin vaccine was tested in hysterotomy-derived 8-week old male calves which were kept in isolation and were free of *Pasteurella* at the time of challenge. Cells of *P.haemolytica* A1 (strain M4/1/2) grown under iron restricted conditions using αα-dipyridyl , were inactivated with formalin and combined with aluminium hydroxide gel (alhydrogel). Five calves were vaccinated subcutaneously in the neck with a 2ml dose on two occasions, 4 weeks apart. The vaccinated calves and a control group of 6 unvaccinated calves were challenged intra-tracheally with 60 mls of a log phase culture of strain M4/1/2 .using an endobronchoscope. Surviving calves were monitored daily for clinical signs and necropsied on the fourth day after challenge. All control calves were removed due to severity of disease before the scheduled necropsy time with a mean total disease score (TDS) of 39.3 ± 1.2. All vaccinated animals survived with a significantly lower (p= 0.01) TDS of 11.8 ± 5.1 . Western blotting showed a strong response to iron-regulated protein (IRP) bands in the sera of vaccinated calves.

P49. Transferrin-binding Protein From *Actinobacillus pleuropneumoniae* Serotype 7: Purification, Characterization and Potential Serodiagnostic Use.

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Transferrin-binding protein from *Actinobacillus pleuropneumoniae* serotype 7 (WF83) was purified by a published method employing affinity-chromatography on immobilised hemin. An essentially pure protein was obtained (as judged from silver-stained SDS-polyacrylamide gel electrophoresis). The protein had an apparent molecular weight of 66 kD in contrast to the previously reported molecular weight for this protein (60 kD); also, we found no difference in its expression in an iron-restricted as compared to a non-restricted culture. The hemin- and swine transferrin-binding activities of the protein was found to be interdependent. We found a preferential reaction with the purified protein in ELISA of sera from a range of experimentally *Actinobacillus pleuropneumoniae*-infected swine as compared to sera from non-infected swine and from swine infected with other agents.

P50. Analysis of the *tbp* Loci in *Pasteurella haemolytica* Serotypes 1 to 16, *Actinobacillus suis* and *A. pleuropneumoniae*.

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Pasteurella and *Actinobacillus* species exhibit an iron uptake system which is capable of obtaining iron directly from host transferrin. This uptake is mediated by specific transferrin binding proteins in the bacterial outer membrane. The gene encoding for the P. *haemolytica* Al transferrin binding protein Tbpl (M.W. 100 kDa) has been cloned and sequenced. A *tbpA* specific probe was used to examine chromosomal DNA digests from the sixteen serotypes of P. *haemolytica, A. suis* and two *A. pleuropneumoniae* strains. The results showed that all 16 serotypes of *P. haemolytica* carry the *tbpA* gene, however, there are considerable differences in the restriction fragments that hybridized with the probe between the A and the T biotypes. Both *A. suis,* and *A. pleuropneumoniae* (CMS and Shoppe) examined also showed hybridization with the probe, though with less homology. A restriction map of the *tbpA* region in some of these strains was constructed to examine the restriction site polymorphism and to compare the degree of relatedness between the *tbpA* genes.

P51. Conservation and Antigenic Cross-Reactivity of the Transferrin Binding Proteins of *Haemophilus* and *Actinobacillus*.

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In extracellular body fluids, *Haemophilus influenzae* acquires iron from the iron-transporting glycoprotein transferrin via a receptor mediated process. This involves two iron-regulated outer membrane proteins referred to as TBPI and TBP2 which show considerable preference for the human form of transferrin. Since the TBPs have attracted considerable attention as potential vaccine components, we used transferrin affinity chromatography of detergent solubilised membranes together with SDS-PAGE to examine their conservation amongst *H.influenzae* type b strains belonging to each of the recognised outer membrane subtypes as well as non-typable strains. Virtually all strains yielded a TBPI of 105 kDa and a TBP2 of 90 kDa which on immunoblots cross-reacted with a monospecific antibody raised against the denatured 68 kDa TBP2 of *Neisseria meningitidis* and with an antiserum raised against the *H.influenzae* native TBPI/TBP2 complex. In addition, these antibodies cross-reacted with the TBPs of the *Actinobacillus pleuropneumoniae* even though this porcine pathogen recognises pig but not human transferrin. These data demonstrate the existence of shared epitopes on the TBPs of

H.influenzae, N. meningitidis and *A.pleuropneumoniae* despite their transferrin species specificity.

P52. Intracellular survival of *Haemophilus somnus* in bovine blood monocytes: effect of cytokine and LPS treatment.

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The interactions between bovine blood monocytes (BBM) and *H. somnus* are known to be complex. In order to study this interaction further, a colorimetric assay using MTT was developed to assess the survival of *H. somnus* within cultured BBM. By using this system, it was found that (1) *H. somnus* was able to survive within BBM *in vitro* and the kinetics of its survival was similar to that seen in BBM isolated from experimentally infected cattle; (2) treatment of BBM with varying concentrations of rBoIFN-γ, rBoTNF-α, rBoIL-1β or rBoGM-CSF had no effect on the survival of *H. somnus;* and (3) treatment of BBM with E. *coli* LPS inhibited their survival. These results suggest that the ability of *H. somnus* to survive in both untreated and cytokine-treated BBM could be an important virulence mechanism, and that LPS is able to trigger BBM to control *H. somnus*.

P53. Inhibition of the Growth of Biotype A Strains of *Pasteurella haemolytica* by Sheep Blood

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Commercial preparations of defibrinated sheep blood inhibited the growth of biotype A strains of *Pasteurella haemolytica* but not T biotypes. This inhibitory activity was demonstrable only in the poured agar medium containing either of two blood lots and was present for up to three months at 4°C. Subsequent batches of plates prepared using the same lots of blood which had been stored an additional two weeks at 4°C, did not retain inhibitory activity. To determine the cause of inhibition, the potential role of serum antibody was examined by the use of immunoblotting and the Rapid Plate Agglutination (RPA) technique. Although inhibitory blood lots contained antibodies to 16 *P. haemolytica* serotypes (representing both biotypes) as shown in immunoblots, the serum of non-inhibitory lots yielded similar results. Serum fractions from inhibitory and non-inhibitory lots were indistinguishable by RPA. Based upon previously documented evidence that A and T biotypes of *Pasteurella haemolytica* are differentiated by their sensitivity to penicillin and observations that the inhibitory lots would not support the growth of other penicillin sensitive organisms such as *Bacillus subtilis* and *Pasteurella multocida*, we suggest that this antibiotic may have been responsible for this observed phenomenon.

P54. Synergy between an ovine isolate of *Bordetella parapertussis* and *Pasteurella haemolytica* A2 in mice.

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When administered intranasally to Swiss-white mice an ovine isolate of *Bordetella parapertussis* caused lung lesions of a severity which was dependent upon the infecting dose. While there were few overt clinical signs of disease, histopathological examination of the lungs revealed a catarrhal pneumonia characterized by hyperaemia, haemorrhage and oedema. *Pasteurella haemolytica* on its own was not pathogenic and was rapidly cleared from the lungs. Pre-infection of mice with 7.5 $x10^6$ colony-forming units (cfu) of *B. parapertussis* followed by intranasal infection with 1.4 x 10^5 cfu of *P. haemolytica* resulted in increased severity of the lung lesions. The timing between the administration of

B. parapertussis and *P. haemolytica* appeared to be important in determining the severity of the disease with the most severe lesions observed when *P. haemolytica* was administered three days after *B. parapertussis*. In addition to the Swiss-whites a second strain of mouse was used in a similar disease protocol. When *B. parapertussis* (6×10^7 cfu total) was administered to Porton mice 33% died within 48h and the organism was isolated from the lungs along with another bacterium. This bacterium (closest identification by API strips: *Actinobacillus ureae*) was also found in the upper respiratory tract, but not the lungs, of control uninfected mice and in lower numbers than observed in the infected groups. Therefore it appeared that the damage to the respiratory tract caused by *B. parapertussis* may have allowed the *A. ureae* to penetrate deeper and to colonise the lung, causing disease.

P55. Pneumonic Pasteurellosis in Sheep and Goats in Malaysia.

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Pneumonic pasteurellosis is a major constraint to the development of the sheep and goat industry in Malaysia. The disease is endemic and is responsible for 39 percent of the mortalities seen in the small ruminants in the country. Imported vaccines were used to control the infection but with limited success. Subsequently, an oil adjuvant combined pasteurella vaccine incorporating local strains of *Pasteurella multocida* and *P. haemolytica* was developed and showed considerable success in controlling the disease in certain areas of the country. Bacteriological study done showed the presence of five serotypes prevailing in different parts of the country. The major serotype seen in the country is A2 (37%) followed by serotypes A7 and A1. Protein profile of the isolates on polyacrylamide gel electrophoresis irrespectively of their origin (animal host, organs and geo-location) showed no distinct difference amongst them. However, on immunostaining revealed 5 to 7 major proteins mainly in the higher molecular weight region that were immunogenic.