EPIDEMIOLOGICAL FEATURES OF PORCINE

PROLIFERATIVE ENTEROPATHY

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CONTENTS

DECLARATION

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ABSTRACT

A polymerase chain reaction (PCR) assay was adapted and optimised for specific detection of *Lawsonia intracellularis* genomic DNA segment in swine faeces. *Lawsonia intracellularis* is the aetiological agent of porcine proliferative enteropathy (PPE) and the PCR represents the first diagnostic test suitable for ante-mortem use in affected swine.

Various methods designed to extract bacterial DNA from faeces were evaluated to establish a convenient and optimum protocol. The PCR was utilised in pig challenge studies to investigate the excretion patterns of *L. intracellularis* in weaner pigs orally inoculated with pure cultures of *L. intracellularis*. This challenge work demonstrated that the PCR was a suitable tool for detection of infection, and indicated that individual animals could excrete *L. intracellularis* organisms for periods of up to ten weeks post-challenge. Such an excretion period has major implications for the transmission of organisms in the field. For example, if infected growers are still shedding *L. intracellularis* organisms upon entry to the breeding population, then this is a possible route for the transmission of disease to younger, susceptible pigs.

A more extensive, two-part investigation of the epidemiological aspects of PPE in the field followed. The investigation comprised a farm sampling study and a questionnaire postal survey. In the farm sampling study, faeces samples were collected serially over a ten month period from breeding gilts and their litters. Samples were subjected to PCR for the detection of infection, allowing estimation of within-herd prevalence, as well as determination of possible transmission patterns. The assay successfully detected the presence of *L. intracellularis* in the weaners and/or growers of three of the five farms selected for this study. The within-herd prevalence for these age-groups ranged from 10 to 30%. The PCR also confirmed infection in several of the adult breeding boars and gilts.

The relative expense of the PCR assay dictates that its practical application, at least for the purposes of research, must be targeted. Thus, the sampling study was coupled with a postal questionnaire survey. Mailing questionnaires to almost 600 commercial production units achieved a 56% response rate. This provided a sufficiently large number of herds to allow statistical analysis of possible risk factors involved in the epidemiology of PPE. This survey indicated that the 1993 to 1995 period-prevalence of PPE in the UK was 31%. Based on the number of sows, herd size was an important risk factor, even when herds with under 50 sows were excluded ($p<0.005$). There was an important link between the occurrence of PE

and nucleus herds, with five out of six nucleus herds in the study having had PE diagnosed in the previous three years. This link was strengthened in that herds obtaining replacement breeding boars from nucleus stock were at an increased risk of PE ($p<0.05$). Factors which could affect the exposure of animals to the faecally-contaminated environment were also significant. Surprisingly, slatted or meshed floors were linked to PE, especially in the younger age-groups ($p<0.05$). Batch movement of pigs on a house basis was significantly protective against PE ($p<0.05$), but batch movement on a pen basis only was neither a protective nor a risk factor.

Epidemiology of PPE is complex, but the PCR has proved to be a valuable tool, capable of screening for the presence of *L. intracellularis* in field conditions. Additionally, its use has permitted some initial conclusions to be drawn regarding the excretion and transmission patterns of this unique organism.

CHAPTER ONE

LITERATURE REVIEW

Introduction

Proliferative enteropathy (PE) is an intestinal disease of pigs, with clinical signs of anorexia and occasional diarrhoea, often accompanied by weight loss or reduced weight gains from six to 16 weeks of age (Rowland and Lawson 1974, 1975, 1992). A more acute form, usually in older pigs, has signs of sudden death with an associated melaena (Rowland and Rowntree 1972). Although the major significance of PE lies in its effects on the economy of the pig industry world-wide (McOrist et al 1993), it has also been found in several other species, including the ferret (Fox and Lawson 1988), foal (Duhamel and Wheeldon 1982, Williams et al 1996), blue fox (Landsverk 1981), rabbit (Schoeb and Fox 1990) and, more notably, the hamster (Stills 1991) which has provided a useful model for studies into PE (Frisk and Wagner 1977, Jasni et al 1994a, 1994b).

The PE complex is now known to be caused by a new genus and species of obligate intracellular bacteria, called *Lawsonia intracellularis* (McOrist et al 1995a). However, although identification has allowed detailed phenotypic and genetic characterisation of this organism (McOrist et al 1995a), its pathogenesis is still very poorly understood.

Pathogenic features of the acute and chronic forms have been comprehensively described. *L. intracellularis* within the intestinal crypt epithelial cells is consistently associated with the typical gross and microscopic lesions (Rowland et al 1973, Rowland and Lawson 1974) which so often are the means of diagnostic confirmation. Thickening of the intestinal mucosa is caused by hyperplasia of these cells and is accompanied by a reduction in goblet cells. Mucosal lesions in the acute form also include petechiations and congestion, accompanied by haemorrhage into the lumen of the intestine with production of clots and fibrin casts (Love and Love 1979). Although eosinophils, lymphocytes and plasma cells are present, extensive inflammation does not tend to be a feature of this disease in either of its forms (Rowland and Rowntree 1972).

Beyond these descriptions, little is known regarding the actual disease process. The epidemiology of PE has not been investigated to any significant extent. Most reports of prevalence have been based on abattoir surveillance (Christensen and Cullinane 1990) and it is unclear as to their true reflection of the situation in the field. Similarly, little is known regarding transmission of disease, shedding patterns or sources of infection. Areas such as immune response and host resistance remain obscure (Holyoake et al 1994c).

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Hitherto, much of this work has not been feasible or practical. Diagnosis has relied upon post-mortem examination, employing histopathological methods which, although specific and reliable, have not been applicable to dynamic studies of the infection within herds. The clarification of the aetiology of PE has now removed one of the main obstacles and helped to overcome barriers in the research of this significant problem.

Aetiology of Proliferative Enteropathy

Early transmission studies with Campylobacter species

The first clue as to the cause of PE was the consistent demonstration of curved intracellular organisms free within the cytoplasm of crypt epithelial cells (Rowland and Lawson 1974). These organisms were initially thought to belong to the *Campylobacter* species, especially as several species (namely, *C. sputorum* ss *mucosalis, C. mucosalis, jejuni and coli*) had been isolated from lesions of PE (Gebhart et al 1983). However, challenge experiments using some of these species, particularly *C. sputorum* ss *mucosalis* and *C. hyointestinalis*, were unsuccessful. Although *Campylobacter sputorum* ss *mucosalis* was capable of producing a short-lived enteric infection experimentally in neonatal pigs (Roberts et al 1980) and gnotobiotic pigs (McCartney et al 1984), neither specific lesions, nor intracellular bacteria were induced.

Reproduction of proliferative enteropathy using intestinal mucosa

The early transmission studies were only successful when filtered homogenates of adenomatous intestinal mucosa, derived from histologically confirmed cases of the chronic form of PE, porcine intestinal adenomatosis or PIA, were used as oral inocula. Nine neonatal piglets from conventional sows were dosed with homogenate at 18 hours of age and killed sequentially from 24 to 66 days post challenge. Clinical signs of reduced weight gains were observed three weeks after dosing and typical microscopic lesions were produced in four of the pigs. In this work, the causative agent was believed to be *C. sputorum* ss *mucosalis* and the intracellular organisms observed were described as resembling *mucosalis*. However, control animals which had also received a suspension containing *mucosalis* failed to develop typical microscopic or gross lesions. Although a small number of *mucosalis* organisms were isolated from the control animals, they had not appeared to induce any epithelial cell proliferation. The only difference between the

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challenge and control pigs was that the latter had not received intestinal homogenate (Roberts et al 1977). Lomax et al (1982a) used intestinal homogenate to produce some microscopic proliferation in the ileal mucosa and intracellular *Campylobacter*-like organisms in 10 week-old pigs derived from specific-pathogen-free (SPF) sows. Very similar lesions were observed in cohort pigs dosed with *C. sputorum* ss *mucosalis*, however. The lesions produced in both cases were rather atypical of PE, consisting of flattened mucosal surfaces and a substantial mucosal inflammatory cell infiltrate. Nonetheless, there was some epithelial proliferation, together with a reduction in the number of goblet cells, even in those pigs dosed with *C. sputorum* ss *mucosalis* alone. While this work did suggest that *Campylobacter* might be involved in the pathogenesis of PE, the source of the pigs was never specified and, although SPF, their PE status was not defined.

Challenge experiments performed on gnotobiotic pigs inoculated orally with intestinal mucosa from natural cases of acute PE also produced typical microscopic lesions associated with the presence of the curved organisms in the enterocytes, demonstrated by indirect immunofluorescence (McOrist and Lawson 1989b). These intracellular organisms failed to fluoresce when stained with antisera prepared against the *Campylobacter* species (*C. hyointestinalis* and C. *coli*), providing further evidence that *Campylobacter* species were not responsible for PE and that the causative agent was antigenically different. Possession of a distinct "omega" antigen (Ω) had been serologically detected using rabbit antiserum in pigs, ferrets and hamsters with PE (Fox and Lawson 1988, Lawson et al 1985) and a monoclonal antibody (IG4) specific to *L. intracellularis* has been manufactured by immunising BALB/c mice with organisms derived from affected mucosa (McOrist et al 1987). This monoclonal antibody specifically reacted with the intracellular organism in the enterocytes of diseased mucosa from hamsters and pigs but did not react with any of the common *Campylobacter* species such as *C. mucosalis, hyointestinalis, fetus, jejuni,* and *coli* or with healthy pig tissue, confirming the unique antigenic status of this elusive organism.

Co-culture of *Lawsonia intracellularis*

One of the main reasons for the difficulty in establishing the aetiology of PE turned out to be that *L. intracellularis* cannot grow in a cell-free medium. Attempts to isolate a

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causative agent had primarily relied on more traditional bacterial culture methods. Therefore, co-cultivation of intracellular bacteria derived from PE lesions on various cell lines, including human foetal intestine (INT-407), rat colonic adenocarcinoma (CRL 1677) and pig kidney (PK-15), was instigated. The rat small intestinal cell line IEC-18 (ATCC CRL 1589) was found to be the most effective for the purpose. The pig intestinal cell lines, including IPEC J2, were not as suitable, achieving poor growth rates and proving difficult to manipulate, although being an intestinal epithelial cell from the target species (Lawson et al 1993). IPEC J2 cells are derived from the jejunum. Since *L. intracellularis* organisms normally infect the small intestine distal to this region, preferably ileal and colonic epithelial cells (McOrist et al 1993), it is likely that the IPEC-J2 cell line lacks certain characteristics required to support *L. intracellularis* organisms. While McOrist et al (1995b) used this cell line (IPEC-J2) to show that cytopathic effects were minimal in porcine intestinal epithelial cells, no improved growth of the organism was observed. Since the IPEC-J2 cell line is slow-growing (Rhoads et al 1994), it is possible that it does not have an adequately high cell turnover to provide sufficient numbers of immature epithelial cells for cultivation of *Lawsonia*. This would also help to explain why the distal small intestine and colon are the sites of infection *in vivo*. To establish primary co-cultures, mucosal filtrates derived from field cases were used as source material. The mucosa from affected intestines was homogenised then incubated in a suspension with trypsin and phosphate-buffered saline (PBS) at $37^{o}C$. Sucrose potassium glutamate buffer (SPG) and 10% foetal calf serum (FCS) were added to the treated mucosa, which was ground down and sequentially filtered through a series of steel mesh, glass fibre and fine membrane filters (Lawson et al 1993), to a final pore size of $0.65\mu m$.

Primary co-cultures were established by inoculating IEC-18 cells, which had been growing for 24 hours, with dilutions of mucosal filtrates. The optimum atmospheric mixture for co-culture is CO_2 and O_2 concentrations of 8.8% and 8.0%, respectively (Lawson et al 1993). These concentrations have proven to be an absolute requirement for the cultivation of *L. intracellularis*. This is perhaps not surprising, bearing in mind that these fastidious intracellular organisms flourish in the microaerophilic environment of the mammalian intestines. The antibiotics neomycin ($50\mu g/l$) and vancomycin ($100\mu g/ml$) are added 3h after infection of the monolayers, to inhibit other bacteria. *Chlamydia* is a common commensal of pig intestines (Szeredi et al 1996) which can contaminate the cell culture

monolayer and its presence is excluded from cultures by immunological testing (McOrist et al 1993).

Quantification of co-culture infection

Monitoring of infection using mouse monoclonal antibody IG4 as the primary antibody (McOrist et al 1987) and anti-mouse IgG-fluorochrome conjugate or peroxidase conjugate is a specific method for confirming the identity of the intracellular organisms as those seen in diseased mucosa from PE cases. It has allowed the level of infection to be estimated by counting the bacteria in cells under light microscopy. The bacteria tend to be concentrated in groups of cells and these infected cells were used as a measure of the level of infection (Lawson et al 1993, McOrist et al 1995c). Monolayer cells containing more than 30 organisms were termed heavily infected cells and groups of these cells were called foci of infection. The number of foci has been used to determine the infectivity of the inoculum, while the number of heavily infected cells gave a measure of bacterial multiplication (Lawson et al 1993).

Passage of infection

Once established, the bacteria co-cultured in one set of cells can be passaged to other cells. This has been attempted in three main ways. Firstly, by lysing IEC-18 cells using potassium chloride (KCl), secondly, by trypsinising the cells or thirdly, by using the supernatant fluid obtained from infected cell monolayers to infect new cell monolayers (Lawson et al 1993). KCl lysis has provided the best method for maintaining the infection over 20 sequential passages. Infected cells are treated with 0.1% KCl, and SPG/FCS. The infected cell monolayer is scraped from the culture flask and passed through a syringe and needle several times. The lysed cells are centrifuged at low speed, to remove the cell nuclei and the supernatant fraction is then centrifuged at 2020 g for 30 min. The bacterial pellet formed is resuspended in SPG/FCS. Fresh IEC-18 cells can then be infected with the bacterial suspension. Using this KCl procedure, bacterial infection of cells peaks at seven days and then slowly declines (Lawson et al 1993). Co-cultures require refeeding with media during this period.

The cell culture system not only offered a method of culturing the fastidious *L. intracellularis*, but it progressed further to provide large volumes of inocula suitable for

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use in challenge trials. McOrist et al (1993) used pure cultures of *L. intracellularis* derived from natural cases to prove that *L. intracellularis* was the primary aetiological agent responsible for proliferative enteropathy.

The main advantage of cell culture is that a known dose of inocula can be produced each time, which allows each animal in the experimental situation to be given identical amounts of organisms. This means that studies in the treatment, control and epidemiology of PE can be repeated and results compared. An additional benefit is that cultures can be screened for the presence of possible contaminants which might interfere with or complicate the disease process (McOrist 1996).

Aetiology and Pathogenesis of Proliferative Enteropathy

Pure cultures of *Lawsonia intracellularis* organisms co-cultured from a natural case of acute PE were used as inoculum to reproduce the disease in four-week old conventional pigs, following oral challenge with the inoculum (McOrist et al 1993). Twenty-two days after challenge, typical microscopic lesions were observed in all the challenged pigs and numerous curved bacteria were demonstrated in the crypt epithelial cells by staining with Young's silver stain (Young 1969) and immunostaining with the specific monoclonal antibody IG4 (McOrist et al 1987). In a later similar challenge trial, all seven conventional pigs challenged developed microscopic lesions typical of PE, while six of the seven developed grossly visible lesions (McOrist et al 1996a). These highly successful experiments were considered the final confirmation that *L. intracellularis* is the cause of PE.

However, challenge work involving the oral inoculation of gnotobiotic pigs at seven days of age with pure culture failed to produce colonisation of the intestinal epithelial cells, and no microscopic lesions were observed (McOrist et al 1993). In contrast, the disease had been previously reproduced in two gnotobiotic pigs dosed at seven days of age with filtered intestinal mucosa obtained from a case of PE. Both microscopic lesions and intracellular organisms were noted in challenged pigs in the latter study. In the same study, identical pigs dosed with a more finely filtered (0.65µm) mucosa failed to develop as convincing lesions and curved intracellular organisms were only demonstrated in the intestinal lumen (McOrist and Lawson 1989b). A later study found that two of three piglets infected with the same non-pathogenic strains of *Bacteroides vulgatus*, followed by oral inoculation with cultured *L. intracellularis* developed microscopic lesions of PE,

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whereas two of two piglets pre-dosed with non-pathogenic strains of *B. vulgatus* and *Escherichia coli* only, failed to develop microscopic lesions (McOrist et al 1994b). Similar studies have been conducted to investigate the pathogenesis of *Isospora suis*, which is the aetiological agent of porcine coccidioisis, capable of causing varying degrees of diarrhoea, and sometimes high mortality, in neonatal piglets. When compared to age-matched and gnotobiotic cohorts, conventionalised piglets, orally dosed at two days of age with a combination of *I. suis* and bacterial flora, succumbed to coccidioisis much more quickly (Harleman and Meyer 1985). The first mortality occurred four days post challenge. Clinical signs were slower to develop in the gnotobiotic pigs, but there was little overall difference in the actual severity or time-course of symptoms, compared to the conventionalised pigs. On average, however, the gnotobiotic pigs survived longer and the first gnotobiotic pig was killed on humane grounds 8.5 days post challenge. Gross and microscopic findings of the gnotobiotic and conventionalised pigs paralleled each other at necropsy. Diarrhoea, malabsorption, villous atrophy and necrotic enteritis was described in both groups. Since there were no differences in pathogenicity or pathological lesions, it was concluded that *I. suis* is a primary pathogen in pigs.

Another extensive study examining the effect of *Trichuris suis* on the mucohemorrhagic syndrome in pigs found significant synergism between this parasite and intestinal microbes. Mansfield and Urban (1996) were consistently able to create typical and severe clinical signs of enteritis and colitis in pigs challenged with embryonated *T. suis* eggs 14 days earlier. Only challenged, untreated pigs developed gross signs but microscopic lesions were noted in challenged pigs, whether treated or not. These lesions consisted of thickening of the muscularis and mucosal layers at the site of worm attachment, destruction of crypt cells and absorptive cells on the colonic surface and an increased inflammatory process. Lesion severity was more pronounced in the untreated, challenged pigs. Perhaps more significantly, lesions progressed to crypt abscessation involving the specialised lymphoglandular complexes in areas of the distal colon, distant from the sites of worm attachment. No bacteria could be cultured from any of the unchallenged pigs. While bacteria were isolated from only two challenged and treated pigs, many species were isolated from challenged, untreated animals, including *Campylobacter jejuni*, *Escherichia coli*, *Pseudomonas* and, interestingly, *Lawsonia intracelullaris*. This work indicated that *Trichuris* infection could promote bacterial invasion and suggested that the severe pathology associated with this parasitic infection is dependent on the presence of

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bacteria. The synergistic effect is taken further by the influence which *T. suis* infection apparently has on bacterial infections in intestinal sites distant from parasitised areas. Mansfield and Urban (1996) speculated a downregulation of immunity to microbial infections by *T. suis*, whereby production of cytokines necessary to combat parasitic infections may be associated with a reduced immune response required for other types of infection, such as interferon-gamma. Whether for similar or different reasons, this synergistic activity appears to be equally important for the pathogenesis of *L. intracellularis*. It is possible that the presence of other bacteria help to minimise a specific immune response to intracellular organisms. Alternatively, *L. intracellularis* may require the assistance of other intestinal anaerobes to gain cell entry. This is apparently the case in other pig enteric diseases, such as swine dysentery. While *Serpulina hyodysenteriae* has reportedly caused lesions in gnotobiotic pigs (Whipp et al 1982), both its ability to colonise and its pathogenicity are enhanced by the presence of other gut flora including *Bacteroides* species (Neef et al 1994). Perhaps these other organisms help to create the optimum intestinal conditions for colonisation by *L. intracellularis*, including pH and oxygen levels. The age difference between the gnotobiotic and conventional pigs used in the first experiment (McOrist et al 1993) cannot be completely discounted as a possible reason for the difference in pathological findings. Little is known regarding the pathogenic effects of *L. intracellularis* but it is possible that neonatal piglets do not possess a necessary factor required for successful infection. An alternative explanation is that age resistance in the neonates is preventing colonisation, perhaps due to failure to express a required cell receptor. A similar phenomenon is believed to occur in enterotoxigenic *E. coli* infections of pigs and has been reported for *E. coli* K99 and 987P (Runnels et al 1980, Dean et al 1989). However, in the case of *E. coli*, piglets are actually more susceptible to the organism in the first few days of life and develop resistance as they get older.

Another explanation may lie in the fact that gnotobiotic pigs have a slower crypt epithelial cell turnover (Savage et al 1981) reducing the opportunity for *L. intracellularis* to invade its most preferred environment. However, PE has been reproduced previously in gnotobiotic pigs dosed at seven days of age, when gut mucosa was used as inoculum (McOrist et al 1989a), demonstrating that colonisation of the intestine of such pigs is possible.

Other pathogenic factors

Common to both forms of PE is the hyperplasia which occurs in crypt lining epithelial cells of the ileum and colon (McOrist et al 1989). The proliferation of these cells is always associated with the presence of curved intracellular organisms and begins shortly after their entry into the crypt cells. Bacteria are thought to enter cells as early as five days post infection (Johnson and Jacoby 1978) and the onset of hyperplasia occurs 10 days post infection (McOrist et al 1989). Hyperplasia is associated with diminished goblet cells and reduced or no apoptosis. The transient nature of the clinical signs observed in chronic PE is reflected in the apparent ability of affected epithelial cells and intestinal mucosa to recover. McOrist et al (1996) observed shrunken, degenerate epithelial cells in affected crypts, the return of apoptotic bodies (a normal feature of healthy porcine intestines) and the reappearance of normal goblet cells in pigs experimentally infected 7 to 9 weeks earlier. Since apoptotic bodies have not been observed in early lesions of PE (McOrist et al 1989), it is thought that *L. intracellularis* is able to influence the normal cell cycle, although the exact mechanism by which it achieves this is not known. The reappearance of apoptosis in regenerating intestine infers that any nuclear response to the presence of intracellular bacteria is transitory and reversible. In itself, apoptosis may be an attempt by the intestine to remove the extra epithelial cells which have resulted from the hyperplasia (McOrist et al 1996a). Similarly, the mode of induction of infected cell proliferation is also unclear. Speculation has suggested bacterial regulation of genes responsible for cell differentiation and apoptosis, or the direct influence on cells by a bacterially-produced mitogen (McOrist et al 1996a).

Little work has been performed to directly assess the effect of type of diet on the outcome of infection with *L. intracellularis*. This may be an area worthy of fuller investigation, considering the apparent relationship which exists between swine dysentery and diet, and the feeding of high fibre diets in the management of non-specific colitis. Pluske et al (1996) demonstrated that clinical signs associated with swine dysentery were linked to the amount of soluble non starch polysaccharides (NSP) in the pigs' diet. They measured pH, ATP and volatile fatty acid production in the gastro-intestinal tract, and performed dietary analysis to assess the levels of NSPs and resistant starch, working on the premise that decreased fermentation in the large intestine protects against swine dysentery. General patterns emerged, indicating that diets containing low amounts of NSP (<5%) reduced the occurrence of clinical swine dysentery. This was complicated slightly by the concentration of resistant starch which could increase the incidence of swine dysentery. Cooked white

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rice was highly effective in reducing swine dysentery, possibly because it reduces spirochaetal colonisation of the large intestine. Its high digestibility minimised the substrate entering the large intestine, reducing both microbial fermentation and levels of VFAs, and increasing the pH. This particular trial was unable to demonstrate a direct link between reduced microbial fermentation in the large intestine and reduced swine dysentery, using pH and VFA levels. Other factors may be involved which are enhanced by highly digestible diets, including effects on mucin production, bacterial mobility, population and colonisation, expression of virulence factors and induction of inflammation. Similar factors may be involved in the pathogenesis of PE, although microbial fermentation in the colon may not be directly significant to *L. intracellularis* infection, which prefers more proximal regions of the intestine. If there is true synergism between *L. intracellularis* and other intestinal organisms, microbial fermentation may still have an indirect influence on the occurrence of PE, through more direct effects either on other micro-organisms or intestinal environment.

Genetic Characterisation of *Lawsonia intracellularis*

Generation of specific DNA probes

As some of the immunohistological work had been unclear (Rowland and Lawson 1974, Chang et al 1984), the realisation that the organism present in the crypt epithelial cells of PE cases was antigenically unique (Lawson et al 1985, McOrist et al 1987) instigated further analytical investigations, principally into its genetic structure. This primarily involved molecular techniques. Specific DNA probes were created from recombinant chromosomal DNA clones, with a view to developing a more reliable and specific means of detecting the causative organism in affected tissues and, if possible, in faeces (Gebhart et al 1991). Chromosomal DNA extracted from PE mucosa-derived *L. intracellularis* (then called *Campylobacter*-like organism, or CLO) was purified by a phenol-chloroform extraction technique combined with an ammonium acetate precipitation. This DNA was digested with *Hin*dIII restriction enzyme and ligated into the plasmid vector pGEM3Zf(+). The vector was transformed into competent *E. coli* and the plasmid DNA from resultant clones was digested with *Hin*dIII again, to ascertain the insert size. 96% of the clones hybridised successfully to ^{32}P-labelled total chromosomal DNA from *L. intracellularis*, while only 3% hybridised with both the CLO and *C. hyointestinalis* DNA. No hybridisation occurred between any of the clones and DNA from *C. coli* or *C. mucosalis*.

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These results reinforced the previous antigenic findings but also established a clear genetic variance. One of these plasmids (p78) was selected and both the plasmid and the actual insert were used as diagnostic probes, after labelling with digoxigenin. These probes hybridised with DNA from faeces inoculated with both infected cellular debris and mucosal filtrate from naturally infected pigs with acute PE, but did not hybridise with DNA from *C. mucosalis* or from non-infected faeces inoculated with non-infected mucosal filtrate (Jones et al 1993b). Similarly, *C. mucosalis*-specific and *C. hyointestinalis*-specific probes did not hybridise with *L. intracellularis* (Gebhart et al 1990). As a sensitivity of 10^7 *L. intracellularis* organisms/g of faeces was achieved, which is comparable to the detection of *Campylobacter* species using similar detection methods, the digoxigenin-labelled probe offered the first possibility of an ante-mortem diagnostic test (Jones et al 1993b).

DNA sequencing

Sequencing of the gene for the 16S segment of the bacterial ribosome is the recognised method for ascertaining the phylogeny of bacteria, particularly intracellular organisms which are difficult to cultivate and study using other means (Weisburg et al 1991). Genomic DNA was extracted from four separate preparations of PE mucosa-derived *L. intracellularis* organisms and phenol/chloroform purified. The polymerase chain reaction (PCR) and three primer pairs capable of amplifying almost full-length 16S rRNA genes from a wide range of eubacteria were used to amplify the gene which codes for ribosomal RNA in *L. intracellularis* (McOrist et al 1995a). Reaction samples from each preparation were pooled and sequenced either by cloning or by direct sequencing (dideoxynucleotide chain termination). Complete or partial sequences were ascertained for each of the pooled reaction products generated by the three primer pairs (Gebhart et al 1993). Clone pCLO28 was part of the genomic library prepared previously from *L. intracellularis* DNA (Gebhart et al 1991) and it was used to establish the full-length sequence of the *L. intracellularis* 16S rRNA gene. On comparison with this full-length sequence, the generated sample sequences were found to be identical. Comparison with rRNA sequences from other bacteria revealed 91% homology with *Desulfovibrio desulfuricans* but only 82% homology with *E. coli*, and less than 80% homology with any of the *Campylobacter* or *Helicobacter* species (Gebhart et al 1993).

The aetiology of PE was therefore defined as a new genus and species of obligate intracellular bacteria, given the name *Lawsonia intracellularis* (McOrist et al 1995a). It has been categorised as belonging to the delta subdivision of the class *Proteobacteria*, also occupied by organisms including *Myxococcus xanthus* and *Desulfovibrio desulfuricans* (Gebhart et al 1993). Although other enteric bacteria, such as *C. jejuni* and *E. coli* are found in this class, they are in the subdivisions epsilon and gamma, respectively. However, it does appear to be the only obligate intracellular organism in this class. Generally, intracellular organisms are not specifically related to each other, often being distributed among different groups. The genus *Rickettsia* and the species *Coxiella burnetii* are found in the same class as *L. intracellularis*, but they tend to gather in the alpha subdivision. More in-depth analysis of the 16S rRNA has indicated that *Coxiella* is actually more closely related to *Legionella* than it is to *Rickettsia*, hampering accurate classification of this genus (Murray et al 1994).

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This classification has been the culmination of work involving immunological techniques and molecular investigations. Assigning *L. intracellularis* as the aetiological agent of PE required the development of the cell culture system, which has also provided a tool to aid both in the development of diagnostic assays and in the investigation of treatment and control protocols.

Phenotypic Characterisation of *Lawsonia intracellularis*

Observations of *L. intracellularis* have been performed in naturally infected pigs (Rowland and Lawson 1974), pigs challenged with pure cultures of pig-derived *L. intracellularis* (McOrist et al 1993) as well as pigs challenged with PE-affected pig mucosa (McOrist et al 1989). Similar studies have been made on hamsters infected with hamster-derived ileal mucosa (Frisk and Wagner 1977), *L. intracellularis* in mucosal cells (Johnson and Jacoby 1978) and hamsters infected with pig-derived cultured inoculum (Jasni et al 1994b).

The organisms are 0.3µm in diameter and 1.0µm in length, usually straight or curved Gram-negative rods (McOrist et al 1995a) which are acid fast when stained with the Ziehl-Neelsen method (Rowland and Lawson 1992). They have a trilaminar outer membrane and an electron lucent zone between the cytoplasmic membrane and the outer membrane (Jasni et al 1994a). There are no flagellae, pili or fimbriae and they appear to be non-motile (McOrist et al 1995a). The layer on the outer surface of the bacteria is antigenically responsive to immunostaining with the monoclonal antibody IG4, which is specific for *L. intracellularis* (McOrist et al 1987). The organism's cytoplasm is granular, although it tends to vary in electron-density and contains no organelles (Jasni et al 1994a, Rowland and Lawson 1974). This bacterium will not grow in cell-free media but only in rat epithelial cells *in vitro* or pig or hamster intestinal crypt epithelial cells *in vivo*. Although *L. intracellularis* has the greatest homology (91%) with *D. desulfuricans* (Gebhart et al 1993), there are many phenotypic differences between the two. *D. desulfuricans* are structurally larger organisms with a polar flagellum. In contrast to *L. intracellularis*, they contain iron pigment, are sulphate-reducing and do not stain with Ziehl-Neelsen (McOrist et al 1995a). Protein electrophoresis of whole cell protein extracts from *L. intracellularis* and *D. desulfuricans* has revealed two completely distinct patterns. Most significantly, *D. desulfuricans* is not an intracellular organism.

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Characterisation of cell culture infection- (a) cell entry

Ultrastructural studies have demonstrated that *L. intracellularis* becomes cell-associated within three hours of inoculation of cell monolayers, but the internalisation pathway is still unclear. There are no other obvious external structures such as pili or fimbriae on infected cells, which may help other enteric bacteria, such as certain strains of *E. coli*, to attach to cells (Bertschinger et al 1992). Electron micrograph studies have not demonstrated any surface patterns to indicate the presence of a surface- or S-layer (McOrist et al 1997a).

In most cases of intracellular parasitism, some adaptation is required to facilitate cell entry. This is either host-directed or parasite-directed and is called endocytosis. Parasite-directed endocytosis is a process common to many intracellular organisms, such as *Shigella* and the malarial protozoa of the *Plasmodium* genus (Moulder 1985). While *Shigella flexneri* does not appear to use pili or other specialised organelles, entry of the host cell can be interfered with using UV light, heat and kanamycin, providing evidence for the parasite's role in internalisation. The adherence of *Plasmodium knowlesi* merozoites to erythrocytes is facilitated by their thick glycoprotein cell coat which forms erect filaments. Subsequent entry only occurs when a subcellular structure, the apical complex, comes into contact with the host cell (Moulder 1985). Host-directed endocytosis is usually associated with a host cell structure such as a receptor or a ligand, allowing parasite-binding to the cell.

There is no confirmatory evidence to indicate the presence of either a host or parasite-associated mechanism or structure to facilitate the *L. intracellularis* entry process. Depressions in the luminal surface of infected hamster epithelial cells have been observed, with an associated bacterium in the concavity and loss or disruption of nearby areas of the brush border, but these have been considered non-specific changes (Jasni et al 1994a). Entry of cells by *L. intracellularis* does not appear to rely on bacterial viability, as formaldeyde-killed and oxygen-damaged organisms are apparently still able to enter epithelial cells (Lawson et al 1995), but host cell viability and function do appear to be necessary. For instance, cytochalasin D, a potent inhibitor of cell microfilament function, markedly reduces cell uptake of *L. intracellularis* (Lawson et al 1995). Entry of many other intracellular organisms, including *Shigella* and *Rickettsia* also requires viable host cells (Moulder 1985), so this finding is perhaps not surprising, but it does suggest that entry of *L. intracellularis* into intestinal epithelial cells is host-directed. It is still not certain whether *L. intracellularis* utilises the host cell's energy or metabolic mechanisms

either for the entry process itself, or for multiplication within the cytoplasm. *Chlamydia* must rely on the host cell's ATP as it is unable to manufacture it (Moulder 1991). *L. intracellularis* may have a similar requirement, especially since it appears to associate very closely with mitochondria and the rough endoplasmic reticulum of the host cell (Jasni et al 1994a).

Recent evidence has indicated the possible involvement of a cell surface ligand in the invasion of epithelial cells by *L. intracellularis*. Treatment of co-culture cell monolayers with the synthetic tetrapeptide, Arg-Gly-Asp-Ser (RGDS), increased the number of *L. intracellularis* organisms within co-culture cell monolayers, compared to Arg-Gly-Glu-Ser (RGES) peptide-treated co-cultures or medium-only controls. The amino acid sequence Arg-Gly-Asp is an important factor in some microbial interactions with host cells, with an ability to increase their invasive capacity (Roivanen et al 1991). Similarly, such peptides have been associated with augmented phagocytic uptake of bacteria and are also important ligands in the interaction between cells and their extracellular matrix, cytokines etc. Nevertheless, this work may indicate a novel mechanism of interaction between the host epithelial cell and *L. intracellularis*, suggesting the presence of a bacterial surface factor which is involved in the invasion of host cells. Further support is added to this argument by the ability of the monoclonal antibody IG4, which recognizes an immunodominant 25 to 27 kDa protein on the outer surface of *L. intracellularis* (McOrist et al 1989) and its Fab fragment to reduce the infectivity of *L. intracellularis* organisms *in vitro* (McOrist et al 1997a).

(b) Bacterial growth, multiplication and release

Bacteria have been visualised in membrane-bound vacuoles up to 24h after entry. Each vacuole is only a temporary home for *Lawsonia*, from which it quickly escapes (Jasni et al 1994a). Possibly, as is the supposition with rickettsial infection, it is attempting to avoid the host cell's defence mechanisms, such as lysosomal enzyme digestion, which occurs upon lysosomal fusion with the phagosome (Moulder 1985). Such an escape process may suggest the existence of a bacterial enzyme capable of disrupting membranes, as phospholipase A is believed to do in rickettsial infections (Silverman et al 1992). *L. intracellularis* freely multiplies in the host cell cytoplasm by binary fission and it does not appear to change its physical structure. Multiplication of many other intracellular organisms results in the *in vitro* death of the cell. Some *Rickettsia* species induce rupture

of the cell (again, possibly phospholipase A mediated). The *Shigellae* species possess many virulence factors capable of causing severe cellular damage, such as the shiga toxin released by *S. dysenteriae* 1 which can have powerful cytotoxic effects, resulting in gross haemorrhage and perforation of the intestine, together with systemic toxaemia (Acheson and Keusch 1994). *L. intracellularis* has not been associated with such a phenomenon. On the contrary, its intracellular presence *in vivo* is almost always associated with a hyperplastic state, which is responsible ultimately for the mucosal thickening observed grossly. Whereas infection by *Chlamydia* can cause rounding up of cells *in vitro* with vacuolation and pyknosis, as well as damage to mitochondria and destruction of organelles generally, no definite cytopathic effect is known to be induced by *L. intracellularis*. In the cell culture monolayer, bacterial numbers peak by seven days post infection before declining again (Lawson et al 1993).

When added to cultures the inhibitor of cell protein synthesis, cyclohexamide, reduces monolayer growth and *L. intracellularis* multiplication, confirming the organism's need for growing cells and indicating the reason for its tropism to dividing cells. It would, therefore, not be in its interests to destroy its host cell.

Spread of *L. intracellularis* bacteria between cells generally requires that the organisms be released from the cells. Possible release mechanisms include physical rupture of the host cell caused by the large number of organisms e.g. *Rickettsia,* or apoptosis e.g. *Shigella* (Zychlinsky et al 1992). While *Chlamydia* can also induce cell rupture, it has also been shown to escape within an extruded host cell or via released cytoplasmic fragments (Moulder 1991). Cytoplasmic protrusions have been observed in ultrastructural studies of infected epithelial cells, both *in vitro* (McOrist et al 1995b) and *in vivo* in experimentally infected pigs (McOrist et al 1996a). Since these protrusions often contain numerous *L. intracellularis* organisms, it has been suggested that this may be a release mechanism which allows spread, firstly, to other host cells and, secondly, to vulnerable animals via the faeces. It is equally possible that large numbers of infectious organisms may be released from the host to the outside environment via infected cells which are naturally sloughed into the intestinal lumen and passed out in the faeces (McOrist et al 1995b). Since the cell turnover for intestinal crypt cells is around three days, this would provide a regular means of escape and short-term protection for infectious organisms against the external environment, before ingestion by a new host.

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Transmission of L. intracellularis

Spread of *L. intracellularis* between hosts is presumably via the faecal-oral route (McOrist et al 1994a). Many intracellular organisms pass horizontally from host to host in a different form to that found within the host cell. *Isospora suis* oocysts and the elementary bodies of *Chlamydia* presumably confer a certain amount of resistance to the external environment, thus aiding transmission to new hosts. In coccidiosis of farm animals this environmental resistance is a significant problem, and often very harsh methods have been employed to try to combat persistent infection, including steam cleaning and phenol disinfection (Ernst et al 1985). Current and Garcia (1991) reported the existence of two different types of oocyst production by *Cryptosporidium parvum*, a thin-walled oocyst which causes autoinfection within the intestinal environment, and a thick-walled oocyst which is excreted in the faeces and is responsible for transmission of infection to another animal. The elementary bodies and reticulate bodies formed by *Chlamydia* are a typical manifestation of the adaptations which some organisms exhibit to facilitate their transfer between hosts. Elementary bodies cannot divide. They are responsible for the transfer of infection, after which they become reticulate bodies which multiply freely in the cytoplasm of the host cell by binary fission but are never able to infect new cells. The increased resistance of the elementary body appears to be due to greater cross-linking via disulfide bonds in the outer membrane proteins (Moulder 1985).

Other organisms, such as *Rickettsiae*, *Plasmodium* and *Leishmania*, require arthropod vectors. *R. rickettsii* replicates in host endothelial cells to cause Rocky Mountain spotted fever in humans, but it is maintained in several tick species, including *Dermacentor* and *Rhipicephalus*, by transovarian transmission and is spread to humans when the infected tick feeds (Murray et al 1994). Most organisms which use a vector for transmission, change their form in order to do so, such as malarial gametocytes, and *Trypanosoma cruzi* trypomastigotes. The *Rickettsiae* species are an exception and do not change their physical form at all throughout their life-cycle. However, most of the bacteria in the *Rickettsiaceae* family are highly susceptible to exposure to the external environment, although *Coxiella burnetii* is different in that it is able to produce a spore-like body which makes it highly resistant to desiccation, allowing it to survive for years in the environment. For this reason, it does not require a vector host, although many animals can act as reservoirs (Murray et al 1994), and transmission between hosts occurs by the inhalation of these

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spore-like particles in the air. To date, there is no evidence to suggest that *L. intracellularis* employs an arthropod vector during any part of its life-cycle.

Target host cells

Even organisms which can easily survive in an extracellular state in their normal form have predilections for intracellular sites because their survival, or subsequent growth, are enhanced by these cells. *Brucella abortus*, one of the causes of infectious abortion in cattle, favours bovine placental cells because they are a source of the growth factor, erythritol (Moulder 1985). *Coxiella burnetii*, although able to multiply in different cell types, can also be found in copious quantities in sheep placenta.

There is a dearth of relevant information, but none of these phenomena appear to apply to *L. intracellularis*. It seems to occur in only one form and transmission *in utero* has not yet been investigated. Many aspects of intracellular parasitism vary widely, even in taxonomically closely related organisms. There are usually some similarities between different organisms in certain areas, however, and the same is true for *L. intracellularis*. Its ability to multiply freely in the cytoplasm by binary fission and to float in a non-motile form is not unique and is a characteristic of *Shigella*, which can cause severe enteritis in humans. *Rickettsia* species also tend to multiply in the cytoplasm, after they break out of the phagosomal membrane which engulfs them. Also like *L. intracellularis*, many other species of intracellular organisms show a definite preference for a specific type of cell. *Rickettsia* species grow almost exclusively in endothelial cells lining small blood vessels. While malarial organisms in the genus *Plasmodium* infect erythrocytes, the genus *Ehrlichia* spares erythrocytes and targets monocytes, lymphocytes and neutrophils instead (Murray et al 1994). *I. suis* and *Shigella* are the most similar to *L. intracellularis* in their choice of intestinal epithelial cells and, while *Shigella* is actually a facultative intracellular organism which can survive extracellularly, it is only pathogenic when it enters the cell (Gyles 1994). It is not clear whether these organisms enter those cells because they are the first with which they come into contact in sufficient concentration or whether these host cells fulfil a specific parasitic requirement. Although it is difficult to be certain why *L. intracellularis* only inhabits epithelial cells of intestinal crypts, the evidence so far points to the bacteria's need for an immature and developing cellular environment.

Virulence Factors of *Lawsonia intracellularis*

Initial observations suggested that the virulence of *L. intracellularis* reduces with the number of *in vitro* passages in cells (McOrist et al 1993). Four of 16 conventional pigs dosed with strain 916/91, passaged six times, developed microscopic lesions typical of PE. Conventional pigs dosed with the same strain, passaged 13 times, developed fewer microscopic lesions. The expression of bacterial virulence factors is thought to be reduced by passage *in vitro*. *E. coli* strains which undergo multiple passages have reduced expression of the fimbrial antigens responsible for adhesion to target cells, thus reducing their pathogenicity (Bertschinger et al 1992). A higher dose of inoculum of *L. intracellularis* has tended to increase the extent of resulting lesions (McOrist et al 1993), an observation common to other enteric diseases of pigs, such as porcine coccidiosis and salmonellosis. Clinical signs and histopathological lesions associated with coccidiosis correlate well with the number of *I. suis* oocysts in the inoculum with 4×10^5 oocysts capable of causing high mortality in young piglets (Lindsay et al 1985). In one study, a similar situation was reported for *Salmonella choleraesuis* infection, where an initial challenge dose of 10^9 organisms produced the longest shedding time of 15 weeks, compared to nine weeks in age-matched pigs dosed with 10^6 organisms (Gray et al 1996). The degree of faecal shedding, in terms of numbers of organisms, followed a similar pattern. Measured in colony forming units per gram of faeces, the higher initial challenge dose resulted in a much greater level of organisms in the faeces. Clinical signs were also more severe in the higher dose group. Pigs in a third group challenged with 10^3 organisms produced no clinical signs and no faecal shedding (Gray et al 1996). Interestingly, though, the initial inoculum dose of *S. choleraesuis* did not affect the actual pattern of excretion observed, the peak of shedding occurring five days post inoculation, regardless of dose.

The difference in pathology between the chronic and acute forms of PE (Rowland and Lawson 1975) may be due to strain variation, accompanied by a difference in virulence factors, such as toxins or haemolysins. An apparent strain variation was observed by Jasni et al (1994b) when 14 hamsters infected with one strain of *L. intracellularis* (1482/89), passaged only twice, failed to develop lesions of PE, while 10 out of 16 hamsters dosed with another strain (916/91), passaged up to six times, did develop typical histological lesions. Clear-cut syndromes due to toxin differences occur with the various and numerous strain of *E. coli* (Bertschinger et al 1992). Oedema disease of weaner pigs is usually caused by not more than four well recognised toxin-producing strains of *E. coli*

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world-wide (Bertschinger and Nielsen 1992). The pathogenesis of oedema disease is orchestrated by a shiga-like toxin, produced by each of these four strains but also relies on the presence of other virulence factors, in particular adherence fimbriae, to produce disease.

Antimicrobial Studies

In vitro testing

Treatment of PE on a herd basis has relied heavily on the use of antimicrobial therapy (Ward and Winkelman 1990, Connor 1991). Tylosin, chlortetracycline and tiamulin have all been widely used, as has carbadox (Winkelman and Hawkins 1996), although the latter's use in the United Kingdom is not legally permitted anymore. These choices have been based on the clinical observations and previous experience of pig practitioners and not on scientifically generated data. However, recent preliminary studies on the antimicrobial sensitivity of *L. intracellularis in vitro* have given credence to these traditional methods of treatment (McOrist et al 1995c).

To achieve this sensitivity testing, the cell culture system was adapted and minimum inhibitory concentrations (MICs) were calculated as the lowest concentration which consistently inhibited bacterial growth to less than 1% of that of control co-cultures. The lowest MICs were seen with penicillin, ampicillin, erythromycin, difloxacin, virginiamycin and chlortetracycline, with tilmicosin and tiamulin achieving moderate MICs (McOrist et al 1995c). Cell culture assessment does have certain drawbacks, the most obvious being the inability to simulate the host's immune response, endocrine system and metabolism, all of which affect the dynamics of each drug. Similarly, in a natural infection, the antimicrobial concerned must be capable of reaching the lower bowel region at the appropriate concentration and still be active in this environment, as well as penetrate the epithelial cells lining the gut crypts (Tulkens 1991). However, the system does provide an accurate *in vitro* method for such evaluations, pinpointing drugs which may merit further *in vivo* investigations.

Diagnosis of Proliferative Enteropathy

Histopathology

The existence of PE is usually suspected on the basis of the associated clinical signs of each form of the disease (Rowland and Lawson 1992). Clinical signs can be confusing, however, and relying on these alone may lead to misdiagnosis, especially as there can be clinical similarities with salmonellosis and swine dysentery. Preliminary diagnosis can be obtained by making mucosal smears and staining them with Gram's stain or the modified Ziehl-Neelsen stain, where organisms are Gram-negative and acid-fast, respectively. These diagnostic methods are not wholly specific and there is the potential for confusion with the presence of other similar micro-organisms, especially the *Campylobacter* species. Confirmatory diagnosis may be achieved by the demonstration of lesions typical of PE in the gut crypts, usually in the terminal ileum, but also at other sites along the intestinal tract. Haematoxylin and eosin stained sections reveal the adenomatous changes caused by the proliferation of enterocytes and the associated thickening and branching of crypts, with goblet cells in the affected crypts often diminished in number (McOrist et al 1989a). Staining paraffin sections with a modified Warthin-Starry silver stain will demonstrate the presence of *L. intracellularis* in the apical cytoplasm of the enterocytes by staining them dark brown/black. Similarly, immunostaining with the monoclonal antibody IG4 is very useful for specific and absolute confirmation (McOrist et al 1987). Histopathological methods give generally indisputable results, but realistically can only be obtained at necropsy. One of the stumbling blocks in PE research has been the inadequacy of the available diagnostic techniques to meet with the requirements of both research and industry. Investigations into the epidemiological patterns of this infectious disease, including prevalence studies and possible modes of transmission, have required a diagnostic assay which is applicable to live animals.

Detection of L. intracellularis bacteria shed in faeces

Stains such as Gram's, Ziehl-Neelsen and Warthin-Starry silver have not been suitable for demonstration of organisms shed in faeces, where the presence of many other faecal contaminants and homogenous material often obscure the diagnosis. The development of *L. intracellularis*-specific mouse monoclonal antibody resulted in an indirect immunofluorescence assay, incorporating the monoclonal antibody and a fluoroscein-

conjugated secondary anti-mouse antibody. This provided the first specific test, suitable for detection of organisms in PE-diseased mucosa, but it was also used to demonstrate the presence of *L. intracellularis* in faeces samples of pigs later confirmed PE-positive at necropsy (McOrist et al 1987). However, the specificity and sensitivity of IFA detection of *L. intracellularis* in faeces samples is questionable and somewhat subjective. Nevertheless, it has helped to prove the validity of bacterial detection in faeces by other methods.

As discussed previously, *in vitro* culture of *L. intracellularis* on a cell-free medium is not a feasible option for detection of organisms derived from faeces. Although inoculation of cultured monkey kidney cells with pancreatin-treated faeces samples has been used as a method for immunofluorescence-based detection of rotavirus antigen (Benfield et al 1982), similar application of such a technique to *L. intracellularis* would be completely impractical. The risk of monolayer contamination with *Mycoplasma* and *Chlamydia* species present in pig faeces samples is extremely high. Therefore, it is improbable that IEC-18 monolayer cells could be maintained for a sufficient period of time to support viable organisms and allow detectable growth. The expense involved in such a technique would also preclude its use.

Detection of Antibodies to *Lawsonia intracellularis*

IgM and IgA responses

At the time of writing this review, no commercially available serological test for the diagnosis of PE exists. Previously, in a three-stage test, *Campylobacter*-like organisms prepared directly from PE-affected mucosa were used as capture antigen and fluorescein-conjugated anti-rabbit or anti-mouse IgG were incorporated as the method of visualisation. Attempts to determine the antibody response to *L. intracellularis* were made by combining anti-pig IgA and anti-pig IgM into the three-stage sandwich assay. Sera from histologically confirmed field cases of PE showed a significant difference in the mean reciprocal IgM titres to *L. intracellularis* when compared to sera from animals with no gross or microscopic evidence of PE (Lawson et al 1988). Agglutination tests were performed in tandem to detect antibody against *Campylobacter mucosalis and C. hyointestinalis* but no differences were observed in the IgM titres between pigs with and without typical PE lesions.

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The IgM response tended to be short-lived (lasting up to eight weeks) but did correlate well with PE lesions found at slaughter. Animals without lesions were, on the whole, seronegative to *L. intracellularis*. It was interesting, however, that nine of 11 finisher pigs in this study had antibody titres to *L. intracellularis* but no lesions, while only two of six weaner pigs with an antibody response showed a significant loss of body condition. This seemed to indicate that overt clinical signs are not always obvious in animals affected with PE. IgA levels have only been briefly studied, and only in sera. They tend to reflect the IgM levels but the titres are lower, although this might be expected as IgA is locally produced at mucosal surfaces (Lawson et al 1988).

IgG response

Efforts to investigate IgG levels have produced disappointing results. Lawson et al (1988) resorted to a three-layer test, similar to that used for detection of IgM, when their indirect test based on fluorescein-conjugated rabbit anti-porcine IgG appeared to be insensitive. The three-layer test, however, produced variable results, depending on the secondary antibody used. Rabbit anti-pig IgG antibodies non-specifically reacted with the *L. intracellularis* antigen, causing back-ground fluorescence, while monoclonal antibody to pig IgG detected no pig antibody at all in sera from known positive cases of PE. There was some success with affinity-purified anti-pig IgG, but at much lower levels than those observed for IgM. Holyoake et al (1994c) confirmed that, although *L. intracellularis*-specific IgG was produced, this was at a lower and more variable level. Nevertheless, they did show that pigs seroconverted between seven and 24 weeks of age on a farm endemically infected with PE. There was also a relatively high level of IgG in three-week old piglets, which suggested that some maternally-derived immunity had been absorbed. This varied widely and declined between three and six weeks of age, coinciding with weaning and mixing. It was concluded that IgG could possibly dictate whether or not weaner pigs would be susceptible to infection at this critical time.

Serological diagnosis has not been a reliable choice open to research. One of the possible reasons for variable IgG-based immunity is that the assays used to date have employed *L. intracellularis* derived from diseased intestinal mucosa and purified through a percoll gradient. This could give misleading results, especially if there was non-specific binding of antibodies to other intestinal flora remaining in the antigen preparation. Serological tests have been used to detect antibodies against other enteric pathogens in pigs, such as *S.*

hyodysenteriae and the coronavirus which causes transmissible gastro-enteritis (Diarra et al 1995, Bernard et al 1990). Mhoma et al (1992) reported the development of an anti-lipopolysaccharide antigen ELISA for swine dysentery. Although there was some apparent cross-reactivity within the three different serogroups tested, there was no reaction with antibodies against weakly beta-haemolytic organisms. The serological microagglutination test used for diagnosis of swine dysentery had improved specificity when conducted using a boiled cell suspension rather than a whole cell antigen preparation. It was also able to differentiate between *S. hyodysenteriae* and *S. innocens*, and was considered a more appropriate test for simple and reliable diagnosis of swine dysentery, at least on a herd basis, when compared to other tests, such as the indirect haemagglutination assay (Diarra et al 1995).

Polymerase Chain Reaction Assay

Introduction

The polymerase chain reaction (PCR) was first described in 1985 by Saiki et al, who used it to recognise the genetic mutation responsible for sickle cell anaemia. They amplified the beta-globin genomic sequence where the mutation occurs, thereby increasing the sensitivity, simplicity and speed of prenatal diagnosis of this genetic disease. The principle behind the PCR is to manufacture identical copies of DNA from small amounts of template DNA, thereby amplifying the template DNA to a level which is more easily detectable by routine laboratory methods (Innis and Gelfand 1990). The PCR is a powerful tool, widely used in research, but it has also, more recently, emerged as a highly specific and potentially extremely sensitive assay for detecting the DNA of infectious agents in clinical samples, such as blood, (Lyamuya et al 1997), urine (Bayer et al 1996) and tissues, including both preserved and fresh samples (Greer et al 1990, Righter et al 1996). Its use with regard to faeces samples has been restricted, however, due to the presence of assay inhibitors (Deuter et al 1995).

The PCR has been thoroughly investigated for bacterial infections caused by organisms which are either difficult to grow in the laboratory, such as *Treponema pallidum* (Burstain et al 1991), or which require assays of greater sensitivity, such as *Legionella* (Paszko-Kolva et al 1995). Furthermore, the relative speed of the PCR has proved valuable in the identification of patients which may require swift treatment (Gumerlock et al 1993).

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The creation of plasmid clones, which are in themselves specific for *L. intracellularis* and can be used in digoxigenin-labelled form for its detection (Jones et al 1993b), led to the sequencing of a 375-bp segment of DNA from one of these clones, given the name p78. Based on this sequence, four oligonucleotides, each 20 nucleotides in length, have been synthesised for use as primers in a highly specific polymerase chain reaction test. Two outer primers correspond to nucleotides 5 to 24 and 304 to 323, respectively, while the inner primers correspond to nucleotides 45 to 64 and 285 to 304, respectively (Jones et al 1993c). Using the outer primers alone, the PCR amplifies a 319-bp fragment of DNA from the genome of *L. intracellularis*.

Extraction of DNA

Extraction of DNA from faeces is hampered by the need to remove faecal contaminants and PCR inhibitors (Deuter et al 1995). The main inhibitors are believed to be bile salts and bilirubin (Widjojoatmodjo et al 1992) and many techniques have been directed at their removal, from "booster" PCR (Saulnier et al 1992) to magnetic immuno-separation of organisms before PCR (Widjojoatmodjo et al 1992, Islam et al 1992).

Most methods have tended to be lengthy and cumbersome (Allard et al 1990). Initial protocols employed for extraction of *L. intracellularis* DNA from faeces and tissue samples have been no exception, based on the binding of DNA to silicates in the presence of high concentrations of guanidium thiocyanate (Jones et al 1993c), an adaptation of a nucleic acid purification method used in human diagnostic assays (Boom et al 1990). Although this can give good detection rates (10^3 organisms/g of faeces), similar rates have been achieved using a much shorter protocol involving dilution and boiling of the inhibitors in the sample (McOrist et al 1994a). Dilution methods may be riskier, however, with respect to false negative results. New techniques are continually emerging, in an effort to reduce the problem of faecal inhibitors (Lou et al 1997).

The theoretically exquisite sensitivity of the polymerase chain reaction is more difficult to achieve in practice, especially when directed at contaminated clinical samples, such as faeces. Although simpler DNA purification methods such as phenol-chloroform extraction may be suitable for some organisms, such as *S. hyodysenteriae* (Elder et al 1994), the same cannot be said for some other pig enteric pathogens. Inexplicable differences were reported between the levels of detection of the PCR for verotoxin-1 (VT-1) and verotoxin-2 (VT-2) genes within different strains of the same species of *E. coli* C600 present in

faeces (Ramotar et al 1995). Detection of the number of colony forming units per 0.1g of faeces was 10^5 times more sensitive for the VT-1 toxin-producing strain (H19B) than it was for the VT-2 toxin-producing strain (933W). It is conceivable that this could have been due to technical difficulties involved in amplifying the gene concerned, but the authors themselves pointed out that gene sequence degradation, or genetic instability, can occur after serial passage of isolates. While the samples concerned had not been passaged, they had been stored frozen for an unspecified number of months prior to PCR testing, which could have led to a certain amount of DNA degradation.

More specialised purification, using immunomagnetic separation (IMS), has helped to extract target organisms, such as *E. coli* F4 (K88), prior to incorporation into the nested PCR which is specific for the heat stable enterotoxin genes ST1a and ST1b. Colorimetric detection of PCR product has further increased the sensitivity (Hornes et al 1991).

Such techniques give very sensitive and specific results and are much quicker than more established laboratory methods. However, they may prove too laborious and expensive for large-scale screening of pig herds, and still require the animal to be shedding at the time of sampling, which may not always be the case. A more practical method, incorporating enrichment cultivation with PCR was used to detect *Salmonellae* in faeces. The enrichment process in brain heart infusion, followed by selective enrichment media, not only increased the number of *Salmonellae*, but helped to dilute inhibitors (Stone et al 1994). Although it had been recommended that this method be used for samples which were negative on normal culture, it did rely on the presence of live organisms, so may have been affected by antimicrobials in animal feed.

Such a method is not applicable to detection of *L. intracellularis* in suspect cases of PE since the growth of *Lawsonia* requires the cell culture environment for growth. Cultivation prior to PCR is not feasible (Gebhart et al 1993).

Reaction components and amplification

The extracted DNA can be incorporated as template DNA into a reaction mix containing an optimised combination of reaction buffer, $MgCl_2$, each of the primers, the four different deoxynucleoside 5' triphosphates (dNTPs), water, and the enzyme *Taq* polymerase. These reagents are the basic formula required for the *in vitro* amplification of DNA. The primers are short lengths of oligonucleotides, usually specific to the sequence of DNA under investigation and the dNTPs are the basic building units of DNA. The PCR owes its

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existence to the discovery of the enzyme *Taq* polymerase, which is unique in that it can withstand temperatures as high as 97°C. It is, therefore, suitable for incorporation into the PCR which relies on very high temperatures.

Amplification is now usually performed in a DNA thermal cycler (Innis and Gelfand 1990) which exposes each reaction mix to a set profile of temperature fluctuations. An initial high temperature (94 to 97°C) is used to denature the template DNA *i.e.* separate the double helix into two separate strands. This allows the primers to anneal or bind to their respective complimentary segments of DNA which flank the target fragment. Annealing temperatures usually lie between 40°C and 60°C and are influenced by a number of factors, including length of primer, length of template DNA and relative concentrations of primer and template. Dimethyl sulfoxide is an additional reagent sometimes required for reaction success and was originally used by Jones et al (1993a). Its main function is to reduce the primer annealing temperature which is beneficial to the efficiency of the *Taq* polymerase (Innis and Gelfand 1990). The $MgCl_2$ is a prerequisite for enzyme function, acting as a catalyst for the polymerisation of new strands of DNA. This is completed in the final temperature stage, the extension phase, which is usually conducted at 72°C. Here, the dNTPs are linked together in a polymerisation reaction which extends from the primers to create a new but identical piece of DNA. Amplified DNA is routinely detected on 1 to 2% agarose gels stained with ethidium bromide.

Application of the PCR to L. intracellularis

Jones et al (1993a) were the first to use the PCR diagnostically on faeces samples from both normal and PE-diseased pigs (experimental and natural cases). In this study, the number of organisms present in infected mucosal filtrate was quantified using specific monoclonal antibody IG4 (McOrist et al 1987) and fluorescein conjugated anti-mouse IgG. DNA was then extracted from the mucosal homogenate and amplified in the PCR. *L. intracellularis*-specific 319-bp PCR product, generated by primers A and B, was detected in reaction mixes containing from 10^4 to 10^1 organisms per reaction. The identity of the PCR product was confirmed as being derived from *L. intracellularis* by reamplification with internal primers C and D. In a similar process, DNA was extracted from faeces samples artificially infected with *L. intracellularis*. The sensitivity of the PCR in this case was 10^3 organisms per gram of faeces. The test was used to show that three of three experimentally infected pigs, which developed histological lesions typical of PE, were also

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PCR positive, as were four of four naturally infected cases. The faeces of an unexposed control pig, normal slaughter pigs and a diarrhoeic pig with no other signs of PE at post-mortem, were all negative on PCR.

Comparison of PCR results for faeces samples from pigs at slaughter with intestinal macroscopic and microscopic findings (including silver staining) and dot-blot hybridisation, indicated that the PCR was the only assay with 100% sensitivity and specificity. Although dot-blot hybridisation also had 100% sensitivity, its specificity was less (91%). This also confirmed the suitability of the PCR for use on field cases of PE (Jones et al 1993a) and helped to validate it as the first assay appropriate for ante-mortem diagnosis of PE in pigs.

Dot-blot hybridisation using the DNA probe has been used to confirm the presence of *L. intracellularis* in preserved intestinal tissue from naturally infected pigs and from the faeces of experimentally infected pigs two weeks post challenge in one trial (Jones et al 1993b) while, in another, the PCR detected *L. intracellularis* DNA in faeces of experimentally challenged pigs 21 days post-inoculation (McOrist et al 1994a). Typical PE lesions in archive sections of formalin-fixed intestines from field cases correlated well with positive PCR results, while the mucosa of recovered pigs which had previously shown clinical signs of PE were PCR negative. Seven of eight animals which developed experimentally induced PE were positive on faecal PCR (McOrist et al 1994a). Additional validation of the test as a post-mortem tool has been undertaken by Jordan et al (1996) who demonstrated a higher sensitivity than that of Warthin-Starry silver staining.

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Epidemiology of *Lawsonia intracellularis* Infections

Introduction

In the first three days of life, infections are responsible for a small fraction of piglet deaths. This is because the piglet gains acquired immunological resistance from its mother, which protects it from pathogens present in the herd, at least under endemic conditions (Vaillancourt and Tubbs 1992). The relative proportion of piglets dying from infectious diseases increases with age and, by three weeks of age, they are responsible for the largest percentage of piglet deaths, as immunity wanes. This is particularly important in pigs, where management usually dictates weaning at three weeks of age, possibly followed by mixing of litters in an intensive production system, often with continuous flow-through of animals. Therefore, at farm level, knowledge of the mode of transmission of enteric pathogens is a vital requirement for control of disease. I will review certain aspects of PE with respect to possible epidemiological factors. Because the experimental reproduction and diagnosis of PE are both very recent advances, there is very little data available relating directly to epidemiology and, in comparison to other faecally-transmitted diseases of pigs, knowledge of excretion levels, patterns of shedding and modes of transmission is extremely poor.

Onset of infection and excretion pattern

Initial reports, based on hamster and pig challenge studies, have suggested that *L. intracellularis* infects intestinal epithelial cells as early as five days after inoculation (Johnson and Jacoby 1978), with the first microscopically detectable lesions occurring as early as 10 days after challenge (McOrist et al 1989). Jones et al (1993d) used the *L. intracellularis*-specific DNA probe to detect DNA in faeces 14 days after challenge. However, shedding had ceased by 21 days after challenge. In later work, higher sensitivity was obtained by the PCR on faeces samples collected from experimentally infected pigs two, four and five weeks after challenge (Jones et al 1993c). Although there was excellent correlation between positive PCR results and the existence of PE lesions, the pattern of shedding over the five-week period was not reported. Piglets in the field normally develop clinical signs between six and 16 weeks of age (Rowland and Lawson 1992), suggesting that piglets must be acquiring infection from around three weeks of age. However, there have been no long-term studies on the excretion patterns of *L. intracellularis*.

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The protozoal parasite, *I. suis*, closely resembles *L. intracellularis*, particularly with respect to its requirement for an intracellular environment for part of its life cycle. *I. suis* also colonises intestinal epithelial cells, although it does so in a different form to that found in the faeces (Urquhart et al 1987). Its life cycle has been well defined (Lindsay et al 1997), including its prepatent period, which ranges from five days to three to four weeks. Patency tends to last about two weeks (Lindsay et al 1992). Although Christensen and Henriksen (1994) reported a cyclic shedding pattern, with several peaks of oocysts appearing in the faeces of infected pigs, Harleman and Meyer (1985) reported a biphasic pattern of shedding in their gnotobiotic and conventionalised experimental pigs, with one peak five to eight days and the second 10 to 12 days after inoculation.

It is possible that the same cyclic pattern of shedding occurs in *L. intracellularis* infected pigs. This has implications for the diagnosis of PE, dictating the collection of a large number of faeces samples to ensure that at least a proportion will be in the excretion phase. Although it is still not certain whether a carrier state exists in proliferative enteropathy, a cyclic shedding pattern could be deceptive and may result in the introduction of potential carriers onto PE-clean units. This is a particular problem in porcine salmonellosis, where sporadic shedding of infectious organisms by carrier pigs has necessitated repeated culture of faeces to ensure identification of carriers (Wilcock and Schwartz 1992).

Transmission of L. intracellularis

It is possible that infection of piglets with *L. intracellularis* occurs before they leave the farrowing house, *i.e.* around weaning time, either from their mother's faeces, or via the environment. This theory has also been investigated in neonatal coccidiosis. The prevalence of *I. suis* oocysts in sows is usually under 5%, which is in contrast to the frequency of *Eimeria* infections in sows, which can be as high as 90% (Ernst et al 1985). Specific testing of sow faeces for *I. suis* oocysts on farms with coccidiosis, has failed to demonstrate significant numbers. In one particular study, no oocysts were found in 77 sows on seven farms with a problem of neonatal coccidioisis (Lindsay et al 1984). It is possible that there may be similarities between *I. suis* and *L. intracellularis* in their epidemiology and transmission patterns. Despite the comparatively broad understanding of *I. suis*, the epidemiology in relation to identification of source animals and modes of transmission is still obscure (Lindsay et al 1997). It has been shown that piglets do ingest their mother's faeces (Sansom and Gleed 1981) so, in the case of both *L. intracellularis*

and *I. suis* infection, the opportunity is there for direct spread from mother to young. However, this has been difficult to demonstrate in porcine coccidiosis, as sows do not seem to shed significant numbers of oocysts (Lindsay et al 1984). Neonatal piglets are known to excrete oocysts between four and eight days of age, so it has been speculated that the source of their infection must be their environment, specifically the farrowing crate, which may also be true of *L. intracellularis*, although it has not yet been established if neonatal piglets in the field can become infected.

Excretion of L. intracellularis and the role of carrier pigs

Normal bacteriological culture methods fail to support the intracellular requirements of *L. intracellularis* (Lawson et al 1993) and the cell culture system is extremely vulnerable to the effects of faecal contaminants such as *Mycoplasma* species, so the problem of accurate quantification of *L. intracellularis* in faeces still needs to be overcome.

In its present form, the PCR for *L. intracellularis* (Jones et al 1993c) can be used diagnostically as a herd screening assay and is suitable for determining prevalence on a national or regional level, but it is not sufficiently advanced to attempt to quantify organisms in individual faeces samples, so the levels excreted in PE remain unknown. Transmission of colibacillosis requires that 10^{10} *E. coli* are present in the faeces, indicating that environmental burdens may be high (Bertschinger et al 1992). Pigs acutely affected with swine dysentery can excrete 10^8 to 10^9 *Serpulina hyodysenteriae* organisms per gram of faeces. In salmonellosis, between 10^6 and 10^7 organisms are excreted per gram of faeces, for *S. choleraesuis* and *S typhimurium*, respectively (Harris and Lysons 1992). Although such large numbers have allowed quick and relatively easy diagnosis of these diseases, in both conditions infected pigs often recover to become clinically normal while still shedding infectious agents. In swine dysentery, excretion of a sufficiently high level of organisms has induced disease in susceptible pigs (Harris and Lysons 1992) and, in salmonellosis, one study showed that over 90% of experimentally infected pigs were still positive for *S. typhimurium* at slaughter four to seven months later. In the latter study, a true carrier state was established, and organisms were isolated from lymph nodes, tonsils, caecum and faeces (Wood et al 1989). Detection of asymptomatic carrier pigs by culture of their faeces is usually unreliable, despite the development of enrichment culture methods for salmonellosis. Wood and Rose (1992) showed that *S. typhimurium* can persist in pigs at a low level for up to 28 weeks after infection, experimentally. Similar problems

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of detection have hampered attempts to control swine dysentery, where a PCR has been developed with the aim of identifying carriers (Elder et al 1994). High sensitivity tests alone may not be sufficient for control, however, since carrier pigs do seem to shed organisms intermittently (Harris and Lysons 1992).

The asymptomatic and otherwise healthy pig still shedding *L. intracellularis* in its faeces is a possible factor worthy of consideration in the transmission of PE, particularly with regard to the introduction of the disease into otherwise clean herds. Acute outbreaks of PE are increasing in frequency, particularly in SPF herds, suggesting that carrier pigs are a possible source of infection (Boeckman 1995).

There is currently no experimental evidence for the existence of a carrier state, either via faecal shedding or in extra-intestinal tissues, but investigations are in the very early stages.

Non-porcine hosts

The asymptomatic shedding pig may not be the only route for the introduction of *L. intracellularis* onto clean units. Other sources of infection have been implicated in the transmission of swine dysentery, especially mice which are reservoir hosts of *S. hyodysenteriae*, not only capable of excreting the organism for at least 200 days (Harris 1984), but also able to induce disease in susceptible pigs exposed to their faeces (Joens 1980). Dogs, birds, rats and flies are believed to be responsible for mechanical carriage only (Harris and Lysons 1992). Carrier pigs are still considered the main means of transmission between herds, as mice tend to remain on their farm of origin (Harris 1984). In salmonellosis, birds and rodents are thought to play a part in transmission between farms, able to contaminate feed which, in itself is a source of infection for the pig, although there is little evidence to suggest that clinical outbreaks can result from ingestion of contaminated feed (Wilcock and Schwartz 1992). In porcine salmonellosis, the infected and excreting pig is the main source of infection, particularly for *S. choleraesuis* infection, where it is capable of excreting large quantities of the organism. This has been further supported by evidence which has indicated that, although *S. choleraesuis* has been the most frequent porcine isolate (Wilcock and Schwartz 1992), it has seldom been found in pig feed or non-porcine reservoirs.

Again, little work has been carried out to specifically examine possible non-porcine hosts which could contribute to the introduction of *L. intracellularis* infection onto a pig unit. PE occurs naturally in hamsters in the USA and disease has been induced using pig-

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derived organisms (Jasni et al 1994b), but no attempts to reproduce disease in rodents have been recorded.

Environmental survival following excretion

Survival of *L. intracellularis* outside the pig has been difficult to evaluate, again owing to the inadequacy of available detection assays. Although other major enteric pathogens of pigs tend to survive well in the pig's environment, they are either in a different stage of their life cycle while inhabiting the external environment, such as *I. suis*, or are otherwise very resistant organisms, given the right conditions, such as *Salmonella* species, which can persist for months or years in a mild and moist environment (Wilcock and Schwartz 1992). *Serpulina hyodysenteriae* has been shown to survive for six days in effluent tanks in the USA, at levels capable of infecting new susceptible pigs (Olson 1995). These slurry tanks are at a very low oxygen tension, so it is doubtful whether they could provide the optimum environment to support *L. intracellularis*. Because *Lawsonia* is an obligate intracellular organism (Gebhart et al 1993), any comparison with epidemiological aspects related to survival should be made cautiously. It is still unknown if *L. intracellularis* is shed within the cells it inhabits as they are naturally sloughed. Unlike *Salmonellae*, multiplication outside the cell is highly unlikely, and has not been observed in the cell-free supernatant fluid of the co-culture system, *in vitro*, nor in the intestinal lumina of infected pigs.

Epidemiological Field Studies

Epidemiological studies of naturally occurring PE have aimed to determine the prevalence of infected herds and of infected pigs within herds and to try to establish the economic effect which PE could have on pig production. Early estimates of the incidence of PE were based on the detection of gross intestinal lesions at normal slaughter. These estimates varied from 0.7 to 1.63% (Emsbo 1951, Kubo et al 1984). In Australia, inspection of carcasses in abattoir surveys indicated that the prevalence of infected herds was actually much higher, sometimes up to 35% (Pointon 1989), while the within-herd prevalence of infection ranged from 5 to 40% (Gogolewski et al 1991).

In another abattoir survey, this time conducted in New Zealand, Christensen and Cullinane (1990) reported that 20 out of 2661 (0.8%) pigs examined at slaughter had lesions typical of PE. These pigs represented five out of a total of 46 (10.8%) herds included in the

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survey. Similarly, Jackson and Baker (1980) estimated that the prevalence of PE, based on observation of clinical signs, was 0.89%. In another Australian study, differences were noted in prevalence based on clinical signs (28%) and prevalence based on examination of pig carcasses at post-mortem (4%), observed in the same region (Holyoake et al 1994b).

It is likely that gross examination of intestinal samples at slaughter is not a particularly sensitive or reliable means of diagnosis, especially as lesions may be confused with other enteric conditions which can cause similar intestinal changes, such as *C. coli* infection (Taylor 1995). Similarly, post-mortem contractions of the mucosa are often observed in the lower small intestine of the pig and these can be misleading with regard to thickening of the ileum at slaughter (Holyoake et al 1994a, Glock 1997). Furthermore, because complete turnover of intestinal epithelial cells takes only three days, resolution of lesions may occur before pigs proceed to slaughter weight (Christensen and Cullinane 1990). Since the chronic form of the disease is almost always transient, with pigs recovering from around four to six weeks after the onset of clinical signs (Rowland and Lawson 1992), it is likely that many attempts to determine prevalence, based on recognition of macroscopic lesions at slaughter or clinical signs, will lead to an underestimation of the occurrence of PE.

More recent surveys of the prevalence of PE have incorporated the PCR as a more sensitive and specific alternative for the detection of *L. intracellularis* in samples of pig faeces (Jones et al 1993c). Lanza et al (1996) reported that 16 out of 73 (22%) Spanish farms sampled for PE were positive for *L. intracellularis* on PCR of faeces samples, with 4% of farms (3/73) presenting a distinct clinical picture typical of the acute form of the disease. In this study, the within-farm prevalence of pigs shedding organisms was 10 to 30%. As over two-thirds of farms sampled were using in-feed medication, it was speculated that the prevalence was actually higher than this.

Prevalence studies

The prevalence of PE in the United Kingdom has not been adequately established, but this is mirrored in many other parts of the world where PE is also a problem, including the USA, Denmark and other pig-rearing countries. Epidemiology requires a reliable diagnostic test which can be performed on a large number of samples and, as discussed above, such a test has been elusive for PE. Serological tests have been popular in national epidemiological surveys of other pig diseases.

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In south-east Spain, the prevalence of the transmissible gastro-enteritis (TGE) virus was evaluated using a monoclonal antibody-based ELISA for serology on field samples (Cubero et al 1993). Analysis of 6,000 breeding sows from 480 farms in four geographical zones indicated an infected farm prevalence of 5%. The within-farm prevalence ranged from 5 to 60%. An anti-lipopolysaccharide ELISA was used to test 106 herds in Australia for the presence of antibodies against *Serpulina hyodysenteriae* and indicated that the prevalence of farms affected by swine dysentery was 33%, the within-herd prevalence ranging from 2.5% to 47.5% (Mhoma et al 1992). This ELISA had a sensitivity and specificity of 77% and 82%, respectively, but this was based on correlation of the serology results with the owners' diagnosis of the disease and recent herd history. Therefore, confusion of swine dysentery with other diseases may have led to inaccurate assessment of the test. Other factors which may have biased these results included antimicrobial therapy, which can reduce the antibody response, too small a sample size if within-herd prevalence was low, and varying swine dysentery status, caused by fluctuations in the composition of individual herds.

The modified agglutination test (MAT) has been incorporated in numerous studies aimed at clarifying the epidemiology of *Toxoplasma gondii*. *T. gondii* is an extremely important protozoal pathogen of mammals, including pigs. It also has major zoonotic implications (Urquhart et al 1987). *T. gondii* spends at least part of its life cycle within cells and shows a predilection for the jejunum and ileum (Moulder 1985, Urquhart et al 1987), so in some ways is similar to *L. intracellularis*. Prevalence studies in the USA indicated that 23% of market and 42% of breeder pigs had been exposed to infection (Dubey et al 1991). These studies were not able to correlate the presence of antibodies with the presence of cysts in the tissues, therefore the test had limited value for diagnosis of existing disease or zoonotic risk.

Cryptosporidium parvum infection in beef cattle has been explored in a more focused way, combining an indirect immunofluorescence assay on adult serum samples with a Ziehl-Neelsen staining method for corresponding adult faeces samples. Using these techniques, the relationship between the specific antibody response to *C. parvum* and oocyst excretion was compared between a farm with a severe *C. parvum* problem and one with no history of the disease (Scott et al 1995). Results implied that there was no difference between the two herds in either the prevalence of infection or the level of excretion. Exposure to *C. parvum* was common in adult cattle, but did not produce disease. The study also suggested

that factors other than infected adults were involved in the development of clinical cryptosporidiosis in calves.

Risk factors

Predisposing factors leading to the infection of pigs with *L. intracellularis* remain to be clearly defined. In one Australian survey, a significant relationship was found between large herds and a veterinary diagnosis of PE, with 83% of the reported outbreaks occurring in herds with more than 100 sows (Holyoake et al 1994b). The source of stock had no effect on the frequency of PE, while farms experiencing clinical PE were more likely to use feed additives. The risk factors and epidemiological features of other enteric diseases have been investigated using various methods for collection of data, depending partly on ease of sampling and availability of reliable diagnostic tests.

In their Spanish survey of transmissible gastro-enteritis, Cubero et al (1993) discovered a strong link between seropositive pigs and herds with more than 50 breeding pigs. This is a common finding in the epidemiology of infectious diseases, partly due to increased stocking densities, which are believed to increase the incidence of shedding (Fedorka-Cray et al 1994). It was speculated that, since larger herds are more likely to stock a greater number of susceptible animals, either due to continuous farrowing or introduction of grower pigs, then the virus would be more likely to become endemic. On smaller farms, the infection was expected to spread more quickly, lasting only a few weeks as the level of immunity increased. There was also a positive association between the presence of infection on a farm and its geographical zone, thought to be due to the dynamics of the viral infection and the limited movement of pigs from less affected zones. An earlier study in the USA concentrated on two farrow-to-finish units affected with TGE. Retrospective analysis of the production records indicated that potential risk factors for the occurrence of TGE in litters included contact with one particular boar at mating, the practice of multiple mating and introduction of replacement gilts from an external source (Siegel et al 1991). While these findings implied that boars, in particular, may be important in transmitting TGE, replacement gilts were believed to represent immunologically naive animals. Regardless of the pathogen concerned, introduction of susceptible animals, such as gilts, to a herd with an endemic infection encourages new outbreaks (Hurnik 1997). Therefore, a continual influx of gilts was considered important in the epidemiology of TGE, as it provided the virus with a constant supply of susceptible animals.

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Some of the prevalence work for *Toxoplasma gondii* infection in pigs has also incorporated investigation of risk factors. Weigel et al (1995) performed two separate studies to confirm the role of the cat in the transmission of *T. gondii* on pig farms. In another study, Dubey et al (1995) detected oocysts in feed and soil, corroborating the role of the environment in the transfer of infection from cats to pigs. They also identified further mammalian species, including mice and rats, as reservoirs of infection, capable of maintaining infection in the farm cat population. In contrast to the TGE study, Weigel et al (1995) found that small farms actually increased the risk of *T. gondii* infection. This was thought to be due to an increase in the ratio of excreting cats to susceptible pigs, as smaller farms would stock smaller numbers of pigs. Many of these investigations have been successful and have helped to clarify several epidemiological points which are useful in the control of toxoplasmosis and TGE.

Although the PCR could be applied to PE prevalence and risk factor surveys, the expense of the assay tends to preclude such large-scale use. Consequently, sampling surveys aimed at determining the prevalence of *L. intracellularis* infection will have to be targeted, either incorporating sample collection from a small number of representative farms or sampling small numbers of animals on a larger number of units.

In the current absence of a reliable serological assay, one alternative for studying epidemiological risk factors in enteric diseases is a questionnaire survey. Pre-existing records can sometimes offer the desired information. If not, then it must be specifically collected for the purpose of the survey (Thrusfield 1995). This could be performed either by telephone interviews (Çetinkaya et al 1994) or by a postal survey of veterinary surgeons or farmers/managers. The latter approach gave valuable information for other conditions in pigs, such as tail biting (Chambers et al 1995) and birth defects (Partlow et al 1991). A postal questionnaire was used to assess the prevalence of PE in Australia (Holyoake et al 1994b). Forty of 71 respondents reported having had PE diagnosed by either a veterinary surgeon, farmer or both, giving a high prevalence of 56%, compared to previous reports. There was a low, but rising, number of laboratory diagnoses, suggesting that farmers and their veterinary surgeons were relying on clinical signs, possibly incorporating abattoir inspection of carcasses, to diagnose PE. The rise in the frequency of diagnoses was probably due to increasing awareness of the disease, although a true increase in absolute numbers of outbreaks may have contributed to some extent.

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CHAPTER TWO

OPTIMISATION OF POLYMERASE CHAIN REACTION ASSAY

FOR *LAWSONIA INTRACELLULARIS*

INTRODUCTION

DNA amplification by the polymerase chain reaction (PCR) has become a powerful tool in molecular biology. Whereas its initial use was restricted to highly purified DNA samples in clean model systems, it has now found a niche in the diagnosis of many bacterial, viral and parasitic infections which require its application to clinical samples, such as blood, urine or tissues. Recently, the PCR has been applied towards faeces samples, which introduced new technical difficulties and challenges. These problems are worth overcoming, especially for diseases which may be difficult to diagnose clinically and where the aetiological agent is either impossible or very slow to culture, such as *Legionella* (Paszko-Kolva et al 1995) and *Mycobacterium paratuberculosis* (Collins et al 1993a).

A PCR for detection of *L. intracellularis* in pig faeces and intestinal mucosa has been described (Jones et al 1993c) using two sets of specific primers designed and constructed from part of the sequence of the *L. intracellularis* genome (Gebhart et al 1991, Jones et al 1993c). Southern blot analysis (Southern 1975) with a digoxigenin-labelled probe confirmed the identity of the 319-bp PCR product generated by those primers as being identical to that of *L. intracellularis* (Jones et al 1993c).

The PCR assay amplifies small amounts of deoxyribonucleic acid (DNA) extracted from micro-organisms already present in the faeces sample. This template DNA is incorporated into an optimised reaction mix which contains the reagents required to manufacture identical copies of the template. The reaction is triggered and processed to completion in a thermal cycler. As discussed in Chapter 1, a pre-set profile of temperatures is designed to denature the template DNA into two separate strands which facilitate annealing of the primers to the ends of each strand. Extension of the primers into new lengths of complementary DNA is catalysed by thermostable enzymes, such as *Taq* DNA polymerase. Each optimised cycle can double the amount of DNA initially present in the sample so that, by the end of the cycle programme, sufficient DNA is created to enable detection using routine laboratory methods. Success of the PCR assay depends on achieving the optimum conditions, including concentration of all the components in the reaction mix and thermal cycler temperatures. Temperature directly affects the activity of *Taq* polymerase, which has a half-life of only 5 min at 97.5°C (Innis and Gelfand 1990).

The final yield of product is also influenced by the composition, concentration and length of the primers, as well as the template DNA concentration.

Before amplification of DNA can begin it must be released from the organism under study. Various techniques described for extraction of DNA from faeces are geared towards its release, separation and purification. While this is a significant challenge in itself, it is compounded by a simultaneous need to remove PCR inhibitors and faecal contaminants, such as bile salts and bilirubin (Deuter et al 1995), which markedly reduce *Taq* polymerase activity. To this end, many extraction techniques have been based on a DNA purification method originally described by Boom et al (1990), involving the incorporation of DNA binding agents, such as diatomaceous earth or silica beads, into a suspension of the test material.

The aim of this study was to optimise a PCR test suitable for further epidemiological applications. As such a range of individual factors needed to be taken into account, the previously described methods were only used as guidelines. The nature of the assay and its vulnerability to inhibitory substances dictated a need to re-optimise each step.

MATERIALS AND METHODS

Validation of the PCR Using Genomic DNA from *Lawsonia intracellularis* Grown in Cell Culture

The PCR protocol was initially optimised using genomic DNA extracted from a pure suspension of *Lawsonia intracellularis* grown in cell culture (Lawson et al 1993). DNA was extracted from the supernatant fluid and the cell lysate of two different strains of *L. intracellularis*, LR 189/5/83 (1) and 916/91, prior to incorporation into the PCR.

1.5 to 3ml of supernatant fluid or lysate was centrifuged at 10,000 *g* for 2 min to form a pellet. The supernatant fluid was discarded and the pellet resuspended in 567µl of Tris-EDTA buffer (TE), 0.1M pH 8.0 . After the addition of 30µl of 10% w/v sodium dodecyl sulphate (SDS) and 3µl of 20mg/ml proteinase K, the contents of the tube were thoroughly mixed and incubated for 1h at 37°C, to facilitate digestion of the bacterial cell wall.

100µl of 5M NaCl was added, followed by 80µl of CTAB (Hexadecyl trimethyl ammonium bromide)/NaCl solution, allowing removal of cell wall debris, denatured proteins and polysaccharides. Removal is facilitated by complexing of these substances with CTAB, in the presence of a high salt concentration (Boom et al 1990). The contents of the tube were mixed and incubated for 10 min at 65°C. To completely remove the CTAB complexes, an equal volume of 24:1 chloroform/isoamyl alcohol was added (approximately 700µl), and the mixture extracted and centrifuged at 10,000 *g* for 4 to 5 min. The supernatant fluid was placed in a fresh tube and 25:24:1 phenol/chloroform/isoamyl alcohol added to remove any remaining precipitates. Again, the mixture was extracted and centrifuged at 10,000 *g* for 5 min before pipetting the supernatant fluid into a fresh tube and combining with 0.6 volumes of isopropanol. This allowed precipitation of the nucleic acids present in the supernatant fluid. The nucleic acids were pelleted by further centrifugation at 10,000 *g*, washed with 70% ethanol to remove residual reagents, and repelleted in the microcentrifuge at 10,000 *g* for 5 min. The ethanol supernatant was discarded and the pellet dried by allowing evaporation of ethanol at 20°C prior to resuspension in 50µl TE buffer. 10µl of this DNA was electrophoresed on a 1.5% agarose gel (Appendix A) and compared with *Hae*III digest of ΦX174 marker bands to estimate the concentration of DNA at 5ng/µl. Optimisation of the PCR was performed using aliquots of the resuspended DNA.

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Reaction components

Initial optimisation of the PCR was attempted on control DNA derived from cultured *L. intracellularis*. The published method by Jones et al (1993a) was adopted as the basic protocol initially, but a number of changes were required to adapt the assay to different laboratory conditions and reagents available in our laboratory. The basic ingredients of the PCR are primers, $MgCl_2$, dNTPs, DMSO, *Taq* DNA polymerase, buffer, water (Appendix C).

Primers

The primers used in the PCR are specific to an uncharacterised but specific segment of the bacterial genome, as established by Gebhart et al (1993). Genomic DNA extracted from purified *L. intracellularis* was cloned into plasmids, allowing sequencing of a 375-bp segment, followed by synthesis of two sets of sequence-specific primers, each 20 base pairs in length. Primers A and B (Jones et al 1993c) are the outer primers which correspond to nucleotides 5 to 24 and 304 to 323, respectively. The resultant product is 319-bp in length.

The stock concentrations of primers A and B used in our laboratory were 50µM and 67µM, respectively. During our optimisation process, 2.5µl of each primer was incorporated into a total reaction volume of 50µl. Five different concentrations were tested for primers A and B: 51µM and 67µM respectively, 5µM and 6.7µM, 3.33µM and 4.5µM, 2µM and 2.7µM and 0.5µM and 0.67µM, respectively.

Primer specificity

The specificity of the primers was tested in a PCR assay incorporating genomic DNA extracted from *Desulfovibrio desulfuricans*. Using phylogenetic studies and primer pairs capable of amplifying 16S rDNA from a wide range of bacteria (Weisburg et al 1991), Gebhart et al (1993) demonstrated that *L. intracellularis* has 91% homology to *D. desulfuricans*. Thus, *Desulfovibrio* DNA would provide a sufficiently stringent test to confirm the specificity of the PCR.

Genomic DNA was extracted from three different strains of *D. desulfuricans* using the same phenol/chloroform/isoamyl alcohol technique described above for *L. intracellularis*. 5µl of extracted DNA from each strain was incorporated into separate reaction mixtures and subjected to the optimised reaction conditions for DNA from cultured *L. intracellularis* (Results). They were exposed to a total of 42 cycles at the profile of temperatures outlined in programme 1 in Table 2.1. A positive control reaction incorporating DNA extracted from *L. intracellularis* and two reactions substituting water for template DNA (negative controls) were included in identical assays subjected to the same conditions.

Magnesium chloride (MgCl$_2$) and deoxynucleoside triphosphates (dNTPs)

The magnesium concentration influences primer annealing, the strand dissociation temperature of template and PCR product, specificity and formation of primer-dimers. It is also required to ensure the accuracy and enzymatic activity of *Taq* DNA polymerase, the normal enzyme used to catalyse the polymerisation of new DNA product. The stock concentration of MgCl$_2$ was 50mM and the volume added to each 50µl of reaction mix was 1.0 to 2.5µl, giving a concentration of 1.0 to 2.5mM per reaction.

Each dNTP was incorporated into the PCR at a final concentration of 200µM. This is the upper limit of the concentration range generally recommended for optimum yield, specificity and fidelity, but is still a comparatively low concentration, which decreases the likelihood of misincorporation of nucleotides (Innis and Gelfand 1990).

Dimethyl sulfoxide (DMSO) and boiling of reaction mix

DMSO was included in the original ingredients described by Jones et al (1993a), therefore 5µl of DMSO was added to each 50µl of reaction mix to allow evaluation in relation to our *L. intracellularis* DNA. It has been reported that DMSO is more likely to inhibit a PCR reaction than it is to enhance it. Innis and Gelfand (1990) found that it can actually reduce the activity of *Taq* polymerase by as much as 50%.

Boiling the reaction mix is one method which could ensure thorough denaturation of the DNA and reduction of reaction inhibitors, but it must be performed prior to the addition of

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Taq polymerase, as this enzyme cannot withstand exposure to boiling. To assess the efficacy of boiling on the reaction mix, the template DNA was added to the mixture which was then boiled for 10 min.

Enzyme source and concentration

DNA polymerase enzyme from different suppliers may have different activities, owing to formula variations and assay conditions, so enzyme from a selection of different manufacturers was incorporated into separate reaction mixes. *Taq* polymerase formulations supplied by Northumbria Biologicals, ICN, Bioline and BRL Life Technologies were evaluated, together with their own PCR buffer. The PCR buffer stock concentration is 10 times the required final concentration, so 5µl was added to each reaction mix. Additionally, the enzyme concentration was varied from 0.5 units to 2.5 units/50µl, as this can directly influence generation of the PCR product. An unnecessarily high concentration leads to non-specific background reactions, while too low a concentration may produce an insufficient amount of product.

Thermal cycler parameters

Table 2.1 summarises the cycle parameters which were tested for the amplification of control DNA derived from *L. intracellularis* grown in cell culture. Recommended extension times advise one minute at 72°C for products less than 2000 bp in length (Innis and Gelfand 1990). Every reaction was exposed to 72°C for 10 min at the end of the entire cycle to ensure complete extension of the primers.

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Visualisation of PCR product

PCR products were visualised on a 1.5% agarose gel stained with ethidium bromide and viewed using a UV transilluminator (Appendix A). After completion of the PCR, 10µl of each amplification product was mixed with 2 to 5µl of gel loading buffer and electrophoresed through a 1.5% agarose gel. The gel was placed in a submarine gel electrophoresis tank, submerged in TAE buffer and connected to a voltmeter set at 60 to 100 volts for 30 to 90 min, depending on the size of the gel. After completion of electrophoresis, the gel was viewed over an ultraviolet light. A DNA ladder, *Hae*III digest of ΦX174, was added to one lane on every gel (see Figure 2.1).

Table 2.1 Cycle parameters used to optimise the polymerase chain reaction

Programme	No. of cycles	Denaturation		Annealing		Extension	
		Temp	Time	Temp	Time	Temp	Time
1	1	93°C	5 min	55°C	45 sec	72°C	45 sec
	33 to 40†	93°C	45 sec	55°C	45 sec	72°C	45 sec
	1	93°C	45 sec	55°C	45 sec	72°C	2 min
2	1	94°C	5 min	55°C	1 min	72°C	1 min
	33	94°C	1 min	55°C	1 min	72°C	1 min
	1	94°C	1 min	55°C	1 min	72°C	2 min
3*	1	93°C	5 min	55°C	30 sec	72°C	30 sec
	35	93°C	30 sec	55°C	30 sec	72°C	30 sec
	1	93°C	30 sec	55°C	30 sec	72°C	5 min

Notes:

* denotes the protocol established by Jones et al (1993a).

† 5µl aliquots of *L. intracellularis* DNA were incorporated into the optimised mixture of ingredients and exposed to a number of cycles ranging from 33 to 40. A 319-bp band was produced at 38 and 40 cycles.

FIGURE 2.1

Opposite is a diagrammatic representation of the ΦX174 *Hae*III Digest (Sigma) used as a DNA marker ladder in all the electrophoresis agarose gels. There are five lighter bands, measuring 271, 234, 194, 118 and 72 base pairs, which are not clearly visible. The position of bands 271 and 234 are marked with arrows.

TOP

1,353
1,078
872
603
310
281
271
234

BOTTOM

49
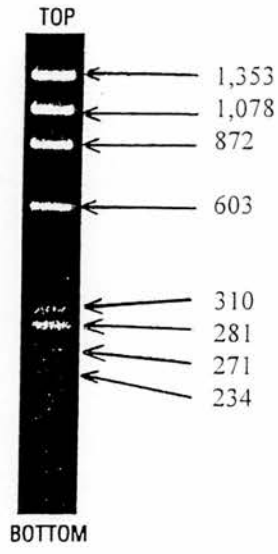
Methods Tested to Extract DNA from Faeces Samples

Several different methods for extraction of DNA from faeces samples were attempted. 5μl of each sample of extracted DNA was assayed in the PCR reaction mix using programme number 1 in the thermal cycler (Table 2.1).

Tween lysis buffer

A swab of faeces was mixed with 1ml of Tween lysis buffer (Appendix A) and vortexed thoroughly. The tube was centrifuged at 200 g for 5 min and the supernatant fluid decanted to a fresh eppendorf tube, then placed in a full 100°C waterbath for 20 min. Each sample was then stored at -20°C prior to PCR. The combination of boiling and dilution utilised by this method aimed to minimise the concentration of PCR inhibitors present in the final sample.

Homogenisation with filtering

A swab of faeces was mixed with 1.5ml of the Tween buffer and vortexed for 60 seconds. 3ml of the buffer was added and the faecal suspension homogenised for 30 seconds, to break down bacterial cell walls and release DNA. The suspension was transferred to a 4ml tube via a coffee filter to separate most of the solid material from the faeces sample and the liquid fraction was centrifuged at 5000 g for 10 min. The supernatant fluid was discarded and the pellet mixed with 1ml of the buffer, prior to incubation for 20 min in the 100°C waterbath. Each sample was then stored at -20°C.

Homogenisation without filtering

A swab of faeces was placed in 4ml of the Tween buffer in a 4ml tube, vortexed for 60 seconds and centrifuged at 200 g for 5 min. The supernatant fluid was removed to a fresh tube and the contents of each tube were homogenised for 2 to 10 seconds and centrifuged again at 5000 g for 10 min. The supernatant fluid was discarded and the pellet resuspended in 1ml of buffer. This was placed in the 100°C waterbath at for 10 to 20 min, then removed.

Guanidine thiocyanate lysis method using laboratory-prepared reagents

Guanidine thiocyanate causes lysis of bacterial cell walls, thus releasing bacterial DNA. A DNA carrier suspension can be added to the lysed material to bind the released DNA. Incorporation of repeated washing stages purifies the bound DNA, while simultaneously reducing the level of reaction inhibitors. This method, originally described by Boom et al (1990), is the basic principle of many commercial kits which are now available.

A swab of faeces was suspended in 0.5ml of 0.1M phosphate buffered saline (PBS), pH 7.3, vortexed, then centrifuged at 200 g for 10 min. The supernatant fluid was decanted and centrifuged at 5000 g for 15 min. The remaining pellet was resuspended in 1ml of guanidine thiocyanate lysis buffer prepared in the laboratory (Appendix A) and incubated for 30 to 60 min at 20°C. The lysis buffer suspension was divided into two tubes, each containing 50µl of diatomaceous earth (DE) preparation (Appendix A). Another 0.5ml of the lysis buffer was added to each tube, vortexed and incubated for another 20 min at 20°C. After vortexing and centrifuging at 10,000 g for 15 to 20 seconds, the supernatant fluid was discarded and washed twice with wash buffer prepared in the laboratory (Appendix A). This was followed by two washes with cold 70% ethanol in distilled water and one wash with acetone. The pellet was then incubated for 15 min at 52°C. 70µl of TE buffer (10mM Tris, 1mM EDTA), pH 8.0 was added and incubated for 15 min at 56°C. The suspension was vortexed and centrifuged, and the DNA-containing eluent drawn off with a pipette for incorporation into the PCR.

DNAce Clinipure Purification System

The DN*Ace* Clinipure Purification System (Bioline, London, UK) is a commercial kit which works on a similar principle to that used in the guanidine thiocyanate method described above but, as it is designed for extraction from cleaner clinical samples such as blood, tissues and urine, several adaptations were necessary to tailor the protocol to faeces samples. The method tested is outlined in Figure 2.2.

The faecal swab was placed in 500µl of TE buffer (10mM Tris, 1mM EDTA), pH 8.0 (Appendix A), vortexed and centrifuged at 500 g for 5 min to separate the heavy, solid material. The supernatant fluid was then incubated in the 100°C waterbath for 10 min and centrifuged at 10,000 g for 20 seconds to pellet any bacteria present. The pellet was washed twice in 500µl of TE buffer, pH 8.0, after which it was incubated for 1h at 20°C in

0.5ml of the lysis buffer provided with the kit (Appendix A). 15µl of carrier suspension (Appendix A) was added to the contents of the tube which was then placed on ice for 5 min to facilitate binding of the DNA. The suspension was vortexed very briefly and centrifuged at 10,000 g for 20 seconds. After discarding the supernatant fluid, the pellet was washed three times in the ethanol wash buffer also provided with the kit (Appendix A). The final wash was aspirated after briefly centrifuging and the pellet dried for 5 min at 52°C. The pellet was then incubated twice with 25µl of elution buffer for 5 min at 52°C, to give a final eluted volume of 50µl, which was stored at -20°C prior to PCR.

FIGURE 2.2 DN*ACE* CLINIPURE PURIFICATION SYSTEM ADAPTED

PROTOCOL

1. Agitate swab of faeces in TE buffer

2. Centrifuge at 500 *g* for 5 min

3. Remove supernatant fluid to a fresh tube and incubate for 10 min at 100°C

4. Centrifuge at 10,000 *g* for 20 seconds

5. Discard supernatant fluid and wash pellet twice in TE buffer

6. Add lysis buffer and incubate for 1h at 20°C

7. Add carrier suspension, vortex briefly and incubate on ice for 5 min

8. Centrifuge at 10,000 *g* for 20 seconds

9. Discard supernatant fluid

10. Wash in wash buffer. Repeat steps 8 to 10 twice

11. Add elution buffer and incubate for 5 min at 60°C

12. Centrifuge at 10,000 *g* for 20 seconds

13. Remove DNA-containing eluent to a fresh tube
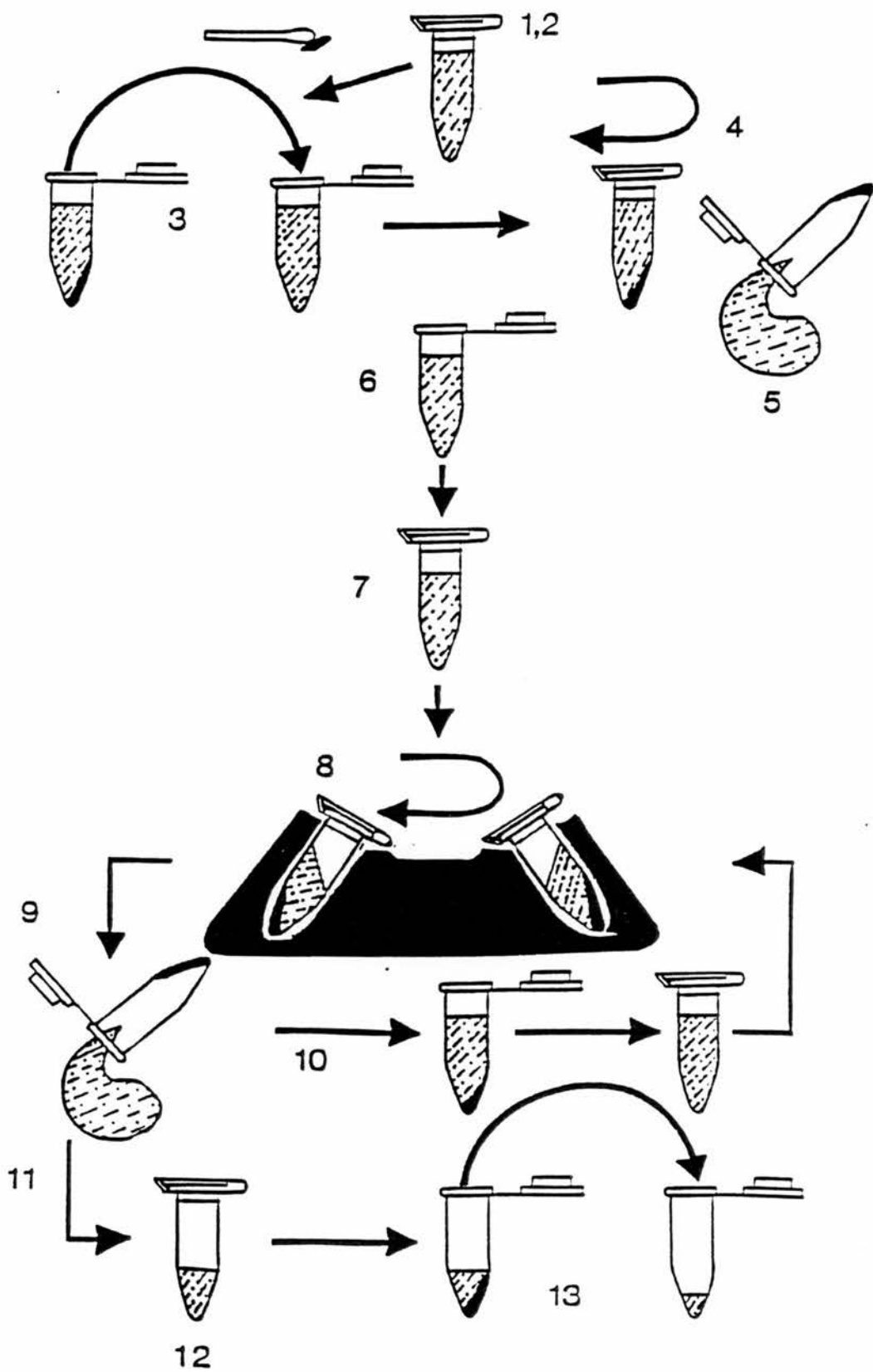
Sensitivity of the PCR and the Effect of Faeces

The DN*Ace* Clinipure Purification System was used to evaluate the detection sensitivity of the PCR for *L. intracellularis* using mock-infected faeces. Pig faeces samples were obtained from a herd with no previous history consistent with the presence of proliferative enteropathy. Individual faecal swabs were placed in 400µl of TE buffer, pH 8.0, and vortexed. They were artificially inoculated or "spiked" with inoculum for which the *L. intracellularis* count was known, to allow calculation of the concentration of bacteria per gram of faeces.

Bacterial inocula (*L. intracellularis* strains 916/91 and LR189/5/83), were thawed and centrifuged at 10,000 *g* for 10 min. Each bacterial pellet was resuspended in TE buffer (10mM Tris, 1mM EDTA), pH 8.0, to obtain a final concentration of 10^6 organisms/100µl TE. Ten-fold serial dilutions were made and added to aliquots of faeces to allow testing of seven dilutions varying from 10^6 bacteria/g faeces to 10^1 bacteria/g faeces. Uninoculated faeces were also tested. Calculations were made assuming one swab carries 0.2g of faeces.

The contents of each tube were centrifuged for 2 min at 500 *g* and the supernatant removed to a fresh tube. After a 10 minute incubation at 100°C in a dri-block, the samples were processed using the DN*Ace* Clinipure Purification System. Finally, 5µl aliquots of purified DNA were incorporated into the optimised PCR protocol using programme 1 cycle parameters (Table 2.1) and a total of 42 cycles.

To try to establish the extent to which the presence of faeces affected PCR sensitivity, 5µl aliquots were taken from each of a range of 100µl volumes of inocula containing 10^5 to 10^0 organisms and incorporated into the optimised PCR. Two negative control samples were assayed under identical conditions. One negative control substituted water for template DNA, while the second incorporated *E.coli*-derived DNA as template. Again programme 1 cycling parameters were used for a total of 42 cycles.

Confirmation of PCR Specificity by DNA Sequencing of PCR Product

The generation of the specific clone p78 allowed sequencing of a 375-bp segment of the *L. intracellularis* genome (Gebhart et al 1991) and subsequent construction of two sets of primers specific to *L. intracellularis* (Jones et al 1993c). The method chosen to confirm *L. intracellularis* specificity was comparison of the published sequence with the sequence of

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the PCR product obtained under our conditions. This was attempted in two ways. Firstly, cloning of the 319-bp PCR product into a plasmid vector to allow later sequencing and, secondly, direct sequencing.

Plasmid Cloning of PCR Product

Cloning of PCR product was attempted using three different vector kits obtained commercially. The advantage of using a vector kit is that it provides assurance that the plasmid and host competent cell will be compatible, *i.e.* the plasmid will be able to replicate in the cell, and it provides a vector system which has been designed to incorporate a method of selection of insert-containing colonies. The TA cloning system (Invitrogen BV, Holland) and the pGEM-T vector system (Promega, Southampton, UK) work on a similar principle. The vectors are pre-cut and presented in a linear form, with an extra deoxythymidine nucleotide added to each 3'-terminal of the DNA molecule. This addition allows the vector design to take advantage of the tendency for PCR polymerase enzymes to add single deoxyadenosines to the 3'-terminal of all PCR products on completion of a reaction. The resultant T and A ends complement each other, encouraging insertion of the PCR product into the plasmid DNA. The third vector kit used was the pCR-Script^{TM} SK(+) Cloning kit (Stratagene, Cambridge, UK), which works on a slightly different basis. Two enzymes are added to the ligation mixture. *Srf* I is a restriction enzyme, which initially predigests the vector, but is also added to the ligation mixture to ensure a sufficient concentration of digested vector is available during the ligation reaction. The second enzyme, T4 DNA ligase promotes incorporation of the PCR product into the vector by reforming the sugar phosphate backbone of the DNA, required for DNA stability, as the strength of the hydrogen bonds alone is not sufficient.

Preparation of template DNA

Bacterial DNA was extracted from the faeces of pig 12 in group 2 (Chapter 3) which had been experimentally infected with a pure culture of *L. intracellularis* 21 days earlier. Extraction from stored frozen (-70°C) faeces was performed using the DN*Ace* Clinipure Purification System and the extracted DNA was subjected to amplification in the final optimised PCR protocol (Results) using the thermal cycler parameters outlined in programme 1 (Table 2.1). Although none of the three vector kits required pre-treatment of

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the PCR product, both purified and non-purified PCR products were used as insert. Purified product was prepared by loading the total 50μl volumes from three identical PCR reactions onto a 1% agarose gel for electrophoresis at 80 volts for 30 min. Bands of the correct size (319-bp) were excised from the gel and purified using the DNA purification kit, Geneclean II (Appendix A). The eluted DNA was suspended in a final volume of 20μl TE buffer (10mM Tris, 1mM EDTA), pH 8.0.

Cloning

Cloning procedures consist of three main steps: ligation of insert (PCR product) into the plasmid vector, transformation of competent cells and plating out for blue/white selection and extraction of plasmid DNA from selected colonies for subsequent restriction enzyme digestion and insert identification.

Ligation

The TA cloning vector is 3.9Kb long and a 1:1 molar ratio of vector to insert was selected for the reaction (TA Cloning System Instruction Manual, Invitrogen, UK). 2μl of vector (50ng) was added to each ligation reaction, therefore 5ng of the 319-bp PCR product insert was required to achieve this required ratio. The approximate concentration of DNA in the purified and non-purified PCR product was estimated from an agarose gel by comparison with the known concentration of bands present in the *Hae*III digest ladder of ΦX174. The purified PCR product and the non-purified PCR product contained approximately lng/μl and 15ng/μl, respectively. Table 2.2 shows the reaction mixes prepared in a total volume of 11μl in a 0.5ml microcentrifuge tube. Each ligation mix was placed at 12°C overnight.

Table 2.2 TA cloning ligation reactions

Reaction mix	Non-purified insert	Purified insert
Water	4μl	2μl
10X ligation buffer	1μl	1μl
pCR^{TM} vector	2μl	2μl
Insert (PCR product)	3μl (1/10 dilution)	5μl
T4 DNA Ligase	1μl	1μl

pGEM ligation

Only non-purified PCR product was used as insert, but two different molar ratios of vector to insert were used, 1:1 and 1:3. The pGEM-T vector is approximately 3.0kb long and the estimated concentration of PCR product DNA was 10ng/µl. The formula below (pGEM-T Vector System Technical Bulletin, Promega, UK) was used to calculate the amount of insert required for 50ng of vector and Table 2.3 summarises the ligation reactions.

$$\frac{\text{ng of vector} \quad X \quad \text{kb size of insert}}{\text{kb size of vector}} \quad X \quad \text{molar ratio of insert/vector} \quad \text{ng insert}$$

Each ligation mix consisted of a total volume of 10µl and was incubated overnight at 12°C. This was followed by a second incubation for 10 min at 70 to 72°C to stop each reaction. Each mixture was cooled to 20°C.

Table 2.3 pGEM cloning ligations

Reaction mix	1:1 ratio	1:3 ratio	Control
Water	6.5µl	5.5µl	5µl
10X ligation buffer	1µl	1µl	1µl
pCR^{TM} vector	1µl	1µl	1µl
Insert (PCR product)	0.5µl (5ng)	1.5µl (15ng)	2µl (8ng)
T4 DNA Ligase	1µl	1µl	1µl

pCR-Script™ SK(+) Cloning kit

4µl of purified PCR product DNA was incorporated into ligation mix A described below. 40µl of purified PCR product DNA was vacuum dried at 50°C and resuspended in 3µl of water. 2µl of this resuspended DNA was incorporated into ligation mix B described below.

	Mix A	Mix B
Water	4.5µl	3.5µl
10X ligation buffer	1µl	1µl
pCR-Script vector	1µl	1µl (10ng)
Insert (PCR product)	2µl	2µl
rATP (10mM)	0.5µl	0.5µl
Srf I	1µ (5U)l	1µl (5U)
T4 DNA Ligase	1µl	1µl

Each ligation reaction was gently mixed and incubated for 1h at 20°C, after which the sample was incubated for 10 min at 65°C.

Transformation

Transformation reactions aim to promote uptake of foreign plasmid DNA containing the PCR product insert by competent *E. coli* cells. Competence refers to the receptive state of these cells, during which they can take up foreign DNA. Treatment of cells with chemical reagents such as dimethyl sulphoxide and reducing agents can help the transformation process. However, most techniques use sudden temperature changes to create a "window" of competency, when plasmid DNA has the greatest chance of entering a cell. Transformations were performed using competent *E. coli* cells provided with each commercial kit. 40 to 50µl of cells were thawed on ice. 1 to 2µl of each ligation reaction was added to cells in either a 1.5ml microcentrifuge tube (TA and pGEM) or a 15ml Falcon 2059 polypropylene tube (pCR-Script). The contents of the tube were incubated on ice for 20 to 30 min, followed by an incubation in a waterbath at 42°C for 45 to 60 seconds. Each tube was removed from the waterbath and placed on ice again for a further 2 min. 450µl of SOC medium (Appendix A) was then added to each reaction tube and

incubated for 1h in a 37°C gyratory shaker-incubator set at 225 rpm. During this time, Luria-Bertani (LB) broth culture plates (Appendix A) were prepared. Each kit had slightly different requirements regarding antibiotic concentration and supplementary reagents for blue/white selection (Table 2.4). After plating out 50 to 100μl of each transformation reaction, the plates were inverted and incubated overnight at 37°C.

Selection of colonies containing PCR product insert

Colonies with or without PCR insert in the plasmid DNA look identical when cultivated on agarose plates, unless there is an in-built "marker" system which enables differentiation and selection of the correct colonies. Usually, the markers come in the form of an antibiotic resistance gene, such as the pUC18 vector which carries the gene coding for β-lactamase. This confers ampicillin resistance. A second pUC18 vector marker, which was used in all of the kits on Table 2.4, is the β-galactosidase gene *lacZ*. The vector only codes for a fragment of *lacZ*, but is subsequently complemented by the host gene on transformation. Host and vector together create an active gene, able to code for and produce β-galactosidase. The importance of β-galactosidase lies in its ability to hydrolyse the chromogenic substrate X-gal, which becomes blue in colour, indicating the presence of the vector in the *E. coli*. Selection of insert-containing colonies is possible because the presence of the insert in the multiple cloning site of the vector results in insertional inactivation of *lacZ*. This allows production of white colonies on a medium containing X-Gal, because there will be no β-galactosidase to hydrolyse the X-Gal. Colonies containing the vector alone (without the insert) will allow blue-white screening.

Table 2.4 Specific requirements for each vector used

Parameters	TA vector	pGEM vector	pCR-Script vector
E.coli cell type	INVαF'	JM109	*Epicurian Coli* XL1-Blue MRF' kan'
β-mercaptoethanol	2µl added to transformation	None used	0.7µl added to transformation
Heat shock (42°C)	60 seconds	45 to 50 seconds	45 seconds
Antibiotics in LB medium	50µg/ml ampicillin	100µg/ml ampicillin	80µg/ml methicillin 20µg/ml ampicillin
Extra media supplements	50µl of X-Gal	40µl IPTG 50µl X-Gal	40µl IPTG 50µl X-Gal

Notes:

X-Gal: 20mg/ml in water stock concentration

IPTG: 0.1M in water

IPTG is an extra requirement in the presence of the *lacI* repressor gene in the *E coli* vector. *LacI* codes for a repressor protein which binds to the promoter region of the *lacZ* gene, preventing transcription of β-galactosidase. IPTG is a synthetic analogue of lactose, which binds to the repressor protein, thereby suppressing its activity on the *lacZ* gene and allowing the production of β-galactosidase.

Plasmid isolation

Single colonies were used to inoculate individual bottles containing 6ml LB medium. These were incubated for 18h at 37°C in a gyratory shaker-incubator. Plasmid DNA was then extracted from cultured bacteria using the QIAprep-spin Plasmid Purification Kit (Appendix A). This system is based on an alkaline lysis method described by Birnboim and Doly (1979). Briefly, cultures were centrifuged at 2000 *g* for 15 min. The supernatant medium was poured off and the pelleted bacterial cells resuspended in 250μl of buffer P1 (Appendix A). 250μl of buffer P2 (Appendix A) was added and the contents mixed by inversion. Buffer P2 is a lysis buffer containing NaOH and sodium dodecyl sulphate (SDS), which denatures cellular proteins. The alkaline pH allows denaturation of plasmid and chromosomal DNA. This reaction was allowed to proceed for no longer than 5 min as longer lysis times can cause the plasmids to form closed circular forms which are resistant to restriction enzyme digestion. 350μl of chilled buffer N3 was added to neutralise the lysate and increase the salt concentration, allowing the denatured proteins, chromosomal DNA, debris and SDS to precipitate. After centrifugation at 10,000 *g* for 10 min, the supernatant fluid was passed through a QIAprep-spin column in a 2ml centrifuge tube. This was re-centrifuged at 10,000 *g* for one minute and the flow-through discarded. The column was washed using 750μl of buffer PE, an ethanol based buffer, and centrifuged for one minute. The bound plasmid DNA was eluted from the column using 100μl of 10mM Tris/HCl (pH 8.5) into a tube.

Each plasmid DNA sample was digested using the appropriate restriction enzyme which was based on the nucleotide sequence flanking the multiple cloning site on the vector map. 10 units of *Eco*RI was used to digest 2μl of the TA cloning plasmid vector by incubating for 1h at 37°C. 5 units each of *Apa* I and *Sac* I were used to digest 4μl of the pGEM plasmid and 4μl of pCR-Script plasmid DNA was digested using 7.5 units of *Not* I and 5 units of *Eco*RI. Each enzyme/DNA mixture was made up to a volume of 11μl using 1μl of buffer provided by the manufacturers and water. After digesting for 60 to 90 min at 37°C, 10μl of each digest was loaded onto an agarose gel, stained with ethidium bromide and viewed using a UV transilluminator at a wavelength of 302nm.

Direct Sequencing of PCR Product

Determination of the sequence of a DNA molecule can be performed using chemical (Maxam and Gilbert 1977) or enzymatic methods (Sanger et al 1977). Enzymatic sequencing relies on the ability of the DNA polymerase to extend a primer after it is annealed to the template of interest. Chain terminating nucleotides, called dideoxyribonucleoside triphosphates (ddNTPs), are included in the reaction. When a ddNTP is added to the chain, it cannot extend any further and extension is stopped. For each region sequenced, a set of labelled, single stranded oligonucleotides is created. Each strand is the same at one end, but differs by each successive nucleotide at the other (Ausubel et al 1994).

Four separate reactions are required to determine the sequence, where only one of the four possible ddNTPs are included at a limiting concentration, along with all the normal dNTPs. This allows the creation of all oligonucleotides which terminate in A, T, G or C. The products are then resolved adjacently in a sequencing gel. Direct sequencing methods rely on the high resolution capabilities of denaturing polyacrylamide gels to allow resolution of these single-stranded oligonucleotides which differ in size by only one nucleotide (Ausubel et al 1994).

To sequence the *L. intracellularis*-specific PCR product, the *fmol* DNA Sequencing System (Promega, Southampton, UK) was used, which follows the Sanger procedure of extension-termination (Sanger 1977). In this method, a sequence-specific oligonucleotide primer was annealed to the 3' terminal of a single stranded template. The annealed template and primer mixture was divided between the four reaction mixes, each containing DNA polymerase, one of the four ddNTPs and all four deoyxnucleoside triphosphates (dNTPs), one of which was radiolabelled.

Extension/termination reaction

Bacterial DNA was extracted from the faeces of a pig experimentally infected with a pure culture inoculum of *L. intracellularis* by the preferred method (Results). The extracted DNA was amplified in the optimised PCR reaction. The combined PCR products from six identical reactions were purified from salts and other small molecules using the Wizard DNA Purification System (Appendix A).

For each set of sequencing reactions, four microcentrifuge tubes were labelled G, A, C or T. 2µl of the appropriate d/ddNTP mix was added to each tube and stored on ice. A set of four

reactions was prepared for sequencing the specific PCR product, while a second set was a prepared for the control sequence provided in the kit. Table 2.5 outlines the reactions.

Sequencing parameters

1µl of Sequencing Grade *Taq* DNA Polymerase (5U/µl) was added to the primer/template mix. 4µl of this mixture was added to each tube containing the d/ddNTP mix and one drop of mineral oil was overlaid. The reaction tubes were placed in a thermal cycler which had been preheated to 95°C. The cycling profile used was 95°C for 2 min, followed by 35 cycles of 95°C for 45 seconds and 70°C for 45 seconds. After programme completion, 3µl of the *fmol* Sequencing Stop Solution was added to each tube and the contents briefly centrifuged to terminate the reactions.

This sequencing protocol was repeated following double purification of the PCR product. A Qiagen tip-5 kit (Appendix A) was used to purify the PCR product initially, and the elution volume was further purified with the Geneclean II kit. The concentration of DNA was estimated by gel comparison to standards at 2ng/µl. 4µl (8ng) was incorporated into a reaction mix containing 5µl of sequencing buffer, 0.5µl of α^{35}S-labelled dATP, 2µl of 1/100 dilution of primer B and 4.5µl of water. This time, a different cycling profile was used: 95°C for 2 min followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 70°C for 1 min.

Immediately prior to loading onto a sequencing gel (Appendix A), both reactions were heated for 2 min at 70°C. 2.5 to 3.0µl of each reaction was loaded into the appropriate well. After completion of electrophoresis, the gels were dried for autoradiography (Appendix A) and placed in radiography cassettes on top of Kodak X-Omat film. Following a three-day exposure, each film was developed using an automatic autoradiograph processor, the radioactive bands visualised and annotated.

Table 2.5 Sequencing reactions for dideoxy sequencing

Reaction mix	PCR product	Control DNA
Template DNA	5μl (8ng)[†]	5μl
Primer*	2μl (0.8M)	2.5μl
α^{35}S-labelled dATP	0.5μl	0.5μl
fmol[R] sequencing 5X buffer	5μl	5μl
Water	3.5μl	3μl

Notes:

* The control primer used was the pUC/M13 forward primer, while primer A from the PCR protocol was used for the PCR product template sequencing reaction.

† The amount of template DNA required was 40fmol, according to the protocol. This was converted to nanograms using the following formula:

ng of template = fmol of template x 6.6×10^{-4} x N (where N = number of template base pairs)

Therefore, ng of template = $40 \times 6.6 \times 10^{-4} \times 319 = 8$ng

Automatic Sequencing

Nested PCR was used to further purify the PCR product. A 1µl aliquot of PCR product was used as template DNA in fresh reaction mixtures. This aliquot was the product of an amplification reaction which had incorporated DNA extracted from the faeces of pig 12 in group 2 (Chapter 3) which had been experimentally challenged with pure cultured *L. intracellularis* 21 days earlier. Nested primers C and D (Jones et al 1993c) were prepared to a stock concentration of 70µM. 70ng of each primer was combined with the template DNA into a reaction mix containing 200µM of each dNTP, 5µl of buffer, 29µl of water, 1.5µl (1.5µM MgCl$_2$) and 0.5µl (2.5 units) of *Taq* polymerase.

The cycle parameters used were 95°C for 5 min (one cycle) followed by 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds (40 cycles). The cycle was completed by one extension cycle at 72°C for 10 min. Six identical reaction mixes were combined and the DNA purified using the Qiagen tip-5 purification kit. A negative control, incorporating water substituted for template DNA, and a positive control, incorporating *L. intracellularis*-derived DNA as template, were subjected to the same conditions at the same time. 5µl of the 40µl purified volume obtained was used to estimate the DNA concentration, via an agarose gel. The remaining 35µl (approximately 1µg DNA) was sequenced by Mr. I. Bennett, using the LI-COR DNA Automatic Sequencer (model 4000L) and protocols established by its manufacturer.

RESULTS

PCR Optimisation

Primers and specificity

The optical density of *L. intracellularis* Primer A was 17/ml (50µM) and Primer B was 22/ml (67 µM). These optical densities were used to calculate the concentration of each of the primers, as follows:

1 O.D. (optical density) = 20µg/ml

Molecular weight (MW) of 1 base =330ng

Molecular weight of primer (20 bases) = 20 x 330ng

Molarity of stock primer = number of µg per ml of primer divided by the molecular weight.

For example,

O.D. of 17/ml = 340µg/ml

MW = 20 x 330/1000 = 6.6

Molarity = 340/6.6 = 51µM.

In reactions also incorporating *L. intracellularis* as template DNA, a 319-bp band was detected when primers A and B were incorporated at 170nM and 220nM, respectively (equivalent to 1:15 stock concentration), or at 100nM and 130nM, respectively (equivalent to 1:25 stock concentration). See Tables 2.6 and 2.7 for optimisation.

A 319-bp band was never detected when *D. desulfuricans* DNA was subjected to this PCR.

Figure 2.3 shows the results of the specificity assay.

MgCl$_2$ and dNTPs

The *L. intracellularis*-specific 319-bp band was produced at all the concentrations of MgCl$_2$ tested, but 1.5mM produced the most consistent results (see Table 2.8). dNTPs were incorporated at 200µM, comparable to published data for *L. intracellularis* (McOrist et al 1994).

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Dimethyl sulfoxide (DMSO) and boiling

None of the reactions which incorporated DMSO resulted in clear PCR product, despite variation of other parameters, such as primer concentration and *Taq* polymerase enzyme source. Therefore, it was not included in the optimised protocol.

All of the reactions which had been subjected to boiling prior to addition of the *Taq* polymerase produced negative results (see Table 2.9). Samples were not subjected to this treatment in the optimised protocol.

Enzyme source and concentration

The ICN *Taq* polymerase enzyme failed to produce specific bands at all, while the enzyme from Northumbria Biologicals did produce a specific 319-bp band (see Table 2.6). Unfortunately, production of this latter enzyme was discontinued, so Biotaq (Bioline, UK) was used. Although this did produce *L. intracellularis*-specific bands, storage for more than two weeks seemed to affect its reliability. On subjecting control DNA from cultured organisms to PCR, Biotaq produced specific bands at concentrations of 1.0, 1.5, 2.0 and 2.5 units per 50µl reaction, but failed to do so at 0.5 units per reaction (see Table 2.10). The most reliable and consistent *Taq* polymerase enzyme for our purposes was manufactured by BRL Life Technologies Ltd. BRL *Taq* polymerase produced specific bands at 2.5 units and 1 unit per reaction. It was not tested at other concentrations.

Thermal cycler parameters

The optimum cycle parameters were provided by programmes 1 and 2. Programme 1 was chosen for continued application to faecally-derived DNA using a total of 42 cycles. Programme 3 failed to produce a 319-bp band when applied to positive control DNA.

Table 2.6 Primer batches, DMSO and enzyme source

	1	2	3	4	5*	6*	7*	8*
dNTPs	8μl	8μl	8μl	8μl	8μl	8μl	8μl	8μl
Primer A	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl
Primer B	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl
Water	24μl	26.5μl	24μl	26.5μl	24μl	26.5μl	24μl	26.5μl
DMSO	2.5μl	0μl	2.5μl	0μl	2.5μl	0μl	2.5μl	0μl
Buffer †	5μl	5μl	5μl	5μl	5μl	5μl	5μl	5μl
DNA	5μl	5μl	5μl	5μl	5μl	5μl	5μl	5μl
Enzyme‡ (2U)	NBL	NBL	ICN	ICN	NBL	NBL	ICN	ICN
Result	-	-	-	-	-	+	-	-

Note:

† the buffer enzymes used here contained $MgCl_2$

* all primers were sequentially identical but tubes 5 to 8 incorporated a different batch. The concentration used was stock i.e. primer A = 51μM and primer B = 67μM, thus the final concentration of primer A and B in the PCR mix was 2.5μM and 3.35μM, respectively.

‡ NBL = Northumbria Biologicals Ltd.

‡ ICN = ICN Biomedicals Ltd.

Table 2.7 Primer concentration

	1	2	3
dNTPs	8μl	8μl	8μl
Primer A (2.5μL)	170nM	100nM	50nM
Primer B (2.5μL)	220nM	134nM	67nM
Water	25μl	25μl	25μl
$MgCl_2$* (1.5μM)	1.5μl	1.5μl	1.5μl
Buffer	5μl	5μl	5μl
DNA	5μl	5μl	5μl
Enzyme (2.5U)[†]	0.5μl	0.5μl	0.5μl
Result	+	+	+ (inconsistent)

Note:

† Enzyme source was BRL Life Technologies (see Appendix C)

Table 2.8 $MgCl_2$ Titration

	1	2	3	4
dNTPs	8μl	8μl	8μl	8μl
Primer A (170nM)	2.5μl	2.5μl	2.5μl	2.5μl
Primer B (220nM)	2.5μl	2.5μl	2.5μl	2.5μl
Water*	25μl	25.5μl	24.5μl	20μl
$MgCl_2$	1.0μM	1.5μM	2.0μM	2.5μM
Buffer	5μl	5μl	5μl	5μl
DNA	5μl	5μl	5μl	5μl
Enzyme	2.5U	2.5U	2.5U	2.5U
(0.5μl BRL)				
Result	+	+	+	-

Note:

*Water volume adjusted to allow for $MgCl_2$ concentrations

(1.5μl $MgCl_2$ = 1.5μM, 2.0μl = 2.0μM, etc.)

Table 2.9 Boiling and primer concentration

	1	2	3
dNTPs	8µl	8µl	8µl
Primer A (2.5µL)	2.55µM	255nM	25.5nM
Primer B (2.5µL)	3.35µM	335nM	33.5nM
Water	16µl	16µl	16µl
DMSO	2.5µl	2.5µl	2.5µl
MgCl$_2$*	2.5µl	2.5µl	2.5µl
Buffer	5µl	5µl	5µl
DNA	10µl	10µl	10µl
Enzyme (BRL 2U)	1µl	1µl	1µl
Result	-	-	-

Note:

* Concentration of MgCl$_2$ used was 2.5µM.

All reactions boiled for 10 minutes prior to addition of enzyme.

Table 2.10 Biotaq concentration (Primer A and B: 170nM and 220nM, respectively)

	1	2	3	4	5	6	7
dNTPs	8µl	8µl	8µl	8µl	8µl	8µl	8µl
Primer A	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
Primer B	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl	2.5µl
Water*	25µl	24µl	23µl	25µl	26µl	25µl	25µl
Buffer †	5µl	5µl	5µl	5µl	5µl	5µl	5µl
DNA (5µl)	1X	1X	1X	1X	0.1X	0.1X	0.01X
Enzyme (0.5µl)	1.0U	1.5U	2.0U	2.5U	0.5U	1.0U	1.0U
Result	+	+	+	+	-	+	faint +

Note:

† Buffer contained MgCl$_2$

* Water volume adjusted to allow for enzyme dilutions

Optimised PCR conditions:

dNTPs	8µl (200µM each)
Primer A	2.5µl (50ng, 170nM)
Primer B	2.5µl (70ng, 220nM)
Buffer	5µl
Water	25µl
$MgCl_2$	1.5µl (1.5mM)
DNA	5µl
Taq polymerase	2.0/2.5 units (BRL)

Extraction of DNA from Faeces

The only extraction method which yielded PCR product of the correct size (319-bp) from the mock-infected faeces samples was the DN*Ace* Clinipure Purification System (Bioline, London, UK). None of the other methods produced a 319-bp band at any of the concentrations tested. The DN*Ace* Clinipure System produced a clear sample of eluted DNA, which was usually colourless, but occasionally pale gold, indicating some residual faecal contamination.

Assay Sensitivity

This extraction method detected 10^5 and 10^6 organisms per swab of faeces, which is equivalent to 5×10^5 and 5×10^6 organisms per gram of faeces or 5×10^3 per reaction. Figure 2.4 shows the results of these sensitivity reactions. Without the presence of faeces, the PCR detected 100 organisms in 100µl of PBS, which is equivalent to 10^3 organisms/ml, or 5 organisms per reaction.

PCR Product Sequencing

Plasmid Cloning

Cloning of the PCR product insert into competent cells was unsuccessful, despite several attempts. The transformation reaction using the TA kit produced a very low number of white colonies (under 15) from the unpurified DNA, and none from the purified template. 10 white colonies were selected for inoculation into LB broth and overnight culture. When the plasmid DNA from 10 selected colonies was digested, only helical and linear forms of plasmid DNA were produced, but no insert DNA was obtained from any of the digests.

The pGEM vector control reaction produced the expected amount of white colonies. However, while white colonies were produced using the *L. intracellularis*-specific PCR product/vector ligation mix, the transformation efficiency was low, although slightly more were obtained from this transformation than the TA transformation. Ten colonies selected for restriction enzyme digestion did not contain any insert. Again, digestion of every colony selected produced two heavy bands of DNA, one approximately 1000bp to 3000bp, indicating the presence of helical DNA and enzymatically restricted linear DNA. There was no evidence of any other bands.

The transformations using the pCR-Script vector produced similar results. In the first attempt, using non-concentrated, purified DNA, only five colonies were obtained. They were all selected for culture. Digestion of three of the samples produced two bright bands between 2000bp and 3000bp with no visible insert, while two of the samples produced one faint band, indicating a low yield of extracted DNA from these original plasmid samples. In the second attempt, using vacuum dried, concentrated, purified DNA, less than 10 white colonies were produced. Four colonies were selected and cultured overnight in LB broth. Restriction enzyme digestion failed to produce bands of a size which would indicate the presence of PCR product. Unrestricted plasmid DNA was run on the same gel, adjacent to plasmid DNA samples which had been restricted with one or other of the two enzymes required, thus serving as controls for enzyme activity. Restriction of three of the four plasmid samples from the selected white colonies produced a different band pattern when compared to these "controls". However, all the bands produced were still in the 2000bp to 3000bp range and no bands under 1000bp were visible.

Numerous blue colonies were also produced in these transformations, although rarely on the scale which would normally be expected. Some smaller white colonies often appeared around the blue colonies. These are considered satellite colonies, which do not have a source of chromogenic substrate X-Gal, due to exhaustion of supply by the larger blue colonies already present. These satellite colonies were never chosen for restriction digests.

Direct Sequencing

Direct sequencing of the control reaction produced visible bands. However, the PCR product DNA originating from the faeces produced a streaked pattern affecting all the lanes and obscuring the resulting signal. This occurred even when the double purified PCR product was incorporated as sequencing template. Therefore, sequencing using this method was not feasible.

Nested PCR and Automatic Sequencing

The nested PCR reaction should produce a 260-bp band on an agarose gel (Jones et al 1993c). On an agarose gel, the PCR product was slightly longer than this, travelling a short distance further down the gel than the 281-bp band of the *Hae*III digest marker ladder (Figure 2.5). The most likely explanation for this was that primer C had annealed properly to the template whereas primer D had not, possibly producing a semi-nested PCR. This would yield a 279-bp PCR product, comprising base pairs 45 to 323 i.e. a segment of DNA flanked by primers C and B, as illustrated schematically in Figure 2.6. If primer D had annealed and not primer C, then this would have produced a segment of DNA flanked by primers D and A and comprising base pairs 5 to 304. This band would have been 300 base pairs in length. Further optimisation would be required to obtain the 260-bp product expected of the nested PCR. However, the purpose of the nested PCR was to purify and concentrate a sufficient amount of PCR product for automatic sequencing. The negative control produced no amplified product.

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Automatic sequencing of the combined purified PCR products using primers C and D finally allowed sequencing of 242 base pairs in total. Comparison with the 375-bp known sequence of *Lawsonia intracellularis* (Genbank CJLDNASEQ, Accession number L08049) produced one mismatch where a dATP replaced a dGTP. Overall, comparison indicated that the identity of the PCR generated DNA fragment was 99% homologous with 242 base pairs of the known sequence. The sequence generated is shown below:

57bp   TTATTGGGAAATATCCCTCATTAATTACTTCATTAGCTC 95bp

           AAGTTAAACAAGCTGCAGCACTTGCAAACAATAAACTT 133bp

           GGTCTTCTTTCTGATAAAAAAGA*AGATGCTATCTCTGC 171bp

           TGCATGTAATGAAATCATAAATGGAGAACTCCTTGATCA 210bp

           ATTTGTTGTGGATTGTATTCAAGGAGGTGCAGGGACAAG 249bp

           TACAAATATGAATGCTAATGAAGCAATTTGTAATCGTGC 288bp

           TCTTGAGCTTA 299bp

* This base should be G and not A.

FIGURE 2.3

Lane 1:   *Hae*III digest of ΦX174 DNA

Lane 2:   PCR product from positive control DNA extracted from co-cultured

            Lawsonia intracellularis (strain LR 189/5/83)

Lane 3:   PCR incorporating DNA extracted from *Desulfovibrio desulfuricans* (strain

            reference CN)

Lane 4:   PCR incorporating DNA extracted from *D. desulfuricans* (strain reference

            RK)

Lane 5:   PCR incorporating DNA extracted from *D. desulfuricans* (strain reference

            BG)

Lane 6:   PCR with water substituted for template DNA (negative control)

Lane 7:   PCR with water substituted for template DNA (negative control)

Lane 8:   *Hae*III digest of ΦX174 DNA

Electrophoresed on a 1.5% agarose gel
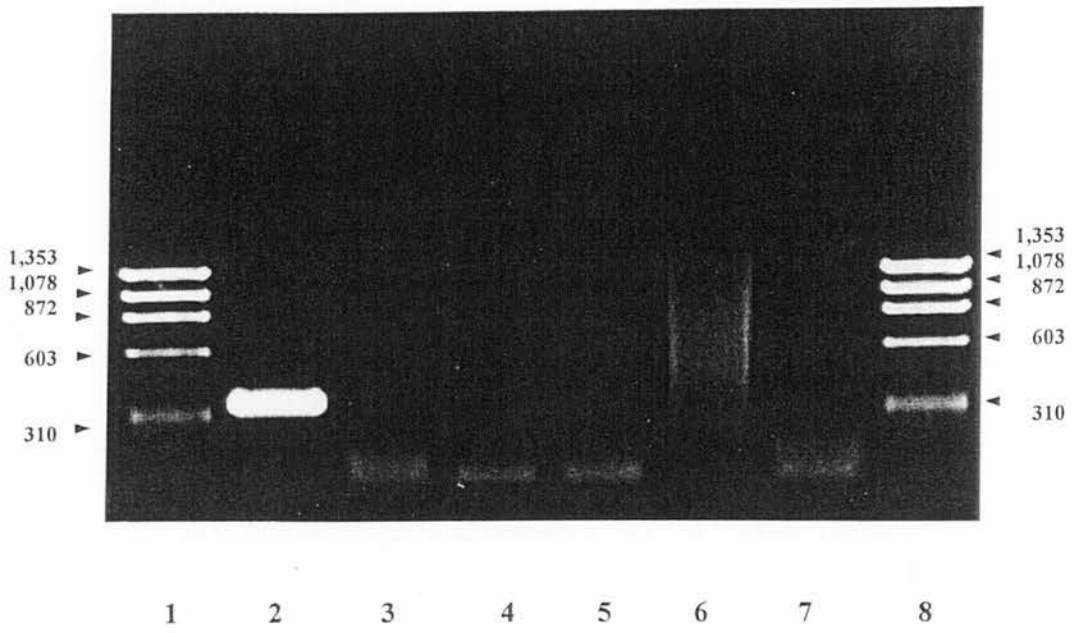
FIGURE 2.4

Lane 1:   *Hae*III digest of ΦX174 DNA

Lane 2:   PCR product from positive control DNA extracted from co-cultured

Lawsonia   *intracellularis* (strain LR 189/5/83)

Lane 3:   PCR with water substituted for template DNA (negative control)

Lanes 4 to 9: PCR product from DNA extracted from "spiked" faeces

Lane 4 10^1 *L. intracellularis* organisms per swab of faeces

Lane 5: 10^2 *L. intracellularis* organisms per swab of faeces

Lane 6: 10^3 *L. intracellularis* organisms per swab of faeces

Lane 7: 10^4 *L. intracellularis* organisms per swab of faeces

Lane 8: 10^5 *L. intracellularis* organisms per swab of faeces

Lane 9: 10^6 *L. intracellularis* organisms per swab of faeces

This agarose gel photograph shows that the sensitivity of the PCR using primers A and B
(Jones et al 1993c) is approximately 5 x 10^5 *L. intracellularis* organisms per gram of
faeces.

Electrophoresed on a 1.5% agarose gel

FIGURE 2.5

Lanes 1 to 6: PCR product form identical nested PCR assays incorporating DNA derived

from the faeces of pig 12 (Chapter 3)

Lane 7: PCR with water substituted for template DNA (negative control)

Lane 8: PCR product from positive control incorporating DNA extracted from

pure co-cultured *Lawsonia intracellularis*

Lane 9: *Hae*III digest of ΦX174 DNA

The band produced in lanes 1 to 6 and lane 8 was slightly longer than the fully nested PCR

product should be, travelling further than the 281-bp marker band shown in Figure 2.5.

Electrophoresed on a 1.5% agarose gel

bp

◄ 1,353
◄ 1,078
872
◄ 603

◄ 310
◄ 281

1　2　3　4　5　6　7　8　9
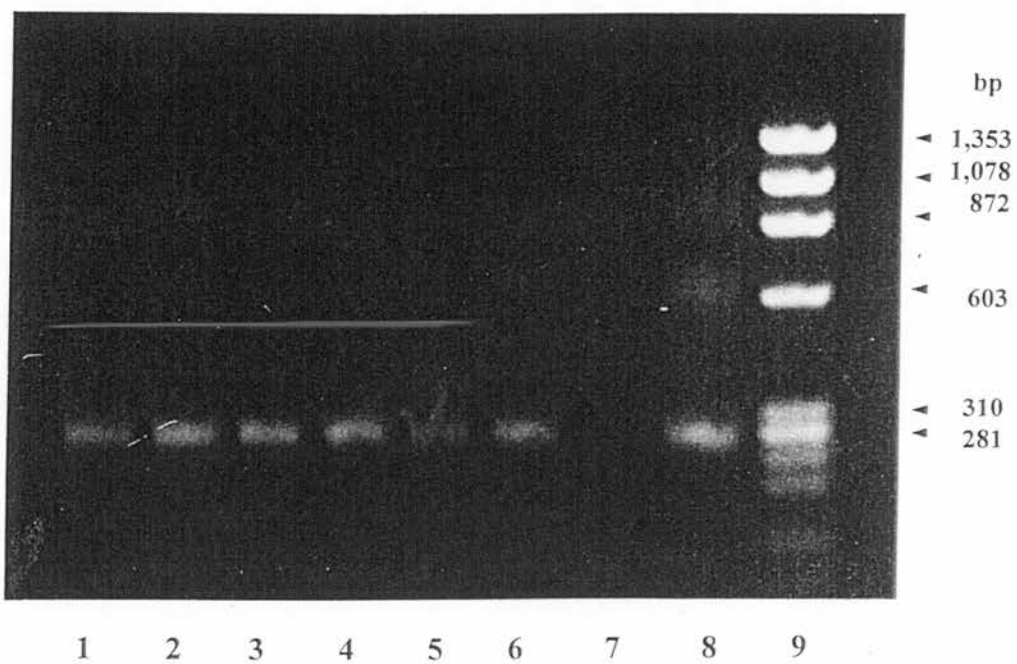
FIGURE 2.6

Shown opposite is a schematic representation of the PCR products generated by the primer sets and the possible explanation for the longer PCR product produced in Figure 2.5. The sequences of primers A, B, C and D are shown below (Jones et al 1993c).
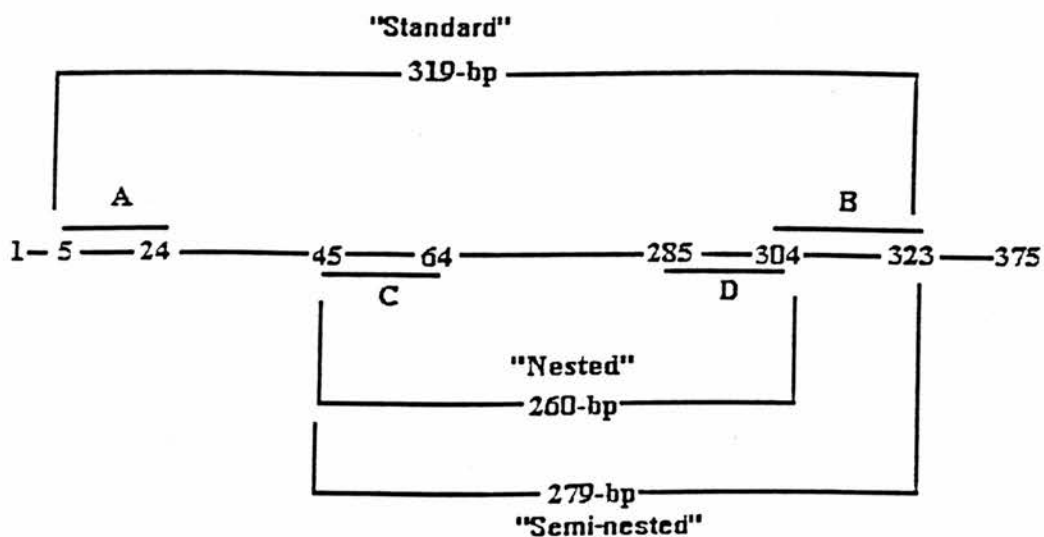
Primer A:	5'- TATGGCTGTCAAACACTCCG - 3'
Primer B:	5'- TGAAGGTATTGGTATTCTCC - 3'
Primer C:	5'- TTACAGGTGAAGTTATTGGG - 3'
Primer D:	5'- CTTTCTCATGTCCCATAAGC - 3'

DISCUSSION

In this study I optimised a PCR assay specific for *Lawsonia intracellularis* which is suitable for use on clinical samples and demonstrated a sensitivity of 5 x 10^5 organisms per gram of faeces. The external primers (A and B) used in this polymerase chain reaction amplified a 319-bp segment specific to the genome of *L. intracellularis* (Jones et al 1993c). This specificity was further verified by the primers' inability to amplify specific product from DNA extracted from various strains of the related bacterium *Desulfovibrio desulfuricans*, not performed in the original PCR description (Jones et al 1993c). It was also confirmed by the high sequence homology which existed between the *L. intracellularis*-specific 375-bp clone (p78) used to generate sequence data and the PCR product amplified from DNA extracted from infected pig faeces.

This PCR assay offers several advantages. Firstly, it provides the opportunity to detect the presence of *L. intracellularis*-derived DNA in live pigs, without the need for animal sacrifice and post-mortem examination, at least on an initial herd screening basis. Secondly, unlike diagnosis based on recognition of clinical signs and many of the other diagnostic tests which have been used to attempt diagnosis from faeces or mucosal samples, such as the Ziehl-Neelsen and Gram stains, it is highly specific. Thirdly, it is potentially an extremely sensitive test. Although Jones et al (1993b) developed a probe which was also specific to *L. intracellularis* and which could be used to diagnose infection from faeces samples, the best sensitivity achieved was 10^7 organisms per gram of faeces. My PCR detected 5 x 10^5 organisms per gram of faeces, equivalent to 5 x 10^3 organisms per reaction, and 1000 organisms per ml of purified *L. intracellularis* DNA, which equates to 5 organisms per reaction.

Another of the attractions of the PCR for diagnosis of proliferative enteropathy in pigs is the relative speed of the procedure. Similarly for the diagnosis of other diseases, such as Johne's disease in ruminants (Collins et al 1993a), PCR can still justify its use, even when other methods of diagnosis, such as faecal culture, are available. Along with excellent specificity, the decreased waiting time for diagnosis is probably one of the major advantages of the PCR to the diagnosis of PE. The rapid diagnosis of acute PE could allow prompt treatment by incorporation of appropriate medication in water, thus helping to minimise deaths in the young adults which are typically affected by this condition.

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Nevertheless, several disadvantages became apparent during the optimisation process. Although the sensitivity and specificity of the PCR was very good compared to previous tests, including the probe, 5×10^5 organisms/g of faeces was still considered low when compared to the extreme sensitivity of "cleaner" PCR systems, despite the fact that this sensitivity is similar to detection levels for other organisms, such as *Clostridium difficile* in human faeces samples (Gumerlock et al 1993). Higher sensitivities were achieved, but only on an inconsistent basis.

This inconsistency is perhaps not surprising, bearing in mind the nature of the sample. I found a 1000 fold difference in the PCR detection level between pure DNA and DNA in the presence of faeces, which underlines the inhibitory effects previously described (Fluit et al 1995, Lou et al 1997). A number of components are known to inhibit the PCR, particularly haem metabolic breakdown products, such as bilirubin and bile acids (Panaccio and Lew 1991) and complex polysaccharides (Monteiro et al 1997). These compounds are major components of faeces, an average human producing 350mg and 500mg per day, respectively. They inhibit the reaction by interefering with the activity of the polymerase enzyme. In attempting to quantify their level of inhibition, Fluit et al (1995) estimated that 10μg/ml of bilirubin and 50μg/ml of bile salts could inhibit the PCR, therefore, the potential for inhibition may be considerable. An obvious disadvantage of inhibition is the production of false negative results, which is an inherent drawback of the PCR.

Faeces is a heterogeneous substance, often varying in texture and consistency. This may make it difficult to recover DNA from small numbers of target organisms. PCR variability has been reported in the detection of *Mycobacterium paratuberculosis* in bovine faeces (Collins et al 1993b) and uneven distribution of organisms was believed to be the cause. In my extraction method for *L. intracellularis*, however, mixing and vortexing of the faecal suspension in the early stages of the procedure should have helped to prevent this. PCR variability and subsequent false negative results may also be caused by reaction components, such as primers and enzymes, differing between batches and manufacturers, which may explain why *Taq* polymerase supplied by BRL worked consistently, whereas the enzyme from Bioline did not. Some of the chemicals and reagents used in the DNA extraction procedure can even interfere directly with the PCR. Examples include SDS and guanidine thiocycanate, emphasising the importance of the washing steps. Thirdly, electrical faults in the thermal cycler may be easily overlooked and can obviously affect

generation of PCR product. Another potential cause of PCR false negatives is sample degradation over long storage periods (Deuter et al 1995), a considerable risk when DNA has originated from heavily contaminated samples like faeces. This was overcome to a certain extent by storing samples at -70°C prior to extraction of DNA and -20°C after extraction of DNA.

In contrast, false positives are also a well-recognised disadvantage of the PCR assay in general. They are usually caused by cross-contamination, to which the assay is vulnerable (Sarkar and Sommer 1990). Contamination occurs when PCR product is accidentally incorporated into a reaction mix, even in very small quantities. This is particularly relevant to the faecal extraction methods previously described, since many of them are protracted, involving several tube manipulations to minimise contamination risk. Many laboratories conduct PCR assays using two separate rooms, one for preparation and one for processing and post-PCR manipulations. The more sensitive the PCR assay, the greater the threat of cross-contamination and, while care should be taken in the routine handling of PCR products, techniques such as nested PCR must be accompanied by even more stringent efforts. Treatment of samples with ultraviolet light irradiation prior to PCR can prevent subsequent amplification (Sarkar and Sommer 1990), and the use of dUTP instead of dTTP in PCR reactions allows the enzyme uracil DNA glycosylase to remove the dUTP, blocking reamplification (Longo et al 1990). I did not use these methods.

The problem of PCR contamination becomes particularly pertinent when nested PCR is used. The best reported sensitivities for PCR on faeces samples have usually involved nested PCR protocols. For example, Collins et al (1993a) achieved sensitivities of 50 *Mycobacterium paratuberculosis* organisms/g of faeces with this method, a significant improvement on the sensitivity of the standard PCR, which was comparable with older culture methods. However, in practice, a compromise has to be made between achieving these high levels of sensitivity and avoiding the very high risk of contamination and subsequent false positive results, especially as the extra precautions which need to be taken with nested PCR can add to the expense of the test.

The DN*Ace* Clinipure Extraction System was the only extraction method which produced positive results in the PCR for artificially infected faeces. *L. intracellularis* is a Gram-negative organism with a cell wall and outer membrane requiring degradation before the DNA is available for the amplification reaction. Several of the protocol adaptations which were made, including the incorporation of a 10 minute boiling step and an increased

incubation period in the lysis buffer, were geared towards maximising release of DNA from the bacteria. A similar method, using individually prepared guanidine thiocyanate lysis and wash buffers in the laboratory, failed to work at all. While perseverance may have been rewarding, these laboratory-prepared reagents may have a short shelf-life, making the use of standardised and tested kit reagents more productive. The use of commercial kits is also likely to reflect ongoing efforts to develop superior extraction methods capable of even higher degrees of DNA purification (Uwatoko et al 1996, Lou et al 1997).

The difficulties encountered in attempting to clone the *L. intracellularis*-specific PCR product into competent *E. coli* cells appeared to centre around a poor transformation efficiency, accompanied by the production of white colonies which did not contain inserts. Failure to obtain inserts of the correct size may have stemmed from poor restriction enzyme efficiency. Restriction enzymes are vulnerable to temperature changes and long-term storage can also reduce their activity. In this work, enzymes were immediately stored on ice when removed from the freezer and several of the reactions used fresh, new enzyme directly from the manufacturer. In addition, comparison of the DNA band patterns obtained from the control plasmid digests and the unrestricted plasmid in the pCR-Script protocol, indicated that each of the enzymes was working.

The transformation efficiency was generally poor. While this can be due to a low PCR yield and, therefore, a low concentration of insert, the poor transformation efficiency occurred regardless of the quantity of cells plated out, the amount of template DNA used as insert or the molar ratios of insert to vector. No PCR product was incorporated into a ligation mix unless it was clearly visible on an agarose gel. Low competent cell efficiency may also reduce the number of white colonies, but cells were stored at the recommended temperature of -80°C and the heat pulse part of the each protocol was meticulously followed.

It is possible that the PCR product had a toxic effect on the competent cells used in the transformation reaction, or that it had a secondary or tertiary structure which made insertion into the vector difficult. Another explanation could be that the competent *E. coli* cells were producing an enzyme such as methylase, in response to the presence of the insert, which was capable of removing part or all of the insert from the vector. If even a small fraction of the insert was still occupying the multiple cloning site, then this may have been enough to block the *lacZ* gene, thus preventing production of β-galactosidase

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and producing a white colony. Furthermore, a very small insert may not be visible on a normal agarose gel after restriction enzyme digestion.

I feel the most probable explanation is that the template DNA was not sufficiently pure for these manipulations. The DNA extracted from faeces or other clinical samples often does not lend itself well to further procedures (Deuter et al 1995). In addition to their inhibitory effect on *Taq* polymerase, contaminants may also be able to reduce the efficacy of other enzymes used in the cloning procedure, such as the T4 ligase. T4 ligase activity is inhibited by high phosphate and salt concentrations (Stratagene Product Catalogue, Stratagene, Cambridge, UK), so may be subject to the effects of other inhibitors too. The manual direct sequencing results indicated that, despite double purification of the DNA, it was still not clean enough to produce a clear signal for sequence determination. The sequence was finally obtained using automatic sequencing on double purified, nested PCR product. As this particular product was not used for manual sequencing or cloning, I could not establish if it would have been pure enough for further manipulations such as these. However, the PCR product sequence produced clearly indicates the identity of the 319-bp product as originating from *L. intracellularis*. This confirms the results of Jones et al (1993a), who used pCLO probes to hybridise to the 319-bp product. Both probes and sequencing are considered accurate indicators of genetic identity in this context. Jones et al (1993a) incorporated a labelled oligonucleotide probe to increase the PCR detection sensitivity for *L. intracellularis* but the procedure is laborious and time-consuming, requiring the generation of a suitable labelled oligonucleotide. Furthermore, optimisation of transfer and immobilisation of DNA onto membranes, followed by hybridisation with a probe, can take a considerable period of time (Ausubel et al 1994), which is impractical for the large number of samples which have to be processed in epidemiological investigations. Where the aim is to confirm the identity of a PCR product by comparison with a known sequence, for example, sequencing methods involving either cloning, manual or automatic sequencing are generally quicker and technically easier.

Despite several drawbacks, the polymerase chain reaction for the detection of *Lawsonia intracellularis* in infected pig faeces has provided a method which allows rapid screening of faecal samples from a large number of animals in a herd, obviating the need to sacrifice selected individuals for post-mortem and histopathological confirmation of PE. This, in itself, is a major step forward as it offers the opportunity for rational monitoring of herds in the UK and world-wide.

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CHAPTER THREE

CHALLENGE AND TREATMENT STUDIES IN PIGS AND HAMSTERS

INOCULATED WITH PURE CULTURED INOCULA OF *LAWSONIA*

INTRACELLULARIS

INTRODUCTION

The development of a cell culture system, capable of satisfying the specific growth requirements of *Lawsonia intracellularis*, led to its cultivation *in vitro*. This development has been a major advance in many ways. Genetic and phenotypic classification of *L. intracellularis* defined it as a novel genus and species of obligate intracellular organism, belonging to the delta subdivision of the class *Proteobacteria* (Gebhart et al 1993, McOrist et al 1995a). It has been instrumental in clarifying the aetiological role of *L. intracellularis* in proliferative enteropathy of pigs (Lawson et al 1993). Pure cultures of *L. intracellularis* were cultivated in sufficient quantities for oral challenge to reproduce lesions typical of proliferative enteropathy in conventional, specific-pathogen-free pigs and in gnotobiotic pigs pre-dosed with minimal gut flora. Gnotobiotic pigs not pre-dosed with gut flora, however, appeared to be resistant to similar challenge (McOrist et al 1993, 1994b). The number of organisms for the oral challenges could be quantified and the inocula screened for the presence of contaminating organisms, thereby introducing a consistency to challenge trials, which could not be achieved when intestinal homogenates were used as a source of challenge inoculum. In pure culture challenges, animals can be dosed with known numbers of organisms, so that it is possible to compare clinical and pathological patterns observed between different strains and doses, in repeatable trials. Also, by avoiding the introduction of intestinal homogenate, there is minimum interference in the passage of *L. intracellularis* to the terminal part of the small intestine and in its entry into epithelial cells, which may otherwise be caused by the presence of cell debris and other micro-organisms (McOrist et al 1997b).

One inherent weakness in the cell culture system is its unsuitability for cultivation of *L. intracellularis* organisms within faecal material. While organisms derived from intestinal lesions can be isolated onto the cell culture monolayer, this is still a cumbersome and time-consuming process, but is justified by the major advantages which its use confers. However, the level of contaminating organisms in faeces samples, such as *Mycoplasma* and *Chlamydiae* spp., means that adaptation of the culture system to detection of infection in faeces is currently unlikely.

The inability to culture *L. intracellularis* from clinical samples has impeded attempts to gather information relevant to the epidemiology of proliferative enteropathy in pigs, including its excretion patterns and modes of transmission. Various approaches are

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possible to combat this clear difficulty. The epidemiology of other difficult-to-culture organisms has been investigated using the host's immune responses. Various serological assays have been developed for porcine toxoplasmosis (Weigel et al 1995) and *Mycobacterium paratuberculosis* in cattle (Goodger et al 1996). A previous serological assay for proliferative enteropathy identified IgM as the prime antibody involved in the host response, and concluded that this response was short-lived (Lawson et al 1988). Another assay, based on an enzyme-linked immunosorbent assay for detection of IgG, lacked sensitivity. As an alternative, molecular-based diagnostic tools, such as specific DNA probes and the polymerase chain reaction (Jones et al 1993c), apparently provided a new approach appropriate to the epidemiological investigation of proliferative enteropathy. Both the specific DNA probe and the polymerase chain reaction (PCR) appeared helpful in monitoring infection patterns in pigs. McOrist et al (1994a) used the PCR to demonstrate the excretion of *L. intracellularis* in the faeces of pigs which had been experimentally challenged 21 days previously, while Jones et al (1993b) used the DNA probe to estimate a likely incubation period of between two and three weeks. No previous challenge trials have been sustained for longer than 21 days, however, so there is no information relating to excretion patterns or correlation with pathological lesions and weight changes over long periods of time. Since proliferative enteropathy frequently appears as a chronic disease on production units, this sort of information would be extremely relevant to future control measures. The challenge trial described in this chapter shows how the PCR was used to monitor infection in susceptible pigs for up to 14 weeks post challenge.

The treatment of proliferative enteropathy, since its identification as a bacterial disease in 1973, has remained arbitrary, as no reliable patterns of antimicrobial sensitivity have emerged from field treatment of the disease. The ability to produce large amounts of *L. intracellularis* in cell culture changed this situation significantly and adaptation of the system to the *in vitro* evaluation of selected antimicrobial agents provided preliminary data, based on minimum inhibitory concentrations (MICs) and bactericidal concentrations, for potentially therapeutic drugs (McOrist et al 1995c). While it is well recognised that *in vitro* evaluation is not wholly representative of the *in vivo* environment, use of challenge trials in pigs or the hamster (*Mesocricetus auratus*) model of proliferative enteropathy may help further assessment of antimicrobial agents.

Although naturally occurring disease has not been reported in British hamsters, the clinical signs and pathology of proliferative ileitis in hamsters is almost identical to those observed in proliferative enteropathy of pigs (Jacoby and Johnson 1981). Histopathological lesions of intestinal crypt epithelial cell hyperplasia and subsequent adenomatous changes with goblet cell reduction in the ileal mucosa have been consistently associated with the presence of curved bacilli in the apical cytoplasm of the enterocytes (Frisk and Wagner 1977). These bacilli react with the monoclonal antibody IG4 (McOrist et al 1987) and, on electron microscopic examination, are structurally identical to those observed in infected mucosa from pigs (Jasni et al 1994a). Such organisms have not been demonstrated in normal hamster tissues. Reproduction of the disease in hamsters with pure cultures of *Lawsonia intracellularis* derived from pigs (Jasni et al 1994b) established the hamster as a model for the condition in pigs. This is particularly useful for the initial evaluation of antimicrobial drugs, since hamsters are easier and cheaper to house, feed and handle.

This chapter also describes a hamster and pig treatment trial. The pig treatment trial was designed to test the pleuromulin derivative, tiamulin, as both a treatment and a preventive measure. Tiamulin acts by attaching to bacterial ribosomes and inhibiting protein synthesis and has achieved MICs of 4µg/ml *in vitro* against *L. intracellularis* (McOrist et al 1995c). It is widely used as a therapeutic drug for other enteric diseases, such as swine dysentery (Dalziel 1996), where antimicrobial susceptibility testing has shown that it is effective against 90% of *Serpulina hyodysenteriae* isolates at 8µg/ml (Buller and Hampson 1994). Tiamulin is also capable of entering cells independently of oxygen levels, whereas other antimicrobials, such as the aminoglycosides, rely on oxygen-dependent mechanisms to enable them to cross cell membranes. When assessed in cell culture, enrofloxacin achieved an MIC of 8µg/ml, justifying further study *in vivo*. Enrofloxacin has been specifically manufactured for veterinary use and is a member of the fluoroquinolones, second generation quinolones which act by inhibiting the bacterial enzyme, DNA gyrase. This enzyme is responsible for catalysing negative supercoiling of chromosomal DNA, allowing the bacteria to accommodate all its genetic material. Inhibition of supercoiling leads to degradation of the DNA and rapid death of the bacteria. The fluoroquinolones have activity against both Gram-negative and Gram-positive organisms and have good tissue penetration (Boothe 1994) but have never been previously tested against proliferative enteropathy. Previously reported hamster trials have tested

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chlortetracycline therapy (Gebhart et al 1995). One of enrofloxacin's metabolites *in vivo* is ciprofloxacin, which is a highly effective drug for human enteric infections.

MATERIALS AND METHODS

Pig Challenge Trial

Thirty Landrace/Large White pigs were weaned from specific-pathogen-free sows, without the use of creep feed in the farrowing house, at 21 days of age. The weaned pigs were split randomly into three challenge groups (1, 2 and 3) and a negative control group (4). Groups 1 and 2 were housed in the same pen but separately from groups 3 and 4, which were also penned separately from each other. Pigs were dosed orally at 24 days of age and fed ad libitum commercial feed based on a wheat/barley breeding sow nut ration consisting of 44.5% barley, 20.5% wheat, 15% wheatfeed and 10% maize with the remaining 10% comprising fat, calcium, vitamins and minerals. There were no added antibiotics and the pigs were not medicated with any immunosuppressive or other drugs.

Pig Treatment Trial

Twenty-seven Landrace/Large White hybrid pigs were weaned from specific-pathogen-free sows, without creep feed in the farrowing house, at 24 days of age, divided into four groups in separate pens and housed in isolation. The pigs in groups T1 and T4 were fed antibiotic-free feed which was a meal consisting of a wheat/barley mixture with soybean added, while those in groups T2 and T3 were given feed containing the test antibiotic, tiamulin (hydrogen fumarate), according to the experimental protocol (see below).

The pigs were examined clinically on a daily basis and weighed weekly. Tiamulin was obtained as a proprietary preparation (Tiamutin 2% premix) from Leo Laboratories, Princes Risborough, Buckinghamshire, UK. Aspland and James, Consultant Analysts, Chatterts, Cambridgeshire, UK, determined the tiamulin levels in the feed given to groups T2 and T3 and confirmed that feed given to groups T1 and T4 was completely free of antibiotics (Certificate Number 31025, dated 21/04/95).

Hamster Treatment Trial

Nineteen Syrian hamsters were weaned at 21 days of age and divided into five groups, A to E. They were housed in cages at 21°C and fed antibiotic-free commercial pelleted feed ad libitum.

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Inocula

Cell culture-derived inocula for oral challenge of piglets and hamsters

The established cell line IEC-18, rat enterocytes (American Type Culture Collection number CRL 1589) were grown to a monolayer in Dulbeccos' modified Eagle's medium (DMEM) supplemented with L-glutamine, amphotericin B (Fungizone) and 10% (vol/vol) fetal calf serum (FCS). These cells were infected with *L. intracellularis* organisms derived from naturally infected British field cases of acute proliferative enteropathy, in the method described by Lawson et al (1993), by the cell culture technician Miss R.A. Mackie. Briefly, following growth of the organisms, passage was performed weekly by treating cells with potassium chloride, removing them from their culture flasks with a cell scraper and rupturing the cells by passing them through a needle. The cell lysate was then used to infect fresh IEC-18 cells. This technique has been described in detail (Lawson et al 1993). Its main function is to expand the cultures both to produce sufficient quantities of inocula for challenge trials as well as to provide inocula for storage on a long-term basis. The supernatant medium was removed from each flask and centrifuged at 4000 *g* for 20 min, after which the bacterial pellet was resuspended in sucrose-potassium-glutamate buffer (SPG) containing 5% FCS, which protects and preserves intracellular bacteria. The cells left in each flask were removed and the cell lysate bacteria combined with the supernatant bacteria to formulate the challenge inoculum. For the pig trials, the final harvest from the co-cultures was performed on the day of challenge and the final inoculate kept chilled until required. For the hamster trial, the inocula was frozen at -70°C and thawed when required. For the challenge trial, two strains were selected for separate groups on the basis of suspected enteropathogenicity. Only one strain was chosen for the treatment trial, to allow direct comparisons to be made between different regimes of drug therapy. The hamsters were challenged with a combined culture of organisms comprising two different strains of three different passages.

Monitoring of inocula

For the count of supernatant bacteria, 1ml of the supernatant medium was diluted 1:80 using DMEM and 1ml of this dilution was added to IEC-18 cell cultures in triplicate for each inoculum. These co-cultures were grown on 13mm diameter glass cover slips in small bottles for five days at the standard atmospheric requirement for growth of *L.*

intracellularis (Lawson et al 1993). Similar co-cultures were set up in duplicate for each of the cell lysates produced during the passage used to prepare the final harvest cultures. After harvesting and washing, the cell cultures were immunostained for *L. intracellularis*, using the specific monoclonal antibody IG4 as the primary antibody (McOrist et al 1987) and an anti-mouse peroxidase conjugate as the secondary antibody in an indirect immunoperoxidase test with haematoxylin counterstaining (Appendix A). The number of heavily infected cells (>30 bacteria per cell, estimated average 50 per cell) was counted by Miss Mackie for each coverslip, using a method previously described in detail (Lawson et al 1993). The count was multiplied by the original dilution and the relative area of the flask cultures, giving an estimate of the number of organisms present in each inoculum used in the trial. One ml samples of each inoculum were incubated on blood agar and nutrient broths in aerobic, anaerobic and microaerobic atmospheres at 37°C. Infected and non-infected IEC-18 cell cultures were stained for the presence of *Chlamydia* and *Mycoplasma* species using commercial antibody kits during the course of the preparation of challenge material (approximately 8 weeks).

Pig Challenge Trial

Trial Protocol

Pigs were dosed using a 20ml syringe attached to a gastric tube directed into the distal oesophagus. The trial protocol is outlined in Table 3.1.

TABLE 3.1 Pig challenge trial protocol

Group	Number of pigs	Challenged with *L. intracellularis*	Strain
1	6	Yes	916/91 (low dose)
2	6	Yes	916/91 (high dose)
3	7	Yes	LR189/5/83
4	11	No	SPG buffer only

Clinical and faecal monitoring

Piglets were weighed and a faeces sample collected at the time of challenge. Subsequently, faeces samples were taken on at least a weekly basis and each piglet was weighed at one week intervals. Piglets were observed daily for clinical signs.

Bacterial DNA was extracted from 0.2g of the faeces sample from each piglet using the DN*Ace* Clinipure Purification System, as described in Chapter 2. 5µl of the extracted DNA was subjected to the optimised polymerase chain reaction in a reaction mix containing 200µM of each dNTP, 2.5µl each of primer A (50ng) and primer B (70ng), 5µl of PCR buffer, 25µl of water and 1.5µl of $MgCl_2$ (1.5mM), also described in Chapter 2.

Each reaction was exposed to an initial cycle of 93°C for 5 minutes, 55°C for 45 seconds and 72°C for 45 seconds. This was continued with 40 cycles of 93°C, 55°C and 72°C for 45 seconds each, followed by one cycle of 93°C for 45 seconds, 55°C for 45 seconds and 72°C for 10 minutes (programme 1 in Table 2.1 of Chapter 2). Positive control DNA derived from co-cultured *L. intracellularis* and negative control DNA derived from *E. coli* were incorporated into separate tubes as controls for every PCR batch. Where possible, a known infected faecal sample was also included from the initial steps. The 319-bp positive PCR product was detected by electrophoresis of reaction product on a 1.5% agarose gel, as described in Chapter 2.

Necropsy

Pigs were euthanased by lethal injection of barbiturate and a full necropsy performed on all pigs at various intervals after challenge. Smaller pigs were euthanased by jugular injection and larger pigs by injection into the ear vein. In group 1, pigs were necropsied 23 (one pig), 55 (two pigs), 76 (two pigs) and 97 (one pig) post inoculation. In group 2, pigs were necropsied 23 (one pig), 76 (two pigs), 97 (two pigs) and 104 (one pig) post inoculation. In group 3, all seven pigs were necropsied 23 days post inoculation and in group 4, eight pigs were necropsied 23 days post inoculation, two pigs were necropsied 55 days post inoculation and one pig 76 days post inoculation. Tissue samples were collected from the stomach, duodenum, jejunum, proximal ileum, mid ileum, terminal ileum, colon, caecum, rectum, mesenteric and ileo-caecal lymph nodes, tonsils, lungs, liver and spleen. These were immediately fixed in 10% formal buffered saline and sectioned routinely at 5µm for histopathology.

Sections were stained by haematoxylin and eosin and Young's silver stain (Appendix A) for intracellular bacteria (Young 1969). Immunostaining with an indirect immunofluorescence assay for *L. intracellularis* incorporating specific monoclonal antibody IG4 and a secondary fluorescein conjugate (Appendix A) was carried out on further sections as described previously (McOrist and Lawson 1989b).

Pig Treatment Trial

Trial Protocol

Pigs in groups T1, T2 and T3 were challenged once with 10ml of the fresh inocula, administered orally with a syringe and gastric tube placed directly into the distal oesophagus. Group T2 pigs were given feed containing premix with tiamulin oral medication, starting two days prior to oral challenge and continuing throughout the trial period, as a "preventive" strategy. The nominal dose rate was 50ppm (2.5mg/kg bodyweight per day). Group T3 pigs were given feed containing premix with tiamulin oral medication, beginning seven days after challenge and continuing throughout the remaining two weeks of the trial period, as a "treatment" strategy. The dose was 150ppm (7.5mg/kg bodyweight per day). Group T4 pigs were dosed with 10ml of sucrose potassium-glutamate-buffer only. Groups T1 and T4 were fed unmedicated feed throughout and none of the pigs were given any other medication. The trial protocol is summarised in Table 3.2.

TABLE 3.2 Pig treatment trial protocol

Group	Pigs	Challenge with *L. intracellularis*	Antibiotic	Strategy	ppm	Dose (mg/kg)
T1	7	Yes	None	-	-	-
T2	6	Yes	Tiamulin	Prevention	50	2.5
T3	7	Yes	Tiamulin	Treatment	150	7.5
T4	7	No	None	-	-	-

Necropsy

Pigs were euthanased with a lethal injection of barbiturate into the jugular vein, 23 days post inoculation, then necropsied immediately. After gross examination of each pig, tissue samples were collected from the proximal ileum, mid ileum, terminal ileum, proximal colon, caecum, spiral colon, ileo-caecal lymph node and tonsil. These were fixed in 10% buffered formalin and processed for staining with haematoxylin and eosin and Young's silver stain.

Hamster Treatment Trial

Trial protocol

Dosing of hamsters was performed using a stainless steel ball-ended canula attached to the end of a 2ml syringe. The canula was placed in the proximal oesophagus to ensure that the inocula reached the stomach. Hamsters were grouped, dosed and treated as outlined in Table 3.3. Groups A, B and D were challenged on day 0 with the prepared inocula. Hamsters in groups C and E received sucrose-potassium-glutamate buffer only, again on day 0. Groups A, C and D were injected daily with enrofloxacin (see below) for five days beginning 10 days after challenge until 14 days after challenge, inclusive, while groups B and E were not medicated at all. The four hamsters in group D were re-challenged on day 21 by oral inoculation with strain 916/91. Each hamster was weighed prior to dosing and at weekly intervals thereafter.

Enrofloxacin

Enrofloxacin (Baytril 5% solution, Bayer plc, Bury St. Edmunds) was administered at a dose rate of 2.5mg/kg to the appropriate hamsters by subcutaneous injection.

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TABLE 3.3 Hamster treatment trial protocol

Group	Number	Challenged	Antibiotic
A	4	Yes	Yes
B	4 (5)*	Yes	No
C	3	No	Yes
D	4	Twice	Yes
E	3	No	No

Notes:

* One hamster was removed from group B to group A on day 2 of the trial to replace a hamster which had been cannibalised.

Necropsy

All but group D hamsters were euthanased by intracardiac injection of barbiturate and necropsied 21 days after challenge. Group D hamsters were euthanased and necropsied five weeks after challenge, 14 days after the second challenge. At necropsy, the abdominal cavity was opened and examined for evidence of gross lesions. The entire gastro-intestinal tract was removed and the intestine, from the jejunum to the colon was dissected from the rest of the tract. The colon and caecum were removed from the ileum at the level of the ileo-caeco-colic junction, opened up and fixed in formal buffered saline. The small intestine was opened along its entire length and rolled onto a short wooden applicator stick, with the duodenum on the inside and the serosa adjacent to the surface of the stick. The tissue was held in place using a small dissection pin and placed in formal saline. This "Swiss roll" technique allows the whole length of the small intestine to be processed on one slide and permits every level of the small intestine to be examined at the same time microscopically.

Sections were prepared and stained with haematoxylin and eosin and immunostained by an indirect immunofluorescence assay including the IG4 monoclonal antibody described by McOrist et al (1987).

RESULTS

Pig Challenge Trial

Inocula

Culture of the inocula onto cell-free media did not isolate any visible organisms. Immunoperoxidase staining of co-cultures set up in parallel to the flasks used to challenge the pigs and hamsters, and of the supernatant fluid from harvested challenge material, both showed many curved bacteria which reacted with the *L. intracellularis*-specific monoclonal antibody. Based on the bacterial counts from each source and assumptions regarding bacteria per cell, dilutions and culture areas, detailed previously (Lawson et al 1993) and outlined in Table 3.4, the piglets in the different groups received different doses of *L. intracellularis*.

Clinical monitoring

One piglet in group 1, three in group 2 and three in group 3 developed mild to moderate diarrhoea, with faeces ranging from pale green to dark red, and a loose or watery consistency. This diarrhoea began two weeks after challenge. All of the pigs in the control group (group 4) remained clinically healthy, except for one which developed a head tilt, possibly due to fighting or an ear infection. It was euthanased early in the trial.

Weight changes

The average weight changes of each of the four groups are illustrated in Figure 3.1, while Figure 3.2 portrays the extreme difference in body condition which was observed six weeks post challenge (nine weeks of age) in two pigs representative of the extreme body conditions observed in the challenged or unchallenged pigs. Their weights are shown graphically in Figure 3.3. The existence of statistical significance could only be calculated in the first three weeks, owing to experimental design which involved sacrificing animals at regular intervals, thus reducing the number of animals in each group. Table 3.5 summarises the mean weekly weight gains and standard deviations for groups 1 to 4 in weeks 1, 2 and 3. The standard deviations of the mean weight weekly weight gains varied within each group to the extent that a t-test was not appropriate. Therefore, the Wilcoxon rank sum was used instead. Briefly this test consists of three basic steps. Observations

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from two comparison groups are ranked in ascending order of magnitude. Any equal values are averaged. The ranks in the group with the smaller sample size are added together and this is compared with critical ranges defined for the Wilcoxon rank sum test (Kirkwood 1988). Group 1 weight gains were significantly lower than group 4 during weeks 1 and 3 ($p < 0.05$), but not during week 2. Group 2 weight gains were significantly lower than group 4 during weeks 2 and 3 ($p < 0.05$) although not during week 1, while groups 3 and 4 were not significantly different at all. Between the challenged groups, group 1 had significantly better weight gains only during week 2, when compared to group 3 ($p < 0.05$). Group 3 had significantly better weight gains over group 1 during all three weeks ($p < 0.05$) but only significantly better weight gains over group 2 during weeks 1 and 2 ($p < 0.05$).

TABLE 3.4 *Lawsonia intracellularis* count and passage number

Group Number	Strain	Estimated Challenge Inocula	Passage Number
1	916/91	1×10^8	12
2	916/91	6×10^8	12
3	LR 189/5/83	3×10^8	9

Notes for Table 3.4:

Strain 916/91

Supernatant portion: 2657 (mean number of heavily infected cells)

    x 50 (average bacteria per cell)

    x 100 (dilution)

    x 5 (aliquot volumes prepared for dosing) = 6.64×10^7 bacteria per 5mls

Cell lysate portion: 100,000 (number of infected cells per coverslip)

    x 50 (average bacteria per cell)

    x 75/1.33 (ratio of flask/coverslip area)

    x 14 (number of flasks) = 3.95×10^9 bacteria in 70mls

Doses

Group 1:   3 x 5mls of supernatant and 10mls of KCl lysis material

    Each of the 5ml volumes of supernatant and 10mls of KCl lysate resuspended in

    15mls SPG and 5% FCS - total volume of 60mls.

    $3 \times (6.64 \times 10^7) + (5.64 \times 10^8)$

    $1.99 \times 10^8 + 5.64 \times 10^8$

    7.63×10^8 bacteria in 60mls volume divided equally between 6 pigs

    1.27×10^8 bacteria per pig

Group 2:   5mls of supernatant and 10mls of KCl lysis material per pig

$$= 6.64 \times 10^7 + 5.64 \times 10^8$$

$$= 6.3 \times 10^8 \text{ in 15mls per pig}$$

Strain LR189/5/83

Supernatant portion: 228.5 (mean number of heavily infected cells)

    x 50 (average bacteria per cell)

    x 80 (dilution)

    x 250 (volume of supernatant in inoculum) = 2.285×10^8 bacteria

Cell lysate portion: 100,000 (number of infected cells per coverslip)

    x 50 (average bacteria per cell)

    x 175/1.33 (ratio of flask/coverslip area)

    x 18 (number of flasks) = 1.18×10^{10} bacteria in 100mls

Doses

The combined counts of these fractions was 1.2×10^{10} bacteria in 350ml = 3.4×10^8 bacteria per 10ml dose.

FIGURE 3.1 Average pig weights of groups 1, 2 and 4
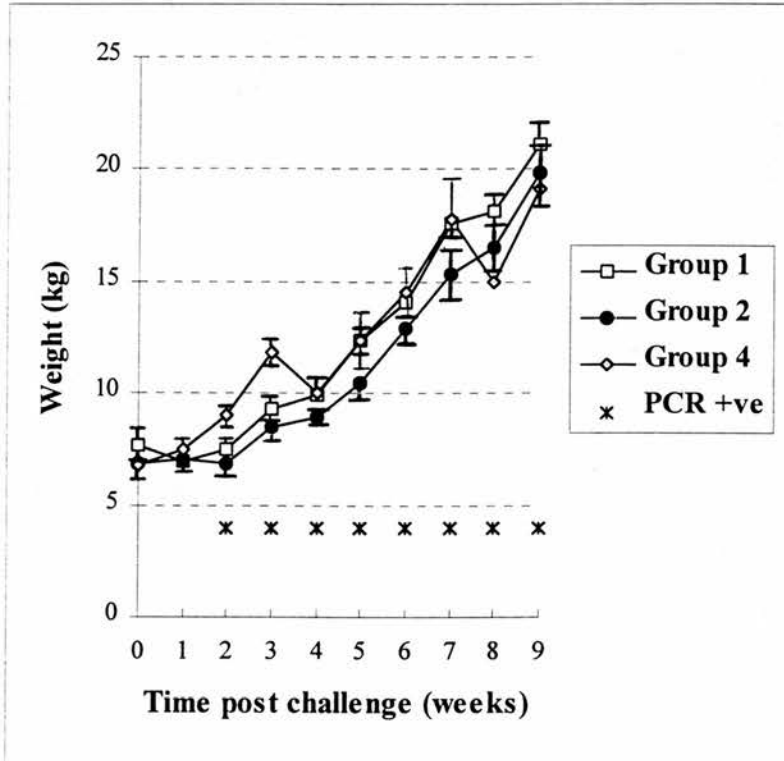
Note: Group 3 pigs not included as this group was terminated 23 days after challenge

TABLE 3.5 Mean weekly weight gains (kg) for first 3 weeks post challenge

GROUP	Week 1 Mean gain* (sd)	Week 2 Mean gain* (sd)	Week 3 Mean gain* (sd)
1	-0.7 (0.55)†	0.52 (0.37)	1.8 (0.32)†
2	0.08 (0.71)	-0.08 (0.43)†	1.6 (0.73)†
3	1.08 (0.27)	2.3 (0.34)	1.8 (1.4)
4	0.8 (0.84)	1.6 (1.16)	2.74 (0.72)

Notes:

* Mean weekly weight gain (kg). The standard deviation is in brackets.

† Mean weekly weight gain significantly different from control group ($p < 0.05$).

FIGURE 3.2 Pigs 12 and 20 six weeks post challenge

FIGURE 3.3 Individual weights of pigs 12 and 20

PCR monitoring

The results for the detection of the 319-bp polymerase chain reaction product, consistent with the presence of the genome of *L. intracellularis* in piglet faeces throughout the course of the trial, are shown in Tables 3.6 to 3.9. All of the pigs were faeces negative prior to challenge. Thirteen days post challenge, one of the group 2 pigs was faeces positive. Twenty days after challenge, one of six group 1 pigs, three of six group 2 pigs and three of seven group 3 pigs were faeces positive for *L. intracellularis*-specific PCR product. None of the control pigs were faeces positive at this time, nor at any of the other times tested, ranging 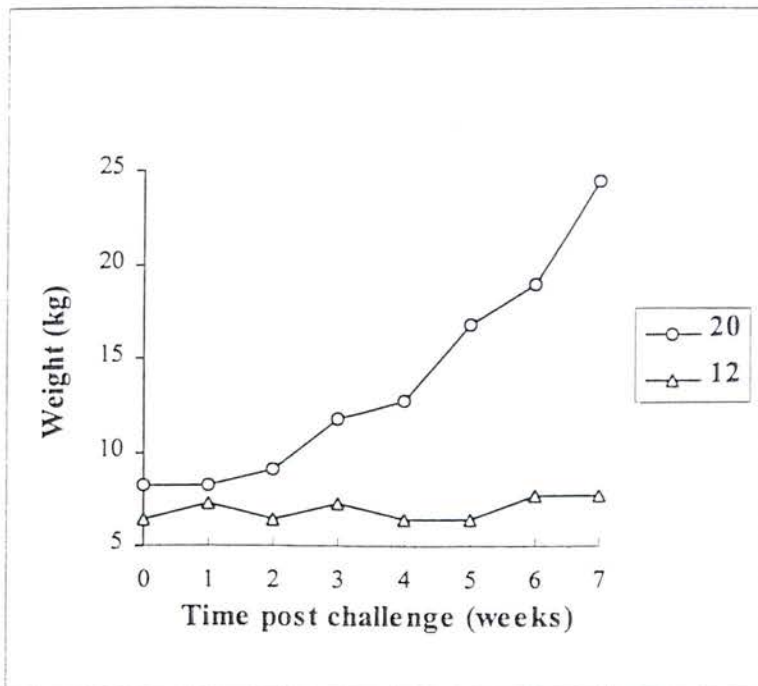from seven to 49 days post challenge. Forty-two days post-challenge, one of the six group 1 pigs and two of the six group 2 pigs were faeces positive, while 49 days after challenge, two of the six group 2 pigs were faeces positive (two different pigs day 42). Sixty-nine days after challenge, one of the pigs from group 2 was still faeces positive. Figure 3.4 illustrates the proportion of animals in each group which were PCR positive at different stages after challenge. Figure 3.5 shows the PCR results from challenged pigs in group 2.

Necropsy

Gross changes typical of PE were observed in one group 1 pig 55 days after challenge, two group 2 pigs at 76 and 104 days after challenge and all seven of the group 3 pigs at 23 days post challenge. In group 1, these gross changes were mild, consisting mainly of thickening of mucosa of the terminal ileum. In the group 2 pigs, the thickening was more pronounced, causing corrugation of the intestinal mucosa. Two sections of terminal ileum from group 2 and group 4 (control) pigs are shown in Figure 3.6. More severe thickening occurred in the group 3 pigs with corrugation and "cobbling" of the mucosal surface (Figure 3.7).

Histopathology

The intestines of pigs showing gross lesions in groups 1 and 2 had healing lesions of proliferative enteropathy, consisting of lengthened crypts, caused by immature crypt epithelial cells and goblet cells extending up from the crypt base, and some mild attenuation of lining epithelial cells, in an attempt to regenerate affected crypts. The intestinal mucosa of group 3 pigs had numerous enlarged crypts, lined by proliferating

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immature enterocytes, particularly in the ileum and colon (Figures 3.8 and 3.9). There were numerous mitotic figures and reduction of goblet cells in the affected crypts. In some crypts, there were no goblet cells at all. In areas affected grossly, these changes were severe and widespread, causing a glandular, or "adenomatous" appearance. The intestines of all other pigs appeared normal. There was a mixed, mild to moderate infiltrate of inflammatory cells, mainly lymphocytes, macrophages and neutrophils in the mucosa and lamina propria of the ileum and large intestine in all pigs. No other lesions were observed in any of the other tissues examined, including the lymph nodes and tonsils. Silver impregnation stains demonstrated numerous, curved and dark staining bacteria situated in the apical cytoplasm of the crypt epithelial cells in affected areas of the intestines of all group 3 pigs (Figure 3.10). Sections of affected mucosa from these pigs treated with antisera to *L. intracellularis*, showed brightly fluorescing curved bacilli in the enterocytes. Intracellular bacteria were not demonstrated either by silver staining or immunostaining in other pigs or in other tissues.

TABLE 3.6 Group 1 PCR results

Days post challenge	Pig No. 1	2	3	4	5	6
13	-	-	-	-	-	-
20	- (+)	-	-	-	-	-
23	-	pm	-	-	-	-
27	+		-	-	-	-
42	+	-	-	-	-	-
49	-		-	-	-	-
55	-		pm	-	pm	-
62	-			-		-
76	pm			-		pm
97				pm		

TABLE 3.7 Group 2 PCR results

Days post challenge	Pig No. 7	8	9	10	11	12
13	-	-	-	-	-	+
20	-	-	-	- (+)	- (+)	+
23	-	-	-	pm	-	+
34	-	-	-		+	+
42	-	-	-		+	+
49	-	- (+)	- (+)		-	-
55	-	-	-		+	-
69	-	-	+		-	-
76	-	pm	-		-	pm
97	pm		-		pm	
104			pm			

TABLE 3.8 Group 3 PCR results

Days post challenge	Pig No. 13	14	15	16	17	18	19
13	nt	nt	nt	nt	nt	nt	nt
16	nt	nt	nt	nt	nt	nt	nt
20	-	+	+	+	-	-	-
23	pm	pm	pm	pm	pm	pm	pm

TABLE 3.9 Group 4 PCR results

Days post challenge	Pig No. 20	21	22	23	24	25	26	27	28	29	30
13	-	-	-	-	nt	nt	nt	nt	nt	nt	nt
20	-	-	-	-	-	-	-	-	-	-	-
23	nt	nt	nt	pm	pm	pm	pm	pm	pm	pm	pm
27	nt	nt	nt								
49	-	-	-								
55	pm	nt	pm								
62		-									
69		-									
76		pm									

Notes for Tables 3.6 to 3.9:

+	positive PCR result	pm	post-mortem
-	negative PCR result	nt	not tested

-(+) positive after repeat amplification of PCR product, where a 5µl aliquot of amplified DNA was incorporated into a fresh assay.

FIGURE 3.4 Percentage of PCR positive pigs in each group
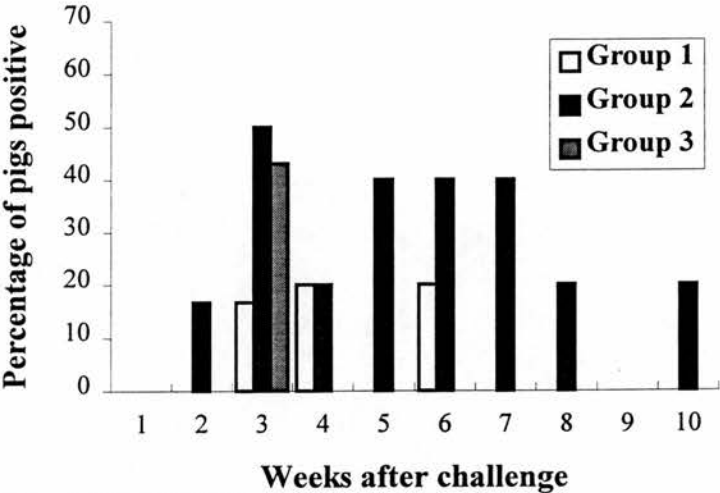
All group 4 pigs were negative when tested throughout

FIGURE 3.5

Lane 1:   *Hae*III digest of ΦX174 DNA

Lane 2:   PCR product from positive control DNA extracted from co-cultured *Lawsonia intracellularis*

Lane 3:   PCR incorporating DNA extracted from cultured *Escherichia coli* (negative control)

Lane 4:   PCR with water substituted for template DNA (negative control)

Lanes 5 to 7:   PCR incorporating DNA extracted from the faeces of group 2 pigs-27 days post challenge (different pigs to those in lanes 8, 9 and 10)

Lane 8:   PCR incorporating DNA extracted from the faeces of a group 2 pig-27 days post challenge

Lane 9:   PCR incorporating DNA extracted from the faeces of a group 2 pig-27 days post challenge

Lane 10:   PCR incorporating DNA extracted from the faeces of a group 2 pig-23 days post challenge

Electrophoresed through a 1.5% agarose gel.

bp

1,353 ►
1,078 ►
872 ►
603 ►
310 ►

1   2   3   4   5   6   7   8   9   10
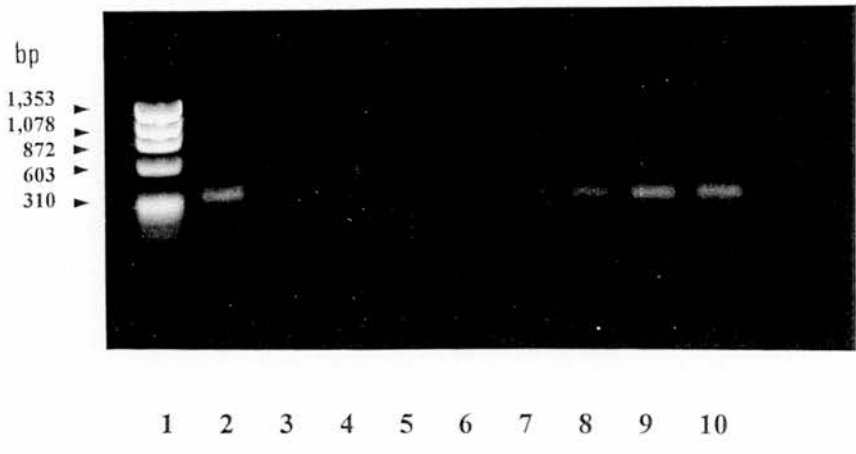
FIGURE 3.6

The segment of terminal ileum along the top of this photograph is from a group 4 (control) pig and shows a normal intestinal mucosa. The segment along the bottom of the picture is from a group 2 pig and has a more thickened mucosa. These samples were taken at necropsy, 71 days post challenge.

FIGURE 3.7

This piece of ileum from a group 3 pig 23 days post challenge shows extensive mucosal thickening which has progressed to severe corrugation, or "cobbling".
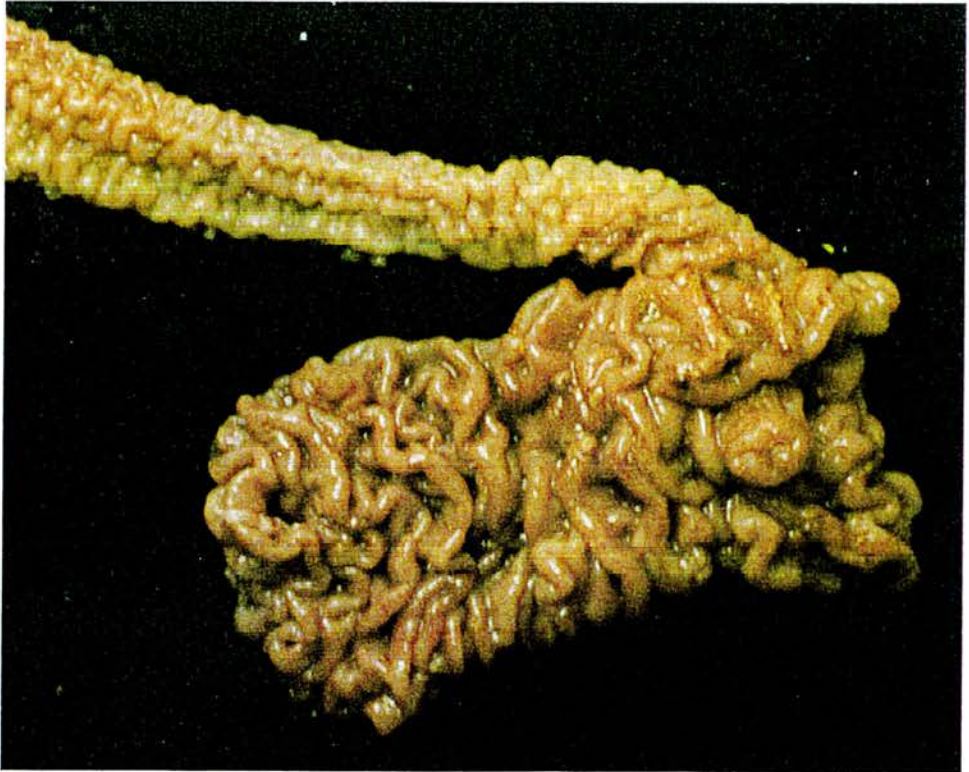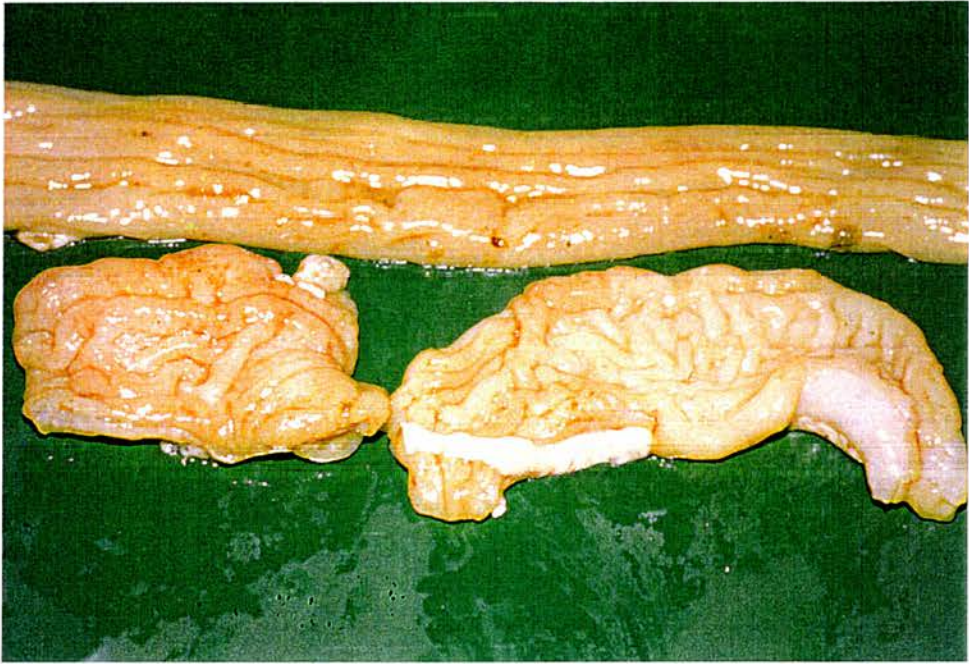
FIGURE 3.8

This section, taken from a terminal ileum from a group 3 pig 23 days post challenge, demonstrates pronounced adenomatous change caused by hyperplasia and proliferation of the intestinal crypt epithelial cells.

Haematoxylin and eosin x 100

FIGURE 3.9

This section is also taken from a group 3 pig 23 days post challenge. It shows a severely hyperplastic crypt with no goblet cells next to several less affected crypts which still have goblet cells present (arrows).

Haematoxylin and eosin x 200
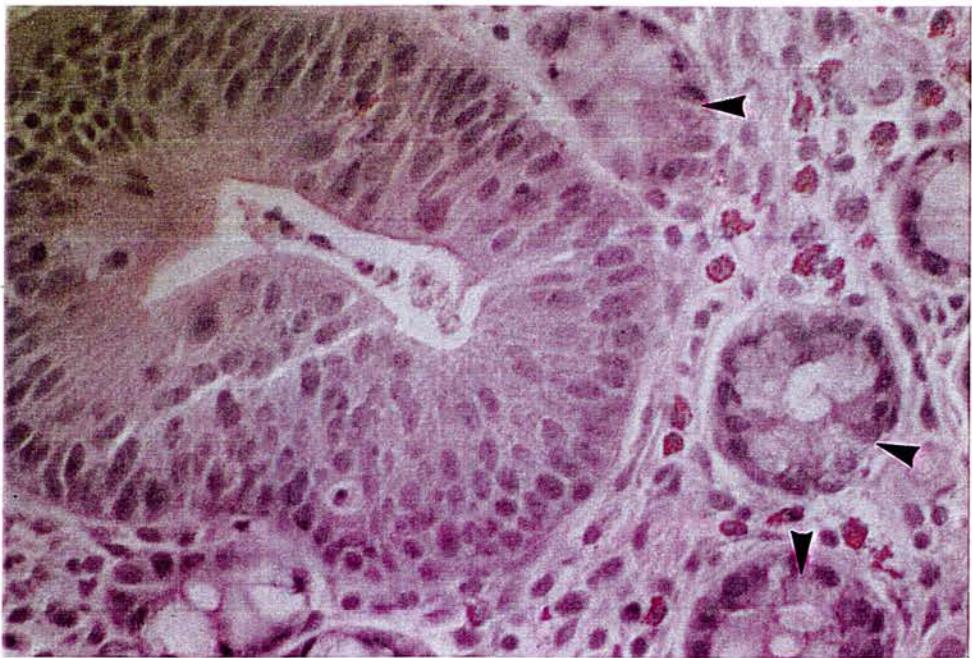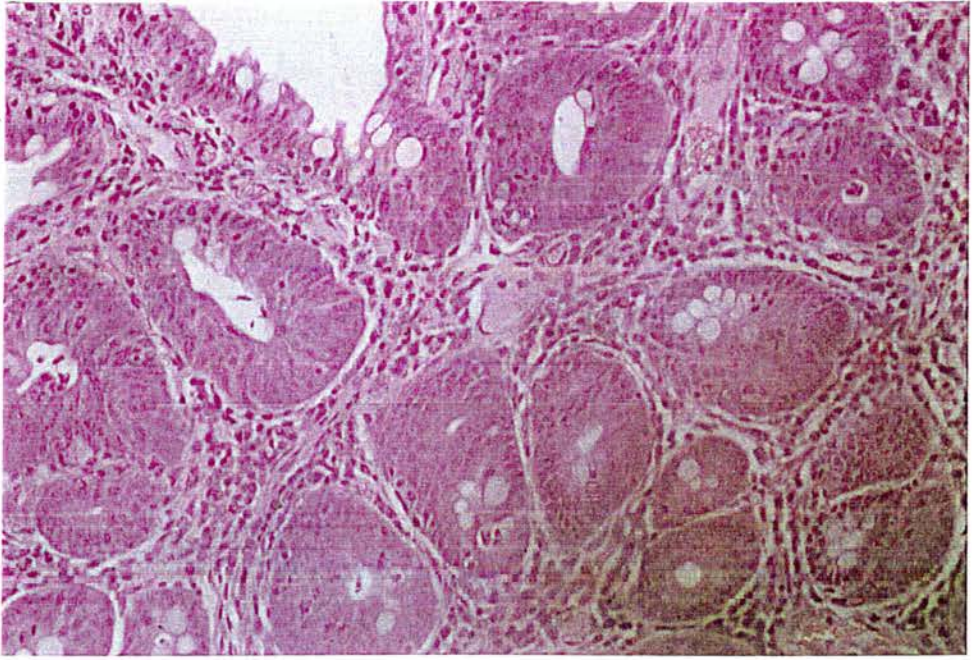
FIGURE 3.10

Opposite is a section of terminal ileum taken from a group 3 pig 23 days post challenge, showing numerous black/brown curved organisms present in the apical cytoplasm of the crypt epithelial cells (arrows).

Young's Warthin-Starry silver stain x 200
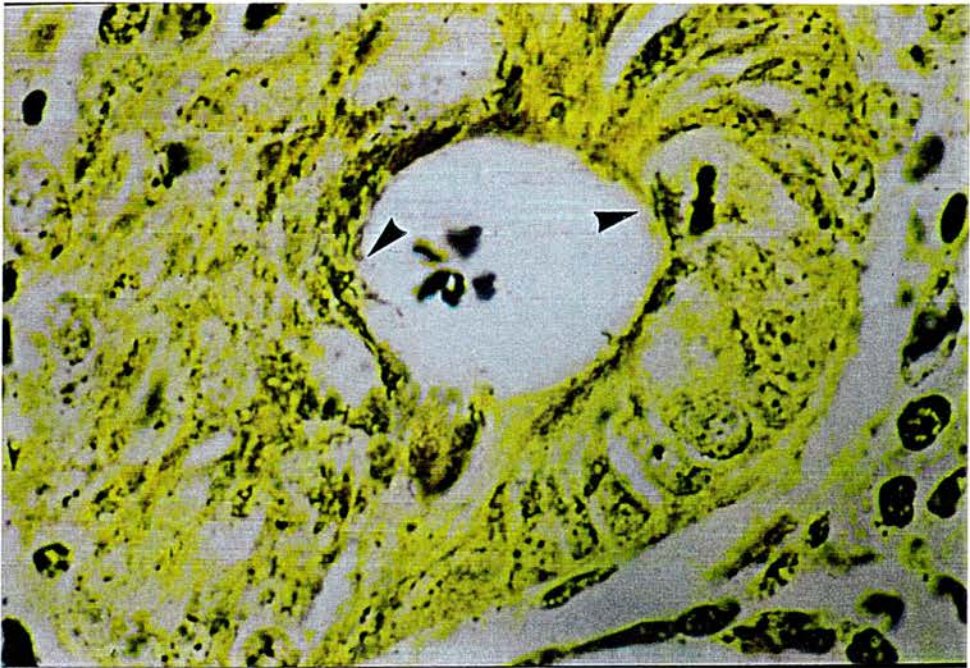
Pig Treatment Trial

Inocula

The bacterial counts of cells heavily infected with *L. intracellularis* organisms in the co-cultures derived from a 1/80 dilution of the supernatant portion of the inoculum are shown in Table 3.10, along with the counts of heavily infected cells in the coverslip co-cultures which were incubated in parallel with the 18 inoculated flasks in the last week prior to harvest of the challenge inoculum. The supernatant and lysate totals were combined to estimate the number of *L. intracellularis* organisms present in the final inoculum at 3.4 x 10^8 organisms per pig. The stained cytospin smears showed numerous bacteria morphologically resembling *L. intracellularis* both within detached cells and independent of them. Cultures of the inoculum onto agar and broth were checked after two, five and seven days incubation, but no cell-free bacteria were grown.

Clinical signs

Three pigs in group T1 developed mild to moderate diarrhoea 2 weeks after challenge, while all the other pigs were healthy throughout. A number of pigs in group T1 failed to gain weight as quickly as those in group T4. The average weight of the pigs in group T1 at necropsy (11.1kg, average daily gain 248g) was 19.6, 9.0 and 14.0%, respectively, below the average weight of pigs in group T2 (13.8kg, average daily gain 362g), group T3 (12.2kg, average daily gain 295g) and group T4 (12.9kg, average daily gain 314g).

Necropsy

All seven of the untreated animals in group T1 developed disease. Six of these had grossly visible lesions, three of which consisted of severe mucosal changes, despite the fact that the pigs had only been infected for 23 days. In affected pigs, the mucosa of the ileum and caecum was grossly thickened and/or hyperplastic. No other sites were affected. One pig had no gross lesions. Microscopic lesions typical of proliferative enteropathy were noted in all seven pigs in group T1. They consisted of enlarged or elongated crypts with or without proliferation of the epithelial lining cells and positive for intracellular *L. intracellularis* organisms, mainly in the ileum and caecum. Associated with this proliferation, there was a reduction in the number of goblet cells which are normally found intermingled at regular intervals with the epithelial cells lining the crypts. These changes have been well described previously (Rowland and Lawson 1975, McOrist

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et al 1993). The percentage of affected crypts in the ileum/caecum of sections examined from each pig varied from 1%/0%, respectively, in one pig, to 10%/50% in another and 100%/100% in two pigs. None of the seven pigs in group T4 (negative control) had any gross or microscopic lesions and intracellular organisms were absent. In group T3 (prevention), no microscopic or gross lesions were apparent in any of the pigs. Two pigs in group T4 (treatment) had mild mucosal hyperplasia which was visible grossly in the ileum, but there were no microscopic lesions in any of the seven pigs in this group.

TABLE 3.10 Treatment trial calculation of number of *Lawsonia intracellularis* organisms in challenge inocula

Inoculum sample and analysis	*L. intracellularis* visualised	Count of heavily infected cells
Supernatant portion: cytospin smear, modified Ziehl-Neelsen	+++	(100%)
1/80 dilution: coverslip culture	+++	244
		269
		214
		187
	Mean	229
Cell lysate portion: parallel coverslip	+++	100,000

Notes:

Calculation of numbers of *L. intracellularis* in inoculum

<u>Supernatant portion</u>: 229 (mean number of heavily infected cells)

    x 50 (average bacteria per cell)

    x 80 (dilution)

    x 250 (volume of supernatant in inoculum) = 2.29×10^8

<u>Cell lysate portion</u>: 100,000 (number of infected cells per coverslip)

    x 50 (average bacteria per cell)

    x 175/1.33 (ratio of flask/coverslip area)

    x 18 (number of flasks) = 1.18×10^{10} in 100mls

Total = 1.2×10^{10} in 350 ml = 3.4×10^8 in 10ml inoculum

+++ = Numerous heavily infected cells present

Hamster Treatment Trial

Clinical signs

There were no abnormal clinical signs observed during the course of the trial. All of the hamsters continued to pass normal faeces and remained active.

Necropsy

There were no gross lesions observed in any of the hamsters and microscopic lesions were not observed on light microscopy and haematoxylin and eosin staining. However, indirect immunostaining with *L. intracellularis*-specific monoclonal antibody (IG4) revealed numerous curved bacilli fluorescing apple-green in the apical cytoplasm of the crypt epithelial cells in one of four hamsters in group A, three of four hamsters in group B (Figure 3.11) and one of four hamsters in group D (Figure 3.12). No fluorescing organisms were demonstrated in any hamsters from group C or group E.

Weights

No major differences were observed in the hamster weights between the five groups, probably due to the short period of time over which the trial was run. Table 3.11 summarises the average weights of each group of hamsters in the post challenge period.

TABLE 3.11 Average weight (grams) of hamster groups

GROUP	*n*	DAY 0	6	13	20	26	33	40
A	4	23.75	31.5	39.2	54	-	-	-
B	4	19.75	30.5	41.5	59.2	-	-	-
C	3	29.3	46	61.7	73	-	-	-
D	4	22.2	36.7	50	65	76	86	95.5
E	3	22.3	33.7	48.7	62	-	-	-

FIGURE 3.11

Photographs a and b illustrate sections of ileum from a group B hamster (challenged and untreated) viewed with an ultraviolet light microscope. Samples were taken at necropsy 21 days post challenge.
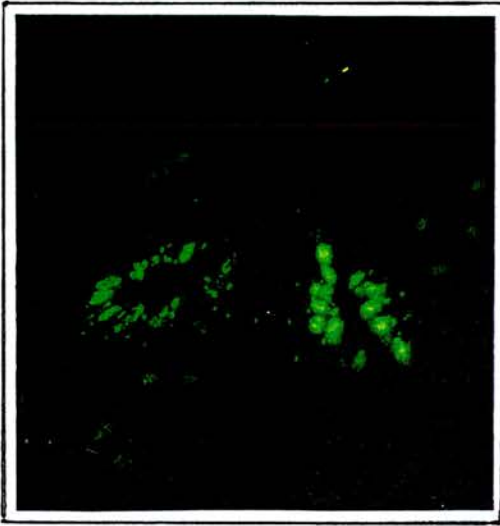
Indirect immunofluorescent assay x 400
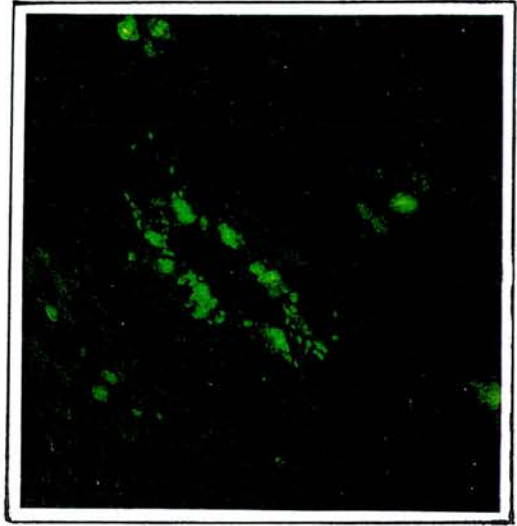
FIGURE 3.12

Photographs a and b (opposite) show sections of ileum taken from a group D hamster (challenged, treated and rechallenged). Samples were taken at necropsy 28 days post challenge.
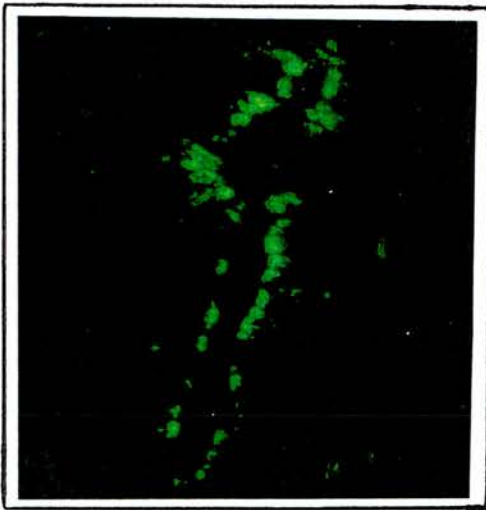
Indirect immunofluorescence assay x 400

(a)   (b)

(a)   (b)
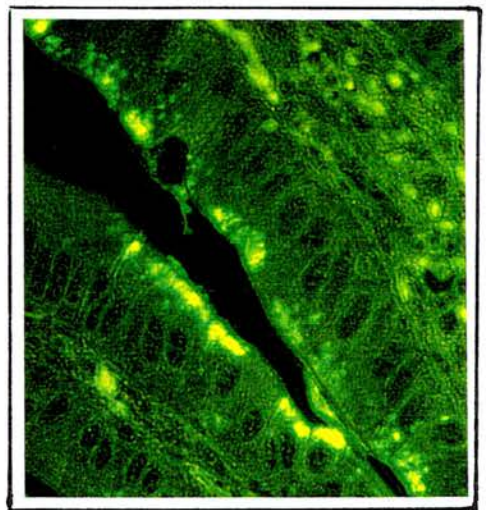
DISCUSSION

Each of these three trials has provided new information relating to many aspects of research into proliferative enteropathy, including the nature and course of infection of pigs with *Lawsonia intracellularis*, and potentially effective treatment protocols. In addition, they have clarified that the cell culture system is a valid method for the cultivation of *L. intracellularis* organisms which are capable of repeatedly reproducing pathological lesions typical of PE.

The pig challenge trial demonstrated that, despite its expense, the polymerase chain reaction is a useful tool for the experimental monitoring of *L. intracellularis* excretion in faeces (see Chapter 2 for PCR development). The PCR first detected *L. intracellularis*-specific product 13 days post-challenge, adding support to the previous reports which have suggested that the incubation period of PE is around two to three weeks (Jones et al 1993b, McOrist et al 1994a). It also demonstrated that individual pigs could excrete *L. intracellularis* organisms for at least 10 weeks after oral challenge.

There is a great deal of information available on the transmission patterns, dose effects and creation of carrier states for other enteropathogens in pigs, such as *E. coli*, *Salmonella* and *Serpulina* species. This is the first challenge study which has allowed at least some of these aspects to be examined, at least on a preliminary level, in relation to *Lawsonia intracellularis*. In this study, the extensive examination of various tissue sites for the presence of infection was intended to establish if healthy animals could also be carriers of infection in areas outside the terminal part of the small intestine. Although the immune response to PE is very poorly understood (Chang et al 1984, Lawson et al 1988), harbouring of organisms in the lymph nodes, spleen or tonsils may explain the sudden and sporadic episodes of acute PE. Such outbreaks have been associated with stressful situations (Bane et al 1997), which may influence the immune system's control of latent infection. No sites outside the small and large bowels, including peripheral lymph nodes and tonsils, were found to be carrying *L. intracellularis* organisms, either by immunofluorescence or silver stain. Preliminary PCR results on the tonsils of PE positive and PE negative pigs were also negative (data not presented). These are only preliminary results and a more sensitive test may reveal small number of organisms previously missed, while a heavier infection may indeed lead to the creation of a carrier animal. In porcine salmonellosis, infected animals can actually harbour organisms in the caecum, tonsils and

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lymph nodes for several months after infection (Wood et al 1989) which is a significant factor in spread of infection, both within herds and between units. Again, stress and intercurrent disease have been associated with outbreaks. A large-scale abattoir survey, incorporating PCR of tonsillar tissue and lymph nodes of healthy slaughter pigs from infected farms, would be an alternative method for the study of carrier pigs in PE, providing more statistically significant results. Nevertheless, initial impressions do suggest that, once *L. intracellularis* is cleared from the intestines, the pig is entirely free of infection. Since it is an obligate intracellular organism with a major preference for epithelial cells, it would perhaps be surprising to find it inhabiting any other area of the body for any length of time.

Strain variation could help to explain how and why two very different conditions, acute and chronic PE, are caused by the same organism. Possibly one strain possesses an antigen which is only recognised by receptors in younger animals, leading to cell entry and the production of the chronic form in weaners and growers. Alternatively, acute PE may be caused by a toxin-producing strain of *L. intracellularis* which is capable of inducing haemorrhage. Unless there is an age-dependent receptor in the host, this would not explain why a more virulent strain does not seem able to produce the acute form of PE in younger animals. The two strains which provided challenge inocula in this trial did vary in their ability to produce disease. LR189/5/83 1 produced moderate to severe disease, with typical gross mucosal lesions occurring only 21 days after challenge in group 3 while, in groups 1 and 2, strain 916/91 produced a much milder picture, producing gross lesions in only two of the six group 2 pigs, which received the higher dose of 916/91. There are several explanations for this. Firstly, pigs in group 2 were necropsied at least 11 weeks after challenge, to allow as long a period as possible post-challenge for the monitoring of infection in live pigs. Such a long period may have allowed resolution of lesions, had any been present in the first few weeks post challenge. Secondly, the inocula used for group 2 had been passaged twelve times in cell culture, while that used for group 3 had only been passaged nine times. If *in vitro* passage affects the *in vivo* virulence of organisms then this may explain the observed differences in mucosal pathology. Finally, there may be a real and absolute difference in virulence between strains, as has been well described in colibacillosis infection of pigs, with a multitude of different antigenic strains exhibiting different virulence factors, from fimbriae and pili, which enhance their ability

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to colonise cells, to plasmid-mediated toxin and haemolysin production (Bertschinger et al 1992, Beutin et al 1994).

In addition to the possible strain difference, there was evidence to suggest the occurrence of a dose effect. Despite challenge, pigs in group 1 achieved reasonable weight gains, comparable to the unchallenged pigs. However, the average bodyweights of groups 1 and 2 were markedly different, group 2 pigs consistently lagging behind. Although they had received the same strain of *L. intracellularis* organisms, pigs in group 2 were challenged with five times the dose used to challenge the pigs in group 1. Such reductions in bodyweight and condition have previously been reported in the field (Gogolewski et al 1991) and experimentally (Roberts et al 1977). Furthermore, the more subtle weight differences in some cases have demonstrated that low grade or subclinical infection may be present in a group of pigs but still be difficult to discern clinically. Dose effects do occur in other enteric infectious diseases of pigs, notably *Isospora suis*, which is also an inhabitant of intestinal epithelial cells. Increasing dose rates are associated with increased morbidity and mortality rates, and decreasing weight gains in pre-weaning pigs. Challenge with less than 5×10^4 organisms only induces mild clinical signs of diarrhoea and no mortality, while 4×10^5 organisms or more can produce high mortality rates (Lindsay et al 1985). Further evidence pointing to a dose effect in PE lies in the proportion of animals in each group where the PCR detected *L. intracellularis*-specific PCR product in the faeces. The PCR results also seemed to detect dose-dependent excretion patterns accompanying the variable clinical changes. In group 1, only one of the six pigs excreted *L. intracellularis* at detectable levels for a period of approximately one week, while five of the six pigs in group 2 excreted detectable levels of *L. intracellularis* for a period spanning eight weeks. Not only do these findings suggest that the likelihood of an individual animal becoming an excretor depends on the initial infectious dose, but also that increasing doses may consequently lead to the development of persistent infection within a group of pigs. The apparent infective dose for *L. intracellularis* is 10^7 organisms (McOrist et al 1993) and both the sensitivity of the PCR for detection of *L. intracellularis* and the estimated numbers of organisms present in the faecal smears, suggest that organisms are excreted at a high level (up to 10^8 organisms per gram of faeces) which, if ingested by a susceptible pig, would also be sufficient to establish a new infection. This agrees with previous findings in the field and experimentally, where numerous organisms were detected in infected pigs and associated *L. intracellularis*

organisms or genome-specific PCR product were detected in faeces by immunofluorescence assays or PCR (McOrist and Lawson 1989b, Jones et al 1993c). While it can be argued that the PCR only detects DNA, in itself not necessarily indicative of viable and undamaged organisms, the presence of morphologically entire organisms in faecal smears stained with the indirect fluorescent antibody test suggests that they are excreted in an identical form to that observed in infected intestinal crypts. Control measures should probably be devised on the principle that excreted organisms are viable.

The detrimental effect which PE has on production economy is primarily caused by the reduced weight gains and carcass quality observed in the chronic form of the disease (Roberts et al 1979, Holyoake et al 1996). As with the challenge trial, substantial weight differences were noted in the pig treatment trial. Challenged pigs which had been medicated with 50ppm tiamulin from two days prior to challenge achieved better weight gains not only compared to the challenged, unmedicated group and the challenged, treated group, as might be expected, but also compared to the unchallenged group. This would suggest that tiamulin used in a preventive manner may have certain growth promoting properties which could be of economic benefit. A similar challenge/medication trial recently used to evaluate in-feed tylosin, not only confirmed growth depression in weaned pigs affected with PE, but also showed that tylosin may also be of similar economic benefit to the farmer, as challenged pigs treated with tylosin produced the most efficient feed conversion ratios (McOrist et al 1997b). Although there have been no controlled reports on the use of tiamulin to treat PE in the field, this trial confirmed its efficacy *in vivo* against *L. intracellularis*. In susceptibility testing in the cell culture system, it had achieved MICs of 4μg/ml, which was considered only moderately effective. The application of the *in vitro* system to drug testing is limited by its inability to impart information relating to host response and conditions. The treatment trial would indicate that tiamulin is able to accumulate in the terminal part of the small intestine and enter the lining epithelial cells at the required concentration, in an active form. Its incorporation in feed prior to exposure to infection also appears to either prevent cell entry of *L. intracellularis*, or inhibits its multiplication within the cell. Similar efficacy has recently been reported for tylosin by McOrist et al (1997). Where *in vitro* assays had indicated that tylosin may not be highly effective, it was able to fulfil the requirements *in vivo*, and maintained a high intracellular activity against *L. intracellularis*.

These trials have shown that the use of cultivated organisms for oral challenge of susceptible pigs is a valid model for medication trials, providing a method of accurate and consistent dosing, without the interference of contaminating organisms. While it is more realistic than *in vitro* susceptibility testing, the *in vivo* challenge/treatment model may not reflect wholly the situation in the field, where compliance with prescribed medication can not always be checked. Nevertheless, preliminary field trials using in-feed tylosin found increased performance compared to control pigs (Moore and Zimmermann 1996). The long incubation period and difficulties associated with recognition of PE will lead to variations in the times of medication and natural challenge. In addition, the difficulties associated with recognition of this condition will, in most cases, dictate that drugs will be used on a treatment basis, after lesions have developed and it is still not known for how long animals should be treated before infection is cleared completely.

The hamster challenge/treatment trial demonstrated the validity of this animal model in investigations into PE. Production of disease in the hamsters was successful, as demonstrated by the presence of curved, fluorescing organisms in the apical cytoplasm of the crypt enterocytes in 75% of the challenge group. It was interesting that neither gross lesions nor microscopic proliferation of epithelial cells, crypt elongation or goblet cell depletion were observed. The presence or absence of intracellular organisms was the only diagnostic feature in infected hamsters. A high concentration of organisms is generally required to elicit PE lesions in hamsters (Frisk and Wagner 1977) and Jasni et al (1994) demonstrated a variation in the severity of disease, depending on the strain used and the passage number. In addition, one of the strains used (1482/89) had been stored frozen prior to use and, despite the fact that these organisms had been passaged only twice, they failed to induce lesions typical of PE. Fresh inocula of a different strain (916/91) had been passaged more often, yet still reproduced lesions. Since the inocula used in this trial was also frozen, this could explain why no gross or microscopic lesions were noted (other than positive fluorescent antibody results). Perhaps the freeze-thawing cycle damages or kills a proportion of the bacteria present in the inocula, thus reducing the infective dose.

Based on the presence or absence of intracellular organisms, neither of the treated groups (A and D) were cleared completely of infection. 25% of each group were still infected at necropsy. Group D had been re-challenged after treatment to investigate if development of resistance to re-infection was possible. The spread of fluorescing organisms deeper into the lamina propria of the intestinal wall suggested that the antibiotic had failed to

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clear the original infection from the intestine. Although these are preliminary studies and are not necessarily representative of a pig challenge/treatment trial, they do indicate that enrofloxacin may have reduced efficacy in a useful model. Enrofloxacin has good tissue penetration, affecting a wide range of both Gram-negative and Gram-positive bacteria (Boothe 1994). Although its effect on obligate intracellular organisms has been reported less widely, its inability to clear *L. intracellularis* from the intestines was surprising, considering its clinical application to enteric diseases. It has been used to successfully eradicate *Shigella flexneri* (which only becomes pathogenic upon cell entry) from primates (Banish et al 1993) and it was reportedly active *in vivo* against canine *Rickettsia rickettsii* infections (Breitschwerdt et al 1991). However, it has been ineffective against human *Ehrlichia* species *in vitro* (Boothe 1994). Reports of the usefulness of quinolones against *Salmonellae* have varied. Wistrom and Norrby (1995) indicated that selected quinolones are of limited use in uncomplicated enteritis caused by *Salmonella*, while other reports found enrofloxacin to be a valuable drug for the treatment of salmonellosis (Froyman 1992). Enrofloxacin accumulates in phagocytic cells, so it is possible that this is why it is generally more effective than the other quinolones, especially against *Salmonella* species, which are facultative intracellular organisms with a tendency to infect macrophages. Interestingly, although it should theoretically be active against obligate intracellular organisms, studies in dogs infected with *Brucella canis* showed that infection became re-established when treatment was stopped (Boothe 1994). In my hamster trial, treatment was also stopped 14 days after challenge and hamsters were necropsied seven days later, which could have provided sufficient time for recrudescence of infection, had it not been eliminated fully.

CHAPTER FOUR

AN EPIDEMIOLOGICAL SURVEY OF PORCINE PROLIFERATIVE ENTEROPATHY

ON BRITISH PIG FARMS

INTRODUCTION

Information relevant to the epidemiology of the proliferative enteropathies is sparse, based mainly on observations during field outbreaks. The elucidation of possible modes of transmission and the establishment of prevalence would depend on a reliable detection assay becoming available. While investigations into many enteric diseases, such as salmonellosis, are facilitated by simple laboratory culture techniques, the obligate intracellular nature of *Lawsonia intracellularis* means that these techniques are not applicable to its detection in clinical samples (Gebhart et al 1993, Lawson et al 1993). Previously, abattoir surveys were used to inspect the intestines of pigs at slaughter. Identification of typical lesions using gross examination provided estimates of prevalence that ranged from 0.8% (Christensen and Cullinane 1990) to between 5 and 40% of pigs (Pointon 1989). This variation is thought to be due to poor methods of detection, lacking in both sensitivity and specificity. Lesions were detected mainly by observation and palpation and depended on the correct recognition of typical pathological signs by the examining veterinary surgeon.

The establishment of a PCR which uses *L. intracellularis*-specific primers (Jones et al 1993c) has now provided the opportunity to undertake both preliminary studies into the excretion patterns of the organism and field prevalence surveys (Lanza et al 1996, Møller et al 1996). The field investigations by Lanza et al (1996) and Møller et al (1996) have used small sample numbers, so correlation with potential risk factors has not been possible. Furthermore, a significant drawback of the PCR is that it is very expensive to perform, making it inappropriate for large sample numbers.

A questionnaire survey offers a cheaper alternative, suitable for gathering information on a large scale from a wide area. Cross-sectional mail surveys have given valuable information on a wide range of diseases in livestock (French et al 1992, Schreuder et al 1993), including enteric ones (Robertson 1992). This method was chosen to investigate possible risk factors for PE in the field.

MATERIALS AND METHODS

Study Design

Survey

The survey was conducted via a postal questionnaire, entitled "A Survey of Porcine Intestinal Adenomatosis (PIA) in Pigs". Prior to the start of the survey, copies were sent to six farmers to assess the ease of completion and to highlight any potentially misleading questions. In the main survey, each questionnaire was accompanied by a letter, giving the reason for sending the questionnaire, which included an explanation of the disease itself and reassurance of data confidentiality (Appendix B). I felt that this particular explanation was needed to clarify the disease I was investigating and to avoid the confusion which has often surrounded the descriptive terminology of proliferative enteropathy (Anonymous 1995a).

The sample size, that is the number of questionnaires posted, was calculated from an expected prevalence of 30% with a 95% confidence interval, and assuming a response rate of 60% (Thrusfield 1995). This was performed using "STATCALC" in EpiInfo, a programme which allows the required sample size to be calculated, providing certain statistical values are given. This study was cross-sectional and, based on a survey population of 20,000 units and an expected prevalence of 30% with a 95% confidence interval, the number of units which were required for sampling was 300. This was multiplied by 100/60 to allow for a reduction in the 100% response rate which this programme assumes. Thus, the required sample size was 500 units.

Study population

The questionnaire was posted to 569 separate pig farms in the United Kingdom, excluding Wales and Northern Ireland. This amounts to 3% of the British total of approximately 20,000 pig farms (MAFF census).

Initially, a number of organisations were approached in an effort to obtain addresses of pig units in the United Kingdom. These included the Pig Journal Magazine (Pig Veterinary Society journal), the National Farmers Union (NFU), the British Pig Association, Pig Farming magazine, the Scottish Office Agriculture and Fisheries Department and two feed compounder companies (BOCM Pauls and Dalgety Agriculture Ltd.).

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Unfortunately, almost all of these organisations do not divulge their clients' addresses or are themselves subject to the Data Protection Act. While the NFU can provide information, they do so at a cost, which was sufficient to preclude this organisation as a potential source of addresses. A list of clients' addresses was finally obtained from Dalgety Agriculture Ltd. (Turriff, UK), under the proviso that they were not divulged to any other parties.

Using these addresses, 262 (46%) questionnaires were distributed directly to farmers throughout Scotland. The questionnaires and explanatory letters were posted in October 1995, along with a stamped envelope addressed to myself. A reminder card was posted to non-respondents two weeks later and a final reminder letter was delivered to non-respondents two weeks after that. Both the questionnaire front page and the reminder card were designed with a small pig motif.

Due to the problems associated with obtaining addresses, delivery to the remaining 307 (54%) pig farms was facilitated by using veterinary surgeons to deliver questionnaires to farms. These were located mainly in Yorkshire, Norfolk and Suffolk. This apparent clustering by region arose because these are the main areas of pig production in Britain, with few pig units existing in other regions. However, small numbers of questionnaires were sent to veterinary surgeons in other areas of the country, such as Oxfordshire, and five questionnaires were sent to County Meath in Ireland.

When veterinary surgeons were involved in the distribution, the questionnaires were either posted with the vets' monthly accounts or handed out during routine visits to pig farms. In each case, a stamped addressed envelope and an explanatory letter accompanied the questionnaire. It was not possible to send individual reminders to each one because the veterinary surgeons did not divulge addresses.

Questionnaire design

The design of the questionnaire and the survey itself followed many of the points made by Vaillancourt et al (1991), including the layout and number of questions, the number and format of pages, and the title. I chose to specify PIA in the questionnaire title because it is the most widely recognised term for PE in the British pig industry (Anonymous 1995a). The term proliferative enteropathy replaced PIA as the preferred term in 1992 (Rowland and Lawson 1992) because it best describes the pathological basis for the various clinical forms of the disease. While this specification of an enteric disease in the title may have

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unintentionally biased the acceptance of positive results by the respondents, the need for recognition of the subject was considered vital.

The final questionnaire consisted of 27 questions on nine sides of A4 paper (Appendix B). The questions were evenly spaced out, as studies by Childers and Ferrell (1979) indicated that the response rate is not always proportional to the number of pages, while Vaillancourt et al (1991) advise spacing questions out to make the questionnaire look easier to complete.

The questionnaire was split into four sections under the following headings: PIA, Farm Details, Birth to Finishing, and Sows & Gilts. Six questions were closed, with every possible answer listed, e.g.

Q1. At what age do you wean your piglets? 21 days or less   ☐

 (Please tick one box)   22-28 days   ☐

             29-35 days   ☐

             36-42 days   ☐

           43 days or more   ☐

Five were semi-open, with a space for the farmer to write his own answer if none of those listed were suitable, e.g.

Q3. Where do your replacement gilts None stocked   ☐

  come from?   Homebred   ☐

  (Please tick one or more boxes) Multiplier herd   ☐

           Nucleus herd   ☐

       Other, please state.....................................

Five questions were split into two or more parts. These related to the medication of feed and water in different age-groups of pigs. In each case, the first part of the question was

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closed, requiring either a yes or no answer, while the second part asked the farmer to clarify his answer by stating the name(s) of the drug(s) used. Three questions asked for numerical answers for completion in a box or on a dotted line. For example:

Q 2: "How many sows do you have?" (Please write the number on the dotted line).

A table format was chosen for seven of the questions, as this was the most compact and simplest way to ask similar questions about different age-groups of pigs, for example

Q5. How is water given to your pigs? (Please tick which applies to each group of pigs. More than one box may be ticked for each group.)

	WEANERS	GROWERS	FINISHERS
INDIVIDUAL TROUGH			
GROUP TROUGH			
NIPPLE DRINKER			
OTHER, PLEASE STATE			

As in the above table, four of these questions were semi-open. Two were completely closed and one table, aimed at medication, was closed but provided a box for the farmer to tell us the drug(s) used.

Finally, the last question asked the farmer to provide or confirm his name and address if he was interested in further involvement with the research. Each questionnaire was given an individual identification number. This also enabled establishment of the farm's region if the farmer chose not to give his address.

Period prevalence of proliferative enteropathy

To ascertain the occurrence of proliferative enteropathy, I asked whether it had been diagnosed on the farm in the last three years, to give the 1993 to 1995 period prevalence.

Farmers were asked who had diagnosed it and how it had been diagnosed. Respondents were asked to indicate, from a list of clinical signs typical of acute and chronic PE, which signs they had observed on their unit.

Risk factors

The following potential risk factors were investigated: type of farm (e.g. nucleus, multiplier, breeder/weaner etc.), size of unit (based on number of sows), source of replacement breeding stock, age at weaning, feeding/medication of creep feed and regimes used to remove faeces. Also, the methods used to provide water to all age-groups (including sows and gilts), and feed to the younger stock were analysed, along with medication practices for each age-group. The types of flooring and flow of pigs through a building were explored (all-in all-out versus continuous flow on both a building level and pen level), as was the frequency of mixing batches of pigs at the weaner, grower and finisher stages of production. Finally, the herd's disease status was analysed, with particular attention to enzootic pneumonia, coccidiosis, swine dysentery and porcine reproductive and respiratory syndrome (PRRS). Three additional diseases were included with this list, specifically mange, erysipelas and parvovirus, to present a varied selection to the producer and thus avoid influencing his reply.

Analysis of Results

Statistical significance

EpiInfo version 6 (Center for Disease Control, Atlanta, USA) was used for the analysis (Appendix B). This is a computer package designed for epidemiological studies and consists of several programmes, allowing questionnaire results to be entered into a data file prior to the analytical stages. For the initial analysis of response rate and three year period prevalence, all the returned questionnaires were included.

To establish a potential relationship between an exposure and the occurrence of PE, the relative risk was calculated. The relative risk gives the best indication of the strength of an association between a risk factor and the disease under study and can be estimated from nearly all study designs, including cross-sectional studies and cohort studies (Vraa-Andersen 1994). The relative risk (RR) is defined as the ratio of the incidence of disease among the exposed to the incidence among the non-exposed (Kirkwood 1988).

For categorical variables, the statistical significance of each relationship was established using the χ^2 test (Yates corrected) on contingency tables which were created by cross-tabulating the exposure in question against the occurrence of PE. In EpiInfo, the statistical significance for continuous variables is calculated using either the analysis of variance (ANOVA) or the Kruskal-Wallis test. Analysis of variance is a parametric test applied to normally distributed sets of data which also have similar variances. The Kruskal-Wallis test is the non-parametric alternative and is the desired method if the two sets of data in question do not have similar variances, whether normally distributed or not. In this study, the only continuous variable was the number of sows. Although normally distributed, the variance between PE positive and negative units was different, therefore the Kruskal-Wallis test was used to calculate the significance of associations.

In the analysis, factors were classed as significant when the probability of an association being due to chance alone was less than 0.05 ($p<0.05$).

Confounding and interaction

The possibility of confounding was considered in the analysis. Confounding is defined as the simultaneous effect which some covariates may have during the analysis of risk caused by one variable. A variable is described as a confounder when it is related to both the variable of interest and the groups being compared (Kirkwood 1988).

The resultant problem of confounding bias is the only type of bias which can be overcome at the analysis stage, providing both the confounder and risk factor have been measured. The two other types, selection and information bias, must be considered during the sampling process and in the design of the questions. In the analysis, confounding is overcome by performing stratified analysis to create separate two-by-two tables and applying the Mantel-Haenszel χ^2 test and weighted odds ratio or summary relative risk. Stratified analysis allows EpiInfo to analyse the risk associated with an exposure factor with and without the presence of the potential confounding factor. Comparison of the weighted relative risk with the crude relative risk will indicate if there is possible confounding or not.

However, stratified analysis does have disadvantages. Firstly, it relies on the initial identification of possible confounders, which may not be immediately apparent. Secondly, in creating separate contingency tables in stratified analysis, there may only be

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a small number of subjects in each strata, leading to unreliable estimates of associations. In some cases, this low number may preclude statistical calculation of risk altogether. Interaction occurs when two exposure factors act together to increase or decrease the relative risk above or below their expected combined effect. EpiInfo tests for interaction during stratification of variables.

Validation

Validity is a measure of the degree to which answers from the sample population reflect the real truth in the general population (Thrusfield 1995). It gives an indication of how well systematic error has been avoided (Vraa-Andersen 1994). Systematic error can stem from poor sample selection (which in turn may lead to a poor response rate), but it can also arise from the presence of unidentified confounders or from what is termed information bias, where questionnaires are either faulty or misunderstood. Respondents may miss questions altogether or even deliberately give the wrong answer, particularly if the subject is a sensitive one, or they feel they have to give a "right" answer (Kirkwood 1988). Validity can be assessed in two ways: internal validity and external validity.

Internal validity

This is the validity of the deductions made as they relate to the subjects within the study. For example, internal validation was performed in this survey by comparing the frequency distribution of herd size seen in different areas of the country (based on the number of sows). The distribution pattern in England was compared to the pattern in Scotland. The majority of the questionnaires in Scotland had been distributed in a blanket manner directly to farms, while the greater portion of questionnaires delivered in England were distributed via veterinary surgeons, so this comparison gave us the opportunity to highlight any major differences between the populations, which may have been due to the selection procedure.

External validity

This is the validity of the deductions made to the general population. Census figures for the number of breeding pigs by size groups in Scotland were obtained from the Scottish Office Agriculture, Environment and Fisheries Department in Edinburgh for June 1995 and the overall frequency of herd sizes, based on sow numbers, were compared to the

survey results for Scotland. Similar data from the Ministry of Agriculture Fisheries and Foods (MAFF) statistics unit in York were obtained for England and Wales and compared to the survey results for England. An overall combination for the two sets of data was compared to the overall survey results for Britain.

RESULTS

Response Rate

From 569 questionnaires distributed, 319 were returned, giving an overall response rate of 56%. This varied from 54% to 66.8% as outlined in Table 4.1. In East Anglia, a relatively small number of questionnaires (n=20), distributed by a leading veterinary surgeon, gave a higher response rate of 85%. Of the 319 replies, 51 of the farmers no longer kept pigs so could not take part in the study. Sixteen were unsure of their PE status and a further four only completed the first two questions, so could not be used in the risk analysis.

Three Year Period Prevalence

83 of the respondents had had proliferative enteropathy diagnosed in their herd in the last three years, which gave a three year prevalence of 31%. As with the response rate, this varied according to region of the country (26% to 53%). The 17 respondent questionnaires from the Norfolk area were excluded from the later risk analysis because of a suspected bias caused by the high response and prevalence rates. Of these 17 farms, nine reported an episode of PE (53%).

Eighty-seven out of the 569 questionnaires had been distributed in smaller batches, usually to practising veterinary surgeons, but also to pig veterinary surgeons working within veterinary investigation laboratories or, in one case, a breeding company. However, the response rates in these cases were very poor, and often none were returned at all. Those which were returned (n=5) were included in the risk analysis but could not be used in a regional comparison of response rate or three-year prevalence rate (Table 4.1). The final risk analysis was therefore based on 231 of 552 (41.8%) questionnaires.

TABLE 4.1   Response rate and three year period-prevalence in main regions analysed

County/Country	Number distributed	Number returned	Response rate	Three year period prevalence
Scotland	262	180	68.8%	29.6%
Yorkshire	100	54	54%	26%
Suffolk	100	63	63%	31.6%
Norfolk	20	17	85%	53%

Note:

The above table does not include the 87 questionnaires which were distributed in small batches of 5 to 20 to veterinary surgeons in scattered regions of the country.

Diagnosis

To investigate methods of diagnosis, 74 (analysis A) or 83 (analysis B - possible sampling bias) PE-positive questionnaires were analysed. In analysis A, 88% of outbreaks had been diagnosed by a veterinary surgeon, whether by a private veterinary surgeon or by a veterinary surgeon at a private or government laboratory. 12% were diagnosed by the farmer, without veterinary help. In analysis B, a veterinary surgeon was responsible for 84% of diagnoses, while the farmer diagnosed 14.4% of outbreaks without veterinary help. Table 4.2 outlines the separate results of these analyses.

Clinical Signs

Clinical signs are often used as an initial indication of the existence of PE and the two forms of the disease are very different in the profile of signs presented. The clinical signs of chronic PE, diarrhoea and weight loss, were noted in 53% of incidents. Reduced weight gains and reduced appetites were observed in 45% and 27% of incidents, respectively. The signs of acute PE, bloody diarrhoea and sudden death occurred individually in 34% and 40.5% of incidents, respectively. These clinical signs were exhibited together in 57% of incidents, although accompanied by other signs, and in 9.5% of incidents, unaccompanied by other signs. In these latter cases, five were diagnosed at necropsy, while the remaining two were diagnosed by a veterinary surgeon. All seven herds exhibiting typical signs of proliferative haemorrhagic enteropathy were breeder/finisher and the pattern of herd size was evenly distributed, ranging from 175 to 920 sows.

In the nine responses suspected of sampling bias, the proportions of sudden death and haemorrhagic diarrhoea were 100% and 55%, respectively, which increased these proportions overall when those nine questionnaires were included in the analysis (analysis B). Figure 4.1 shows the spread of clinical signs observed in both analyses. These findings suggested the possibility that the nine units concerned had been selected on the basis of a recent diagnosis of PE by the veterinary surgeon who had distributed the questionnaires for us. Therefore, the suspicion of selection bias still remained and, for this reason, analysis A was used to investigate potential risk factors.

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TABLE 4.2 Individuals responsible for the diagnosis of PE

Variable	Analysis A *n = 74*	Analysis B *n = 83*
Veterinary surgeon alone	44 (59%)	50 (60.2%)
Producer alone	9 (12%)	12 (14.4%)
Laboratory alone	14 (19%)	13 (15.7%)
Vet* & Producer	3 (4%)	3 (3.6%)
Vet* & Laboratory	2 (2.7%)	2 (2.4%)
Producer & Laboratory	1 (1.3%)	1 (1.2%)
Producer, Vet* & Laboratory	1 (1.3%)	1 (1.2%)
		One non-reply

Note:

* vet = veterinary surgeon

FIGURE 4.1

Comparison of distribution of clinical signs between analyses A and B
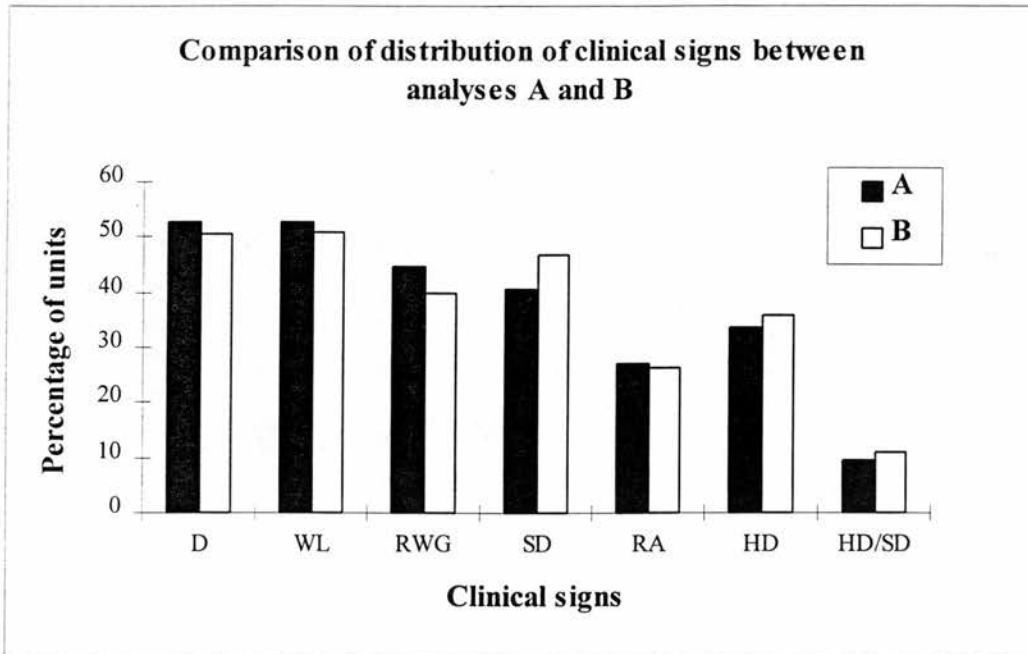
D = diarrhoea

WL = weight loss

RWG = reduced weight gains

SD = sudden death

RA = reduced appetite

HD = haemorrhagic diarrhoea

HD/SD = haemorrhagic diarrhoea and sudden death (no other accompanying symptoms)

Figure 4.2 illustrates that, in the final analysis of 74 questionnaires, 70.2% of outbreaks had been diagnosed using post-mortem techniques, with a veterinary surgeon responsible for over two-thirds of the diagnoses which had been based on the grounds of clinical signs alone.

Other Risk Factors

Herd size

Herd size was based on the number of sows kept. The average number of sows in herds where PE had been recorded was 343 (range 0 to 2,600). In those herds where PE had not been diagnosed, the average number of sows was 197 (range 0 to 1800). This indicated a strong association between larger herd size and the presence of PE. The number of sows was the only continuous variable in the questionnaire and, when finisher herds (no sows on farm) were excluded (n=14), it followed a Normal distribution. However, the variance between each population differed, so the Kruskal-Wallis test was used as the non-parametric alternative, to evaluate the significance of the difference between the means.

This test was performed initially including all herds, where the difference between the mean number of sows on PE positive and PE negative units was significant at $p < 0.0005$, but it was also used to analyse units with 50 sows or more, in order to avoid the influence of herds of grower-finisher pigs which had no sows. The difference between these means was still significant at $p < 0.005$. The full results of these tests are illustrated on Table 4.3.

The significance of herd size as a risk factor was further confirmed by creating a new variable, which divided herds into units of ≥ 500 sows or <500 sows. This allowed the production of a two-by-two contingency table to determine relative risk (RR) of this new variable in relation to the occurrence of PE. Significance was calculated using the χ^2 test for contingency tables. Again, this was performed including all herds ($p < 0.001$) and on herds with 50 sows or more ($p < 0.005$). The results of these tests are illustrated on Table 4.4.

FIGURE 4.2 Method of Diagnosis

Clinical
signs (self)
9.5%

Clinical
signs (vet)
20.3%

Postmortem
70.2%
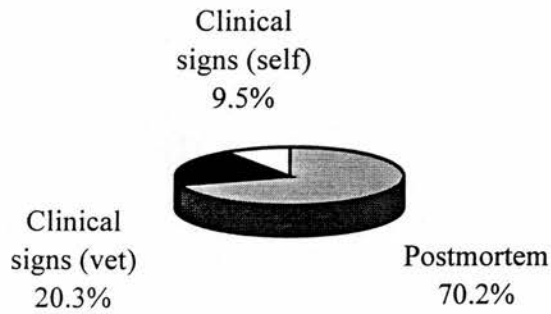
TABLE 4.3 Analysis of herd size (Kruskal-Wallis χ^2)

	All herds analysed	$\geq$50 sows only
PE positive median[†]	220	220
PE negative median[†]	150	200
χ^2 *	12.7	8.2
p value	p<0.01	p<0.01

Notes:

* Kruskal-Wallis χ^2

† median number of sows in herds

TABLE 4.4 Analysis of herd size as risk factor using χ^2

	All herds analysed	≥50 sows only
RR (range)	2.15 (1.51-3.06)	1.99 (1.39-2.85)
χ^2	10.36	8.32
p value	p < 0.001	p < 0.005

Nucleus herds

Nucleus herds were significantly associated with PE in two instances. Firstly, they were the only type of unit associated with an occurrence of PE (p<0.05). There were a total of six nucleus herds in the analysis and five of these had recorded an episode of PE. The only nucleus unit which had not recorded PE only had 80 sows. Secondly, although the source of breeding gilt did not appear to be an influential factor (p>0.05 for all categories), units which obtained replacement breeding boars from nucleus herds were more likely to have had PE in their pigs (p<0.05), with 43% (32/74) positive herds and 27% (37/135) negative herds acquiring replacement boars from this source.

Creep feed

Offering creep feed was not associated with PE, with 64% of PE positive herds and 67% of PE negative herds giving their piglets creep feed prior to weaning. The medication of creep feed was neither a risk factor nor a protective factor. 29 of 47 (61%) PE positive herds medicated their creep feed, compared to 49 of 92 (53%) PE negative herds. 21 of 29 (72%) PE positive and 39 of 49 (80%) PE negative herds stated the drug they used for incorporation into creep feed. The main antibiotic used by both PE positive and negative herds was chlortetracycline (57% and 64% of PE positive and PE negative herds, respectively). Of those farms which medicated creep feed with antibiotics other than chlortetracycline, no difference was observed between positive and negative herds in the general trend or pattern of drugs incorporated. Table 4.5 illustrates these results.

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TABLE 4.5 Comparison of drugs used for incorporation into creep feed on PE positive and PE negative herds

ANTIBIOTIC INCORPORATED	PE POSITIVE HERDS	PE NEGATIVE HERDS
Chlortetracycline*	62%[†]	66.6%
Lincomycin	9.5%	10.2%
Trimethoprim/ Sulphonamide	14.3%	2.6%
Tylosin	0%	5%
Other	14.3%	15.5%

Notes:

* chlortetracycline on its own or in combination

† this is the number of units incorporating each antibiotic as a percentage of those who stated a drug

Medication of Stock

The medication of weaner and finisher feedstuffs was not linked to the occurrence of PE. Similarly, the medication of sows and gilts, either in water (none of the sows were medicated via water, gilts p>0.05) or in feed (gilts and sows p>0.05), had no effect on the presence of PE. However, as illustrated in Figure 4.3, PE negative herds were almost twice as likely to have never medicated their growers' feed than PE positive herds (p<0.05).

Weaners

Chlortetracycline was the most frequently used drug for addition to weaner rations. 63.5% and 62.5% of PE positive and PE negative herds used it all the time, 80% and 70%, respectively, used it occasionally and 76% and 62%, respectively, used it regularly. The differences in drug usage between PE positive and PE negative herds were not significant. Lincomycin, trimethoprim/sulphonamide and tylosin were the next most frequently used drugs and, although there were some differences in their inclusion between herds which had recorded episodes of PE and herds which had not, the numbers involved were too small to reliably assess the significance of these differences (Tables 4.6, 4.7 and 4.8).

Growers

A similar pattern occurred in the growers, with chlortetracycline the most frequently incorporated drug, although tylosin was also used for regular or permanent incorporation. Similar patterns within these two regimes (regular or permanent use) were observed both in PE positive and negative herds. However, the addition of chlortetracycline on an "occasional" basis did point to some differences, with 94% (15/16) and 58% (24/41) of PE positive and negative herds, respectively, medicating their growers in this way. This difference between PE positive and negative herds was significant (p<0.05). Although a higher proportion of PE negative herds tended to use chlortetracycline on a permanent ("all the time") basis, the numbers were too small to test for any statistically significant difference (Tables 4.6, 4.7 and 4.8).

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FIGURE 4.3 Comparison of medication regimes used in the grower stock of PE

positive and PE negative herds
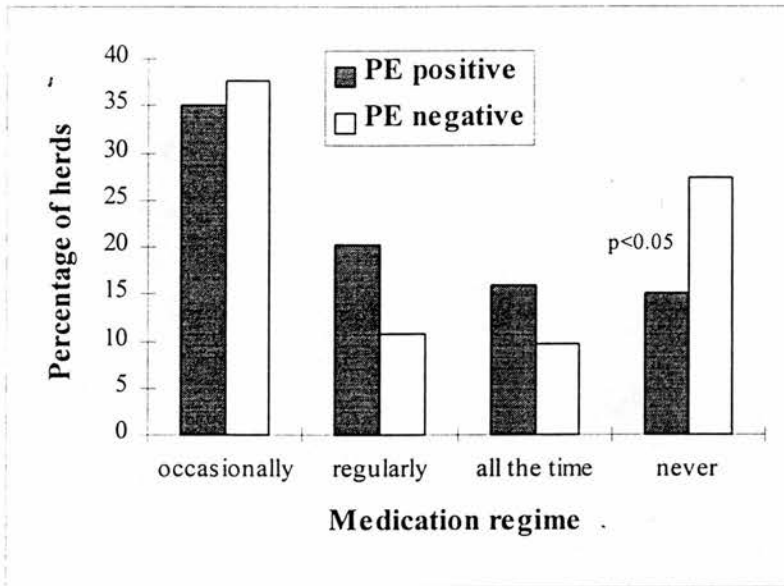
Finishers

The general trend in the weaner group was repeated in the finisher group, with few differences between PE negative and PE positive herds. Although chlortetracycline was still frequently used, tylosin replaced it as the medication most frequently incorporated. Again, the numbers involved were too small to test for any statistically significant difference (Tables 4.6, 4.7 and 4.8).

Housing

Floor type

Fully slatted ($p<0.05$) and fully meshed ($p<0.01$) floors in the weaner accommodation were associated with an increased prevalence of PE. When these two variables were combined to form one category (full slats/mesh), this was a highly significant risk factor ($p<0.0005$). Conversely, these herds were less likely to use straw ($p<0.0005$) or solid flooring ($p<0.001$) in their weaner accommodation (Table 4.9) *i.e.* straw appeared to have a protective effect.

Similarly, when fully meshed and fully slatted floors were grouped into one variable in the grower accommodation, this was also significantly linked to PE ($p<0.05$). In the finisher accommodation, both fully slatted ($p<0.05$) and partially slatted ($p<0.05$) floors were individual risk factors. When combined to form one category (slatted floors), this risk factor increased substantially ($p<0.01$) and again, straw was less likely to be used ($p<0.05$) for finisher pigs in PE positive herds, with an apparent protective effect in PE negative herds.

Nipple drinkers

The use of nipple drinkers was highly associated with an increased occurrence of PE in both growers and finishers, while in the weaner accommodation, there was only a marginal association (Table 4.10).

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TABLE 4.6 Pattern of drugs permanently incorporated ("all the time")

Drug	Weaners		Growers		Finishers	
	PE + *n=22**	PE - *n=32**	PE + *n=11**	PE - *n=14**	PE + *n = 7**	PE - *n=6**
Chlortetracycline	14 (63.5)	20 (62.5)	4 (33)	8 (57)	1 (14)	1 (16.7)
Lincomycin	1 (4.5)	3 (9.4)	-	-	-	-
Trim/Sulph†	5 (23)	2 (6.3)	-	-	-	-
Tylosin	-‡	3 (9.4)	1 (9)	3 (21)	4 (57)	3 (50)
Others	2 (9)	4 (12.5)	6 (54.5)	3 (21)	2 (28)	2 (33)

Notes:

*   the number in brackets is the respondents incorporating each drug shown as a percentage of those   who stated a drug

†   trim/sulph = trimethoprim sulphonamide

‡   one respondent used tylosin in combination with chlortetracycline

-   not stated

TABLE 4.7 Pattern of drugs incorporated on a regular basis

Drugs	Weaners		Growers		Finishers	
	PE + *n=17**	PE - *n=29**	PE + *n=13**	PE - *n=12**	PE + *n=4**	PE - *n=11**
Chlortetracycline	13 (76)	18 (62)	6 (46)	7 (58)	1 (25)	4 (36)
Tylosin	0 (0)	4 (14)	3 (23)	3 (25)	2 (50)	6 (54.5)
Others	4 (24)	7 (24)	4 (31)	2 (17)	1 (25)	1 (9)

Note:

*   the number in brackets is the respondents incorporating each drug shown as a percentage of those   who stated a drug

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TABLE 4.8 Pattern of drugs incorporated on an "occasional" basis

Drug	Weaners		Growers		Finishers	
	PE + *n=10**	PE - *n=17**	PE + *n=16**	PE - *n=41**	PE + *n=15**	PE - *n=22**
Chlortetracycline	8 (80)	12 (70)	15 (94)	24 (58.5)	8 (53)	9 (41)
Lincomycin	-	-	-	4 (10)	2 (13)	2 (9)
Tylosin	-	-	-	-	4 (27)	5 (23)
Trim/Sulph†	-	-	-	3 (7)	1 (7)	1 (4.5)
Others	2 (20)	5 (30)	1 (6)	10 (24)	0 (0)*	5 (23)

Notes:

*   the number in brackets is the respondents incorporating each drug shown as a percentage of those   who stated a drug

†   trim/sulph = trimethoprim sulphonamide

‡   a small number reported using other drugs (penicillin and apramycin) in combination with tylosin and chlortetracycline

-   not stated

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TABLE 4.9 Significance of floor type

Age group	Type of floor	RR (range)*		p value
Weaners	Full slats	1.57	(1.08-2.3)	p<0.05
	Full mesh	1.7	(1.2-2.5)	p<0.01
	Full slats/mesh	2.2	(1.5-3.2)	p<0.01
	Straw	0.43	(0.27-0.69)	p<0.01
Growers	Full slats/mesh	1.6	(1.1-2.4)	p<0.05
Finishers	Full slats	1.8	(1.2-2.6)	p<0.05
	Partial slats	1.6	(1.07-2.25)	p<0.05
	Full/partial slats	2.43	(1.6-3.8)	p<0.01
	Straw	0.6	(0.4-0.88)	p<0.05

* 95% confidence interval

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TABLE 4.10 Significance of nipple drinkers

Age Group	RR (range)	p value
Weaners	1.85 (0.97-3.52)	p=0.06
Growers	1.97 (1.15-3.37)	p<0.01
Finishers	3.85 (1.66-8.95)	p<0.01

Grouping of pigs and waste removal

The use of all in-all out regimes had no effect on the occurrence of PE when carried out on a pen basis. All-in all-out on a house basis in the grower accommodation, however, did reduce the likelihood of PE (p<0.05).

There was also a difference between PE positive and negative herds in their faecal waste removal methods. Slurry systems were consistently used in PE positive herds in all three age-groups studied *i.e.* weaners (p<0.05), growers (p<0.01) and finishers (p<0.01). The other methods of waste removal, automatic, manual and "other" were not linked to PE, although the manual removal of faeces in negative herds appeared to have a protective effect (RR= 0.56, p<0.05).

Other factors recorded or measured in questionnaire

The following factors did not have a direct relationship with PE:

- age at weaning
- frequency of mixing pigs
- feeding creep feed with or without medication
- movement of pigs on a pen basis only
- methods of administering water to any age-group of pig, barring nipple drinkers in the finishing accommodation
- methods of feeding any age-group of pig
- medication of sow or gilt feed
- medication of sow or gilt water

Herd health

When farmers were asked about the existence of other diseases on their units, both enzootic pneumonia and PRRS were significantly linked to PE, with a significance of $p<0.01$ and $p<0.05$ respectively (Table 4.11). Coccidiosis was marginally significant ($p=0.07$), while swine dysentery, mange, erysipelas and parvovirus were not risk factors for PE. Enzootic pneumonia and PRRS also both had a similar significant relationship with herd size (Tables 4.12 and 4.13).

TABLE 4.11 Relationship between other diseases and PE

Disease	PE +ve *n = 74*	PE -ve *n = 137*	RR	(RR range)*	p-value
Enz. pneumonia	57 (77)	81 (59)	1.9	1.2-3.1	0.008
PRRS	44 (59.4)	61 (44.5)	1.53	1.04-2.25	0.04
Coccidiosis	16 (21.6)	16 (11.6)	1.6	1.04-2.35	0.07
Mange	34 (46)	82 (59.8)	0.7	0.48-1.02	0.08
Erysipelas	39 (52.7)	83 (60.6)	0.8	0.57-1.2	0.38
Swine dysentery	18 (24.3)	37 (27)	0.92	0.6-1.42	0.8
Parvovirus	26 (35.1)	45 (32.8)	1.08	0.74-1.6	0.8

* 95% confidence interval

TABLE 4.12 Relationship between selected diseases and herd size

Disease	RR (range)	χ^2	p value
PE	2.2 (1.5-3.06)	10.36	$p < 0.005$
PRRS	1.5 (1.2-2.06)	4.6	$p < 0.05$
Enzootic pneumonia	1.4 (1.2-1.7)	5.88	$p < 0.05$

TABLE 4.13 Relationship between selected diseases and herd size (herds with less than 50 sows excluded)

Disease	RR (range)	χ^2	p value
PE	2 (1.4-2.8)	8.3	$p<0.005$
PRRS	1.4 (1.1-1.9)	2.96	$p=0.08$
Enzootic pneumonia	1.4 (1.15-1.6)	4.4	$p<0.05$

Note:

Two categories were compared:

large herds were defined as having 500 or more sows

small herds were defined as having less than 500 sows

Confounding

Two variables, herd size and PRRS, not only had a significant effect on the occurrence of PE, but also on each other. For this reason, stratified analysis was undertaken to try to establish if either factor was confounding the other's relationship with the disease. The same analysis was required for enzootic pneumonia. In both cases, only herds with sows were included in the analysis *i.e.* finisher only herds were excluded. The results on Table 4.14 indicate that PRRS was only significantly linked to PE because of its relationship with herd size, which was also linked significantly to PE, *i.e.* herd size was a confounding factor for PRRS. In contrast, enzootic pneumonia appeared to be significantly linked to PE, regardless of herd size.

There was a small number of nucleus herds in the study but, since this type of herd is often large, the possibility that herd size was confounding the relationship between nucleus herds and PE was investigated. These results are illustrated in Table 4.14. Briefly, herd size was still significant ($p<0.01$) when nucleus herd was investigated as a possible confounding factor. In contrast, when herd size was analysed as a possible confounder, nucleus herds were no longer significant ($p>0.05$), suggesting that herd size was confounding the relationship between nucleus herds and the occurrence of PE.

There was a high correlation between nipple drinkers and slatted/meshed flooring. Of the 88 herds which housed their weaners on fully slatted or fully meshed flooring, 86 were also administering water via nipple drinkers, as were all 38 herds housing growers on partially meshed or slatted flooring, and 83 of 91 herds housing finishers on slatted flooring. This tended to hamper the investigation of confounding between these variables, due to marginal zero values (Table 4.15). Nevertheless, some general patterns emerged. In summary, although there was apparent confounding between nipple drinkers and slatted flooring in the weaners, fully meshed flooring was significant, independently of nipple drinkers in this age-group ($p<0.05$) and fully interrupted flooring (*i.e.* fully slatted and/or meshed flooring) was highly significant ($p<0.01$).

There was also a high degree of correlation between the use of slurry pit systems and fully slatted/meshed flooring. In the weaner accommodation, 82 of 86 herds using fully slatted/meshed flooring were also using a slurry system. In the growers, this ratio was 38 out of 38 and, in the finishers, 38 out of 39. In the weaner accommodation, slatted and/or meshed flooring was significantly linked to PE, independently of the existence of a slurry

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system (p<0.01). In contrast, the relationship between slatted flooring in the finisher accommodation and PE appeared to be confounded by the use of a slurry system.

The general pattern of these results indicated that fully interrupted flooring was more likely to be a risk factor when used in the weaner accommodation than in the grower or finisher accommodation (Table 4.16).

Interaction

Demonstration of interaction between herd size and PRRS, or between nipple drinkers and slatted flooring in the finisher accommodation was not possible. Interaction between other factors were investigated, including between nucleus herds and herd size and nipple drinkers and slatted or meshed flooring in the weaner accommodation but, due to small sample numbers, testing was again not possible.

Table 4.17 is a summary list of risk factors for PIA, including the relative risks and p values.

Univariate Analysis

Although univariate analysis may have helped to clarify the relative importance of certain risk factors in this study, the use of the regress command (regression analysis) in EpiInfo is not possible with non-numerical data. As most of my data was categorical, I was not able to study this particular area in greater depth.

TABLE 4.14 Stratified analysis for herd size and PRRS, enzootic pneumonia and nucleus herds

Exposure Factor	Possible confounder	Crude RR	Summary RR (95% C.I.)†		p-value*
Herd size	PRRS	2.1	1.94	(1.33-2.82)	p<0.01
Herd size	Enz. pneumonia	2.1	1.86	(1.28-2.72)	p<0.05
PRRS	Herd size	1.53	1.4	(0.95-2.07)	p>0.05
Enz. pneumonia	Herd size	1.9	1.74	(1.04-2.91)	p<0.05
Nucleus herd	Herd size	2.48	1.82	(1.24-2.67)	p>0.05
Herd size	Nucleus herd	2.15	1.95	(1.36-2.81)	p<0.01

Notes:

† 95% C.I. = 95% confidence interval given in brackets

* p-value of the Mantel-Haenszel chi-square for the summary RR (Appendix B)

TABLE 4.15 Stratified analysis for flooring and nipple drinkers

Exposure Factor	Potential confounder	Crude RR	Summary RR (95% C.I.)†		p-value*
Nipple (weaners)	Full slats	1.77	1.59	(0.86-2.95)	p>0.05
Full slats	Nipple (weaners)	1.56	1.44	(0.99-2.09)	p>0.05
Nipple (weaners)	Full mesh	-	-		p>0.05
Full mesh	Nipple (weaners)	-	-		p<0.05
Nipple (weaners)	Full slats/mesh	1.77	1.17	(0.63-2.2)	p>0.05
Full slats/mesh	Nipple (weaners)	2.17	2.01	(1.33-3.05)	p<0.01
Nipple (finishers)	Slats	3.86	3.06	(1.24-7.54)	p<0.01
Slats	Nipple (finishers)	2.82	1.85	(1.18-2.88)	p<0.01

Notes:

† 95% C.I. = 95% confidence interval given in brackets

- unable to test

* p-value of the Mantel-Haenszel chi-square for the summary RR (Appendix B)

TABLE 4.16 Stratified analysis of flooring and slurry systems

Exposure Factor	Possible confounder	Crude RR	Summary RR 95% C.I.[†]		p-value*
Slats/mesh (weaners)	Slurry	2.2	2.0	(1.27-3.15)	p<0.01
Slurry	Slats/mesh (weaners)	1.68	1.0	(0.64-1.64)	p>0.05
Slurry	Part mesh/slats (growers)	1.64	1.71	(1.17-2.5)	p<0.05
Part mesh/slats (growers)	Slurry	1.08	0.8	(0.56-1.15)	p>0.05
Slats (finishers)	Slurry	2.42	1.26	(0.75-2.11)	p>0.05
Slurry	Slats (finishers)	2.73	1.82	(1.06-3.11)	p<0.05

Notes:

† 95% C.I. = 95% confidence interval given in brackets

* p-value of the Mantel-Haenszel chi-square for the summary RR (Appendix B)

TABLE 4.17 Summary of risk factors for PIA in Great Britain

Significant Factors	RR (range)*	p-value
Large herds (>499 sows)	1.99 (1.39-2.85)	p<0.005
Nucleus herds as source of breeding boars	1.55 (1.08-2.21)	p<0.05
Growers never medicated	0.55 (0.31-0.98)	p<0.05
Fully slatted floors (weaners)	1.57 (1.08-2.29)	p<0.05
Fully meshed floors (weaners)	1.7 (1.19-2.46)	p<0.01
Straw bedding (weaners)	0.43 (0.27-0.69)	p<0.001
Full slats (finishers)	1.77 (1.22-2.56)	p<0.05
Part slats (finishers)	1.55 (1.07-2.25)	p<0.01
Enzootic pneumonia	1.9 (1.16-3.1)	p<0.01
Batch movement of growers by house	0.49 (0.25-0.97)	p<0.05
Slurry system in growers	1.8 (1.17-2.76)	p<0.01
Slurry system in finishers	2.93 (1.77-4.85)	p<0.001
Manual removal of faeces in weaner house	0.56 (0.33-0.97)	p<0.05

Note:

* 95% confidence interval

Validity

Internal validity

Figure 4.4 illustrates the comparison of herd size in England and Scotland obtained from the survey results. While the general trend was similar, there was some variation. In Scotland, there was a higher percentage of smaller herds in the sample than there was in the sample from England. The same comparison was made between Scottish and English/Welsh herds in the census results (Figure 4.5). In this case, the trends were closer, and there was a higher proportion of small herds (under 50 sows) in each population. In the general census of Scotland, 44% (219/493) of herds had below 10 sows and 60.4% (298/493) of herds had under 50 sows, while in England and Wales, these same figures were 43% (2579/6014) and 65% (3911/6014), respectively.

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External validity

An overall comparison was made between the herd size distribution in the survey and the distribution in the combined census of Great Britain. This helped to assess how the survey sample related to the general population and indicated that a higher proportion of larger herds had been included in the survey than appeared in the census. Again, this was caused by the apparently very high number of small units (*i.e.*<50 sows) currently operating in Britain. Consequently, an overall comparison was made on two levels. Firstly, including all sizes of unit (Fig 4.6) and, secondly, excluding units with under 50 sows (Fig 4.7). The distribution pattern still showed that the survey sample included a higher proportion of larger herds.

FIGURE 4.4

Internal validation - comparison of herd size in England and Scotland (survey)

NR = no reply

FIGURE 4.5

Comparison of herd size in England/Wales and Scotland (census)

FIGURE 4.6

Comparison of herd size between survey and census results (Great Britain)

FIGURE 4.7

Comparison of herd size between census and survey in Great Britain (herds <50 sows excluded)
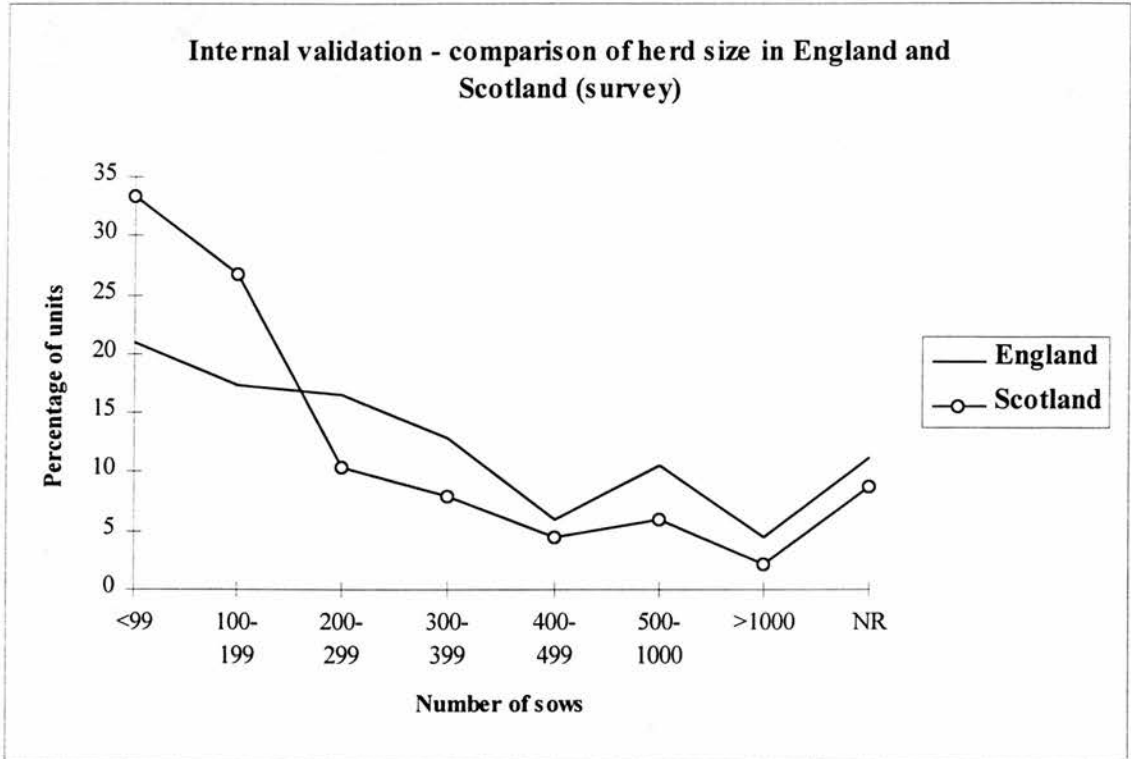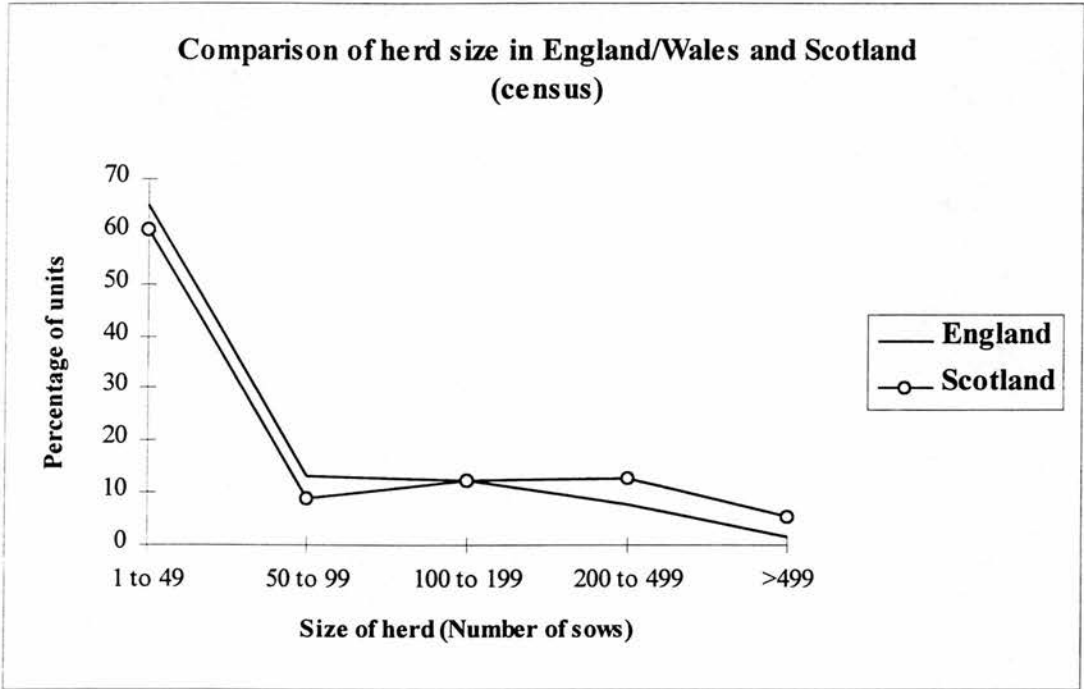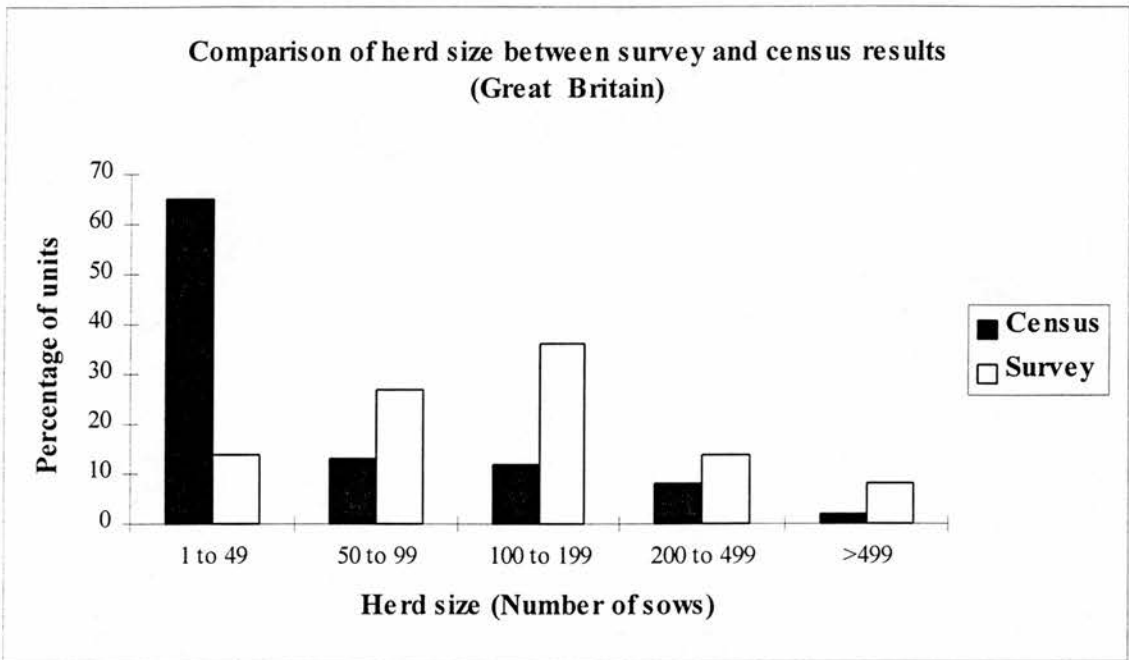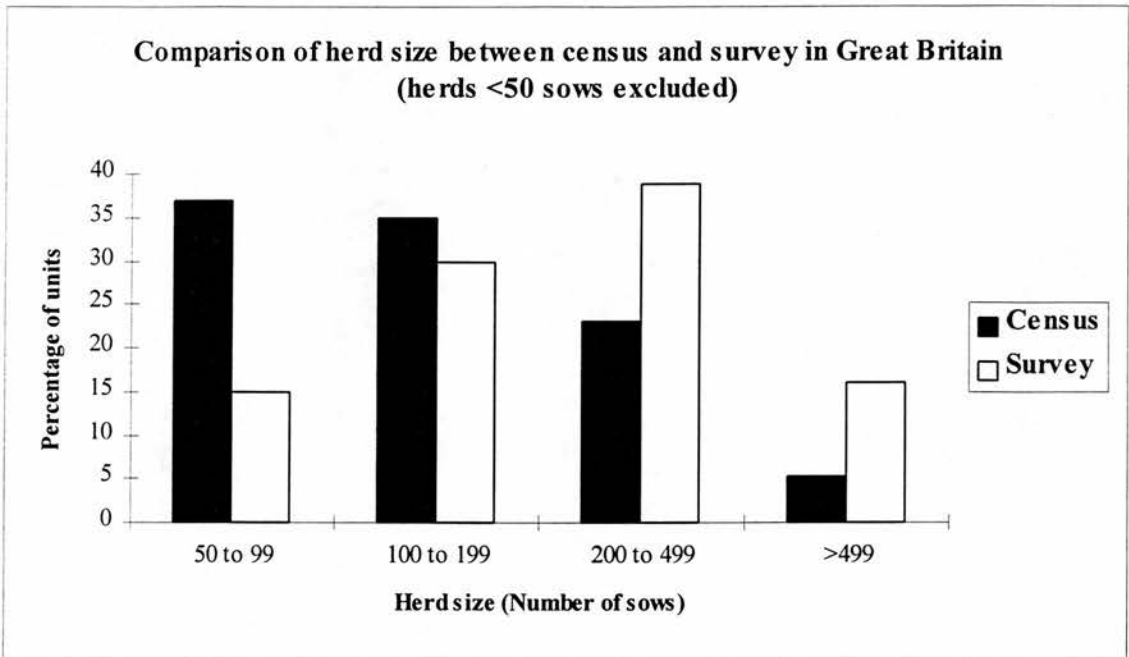
DISCUSSION

This survey is the first to specifically investigate prevalence and potential risk factors of proliferative enteropathy in Great Britain. It indicates that the 1993 to 1995 period prevalence of PE in British herds was 31%, with some regional variation. Although the questionnaire asked whether or not the disease had been diagnosed in the previous three years, this percentage could not be considered an incidence rate as this would imply that all cases had first appeared on the farm around the time of diagnosis. The problems associated with clinical recognition and diagnosis of PE, particularly in its chronic form, and the strong possibility that it may be present on a unit for years prior to diagnosis (Roberts et al 1979), means that it is more accurate to describe this rate as a prevalence. Similarly, clinical recognition by non-veterinary surgeons, *i.e.* self (producer) diagnosed outbreaks, should be treated with caution, since the proliferative enteropathies could be confused with conditions such as swine dysentery or post-weaning *E. coli* diarrhoea.

Outbreaks of infectious disease are generally more severe, of longer duration and more difficult to control in larger herds (Alexander and Harris 1992). In this study, herd size was strongly associated with PE. The impact of some infectious diseases can increase with the size of the unit, such as transmissible gastro-enteritis (Cubero et al 1993), probably due to an increased frequency of pig-to-pig contact, although stocking density does not necessarily increase with increasing herd size. Larger herds may also have a greater number of naive stock which are capable of sustaining infection and transmitting disease, while infections in smaller herds are more likely to manifest as epidemics which die out as the availability of susceptible animals decreases. Enzootic pneumonia and PE status were linked, independently of herd size, while the link between PE and PRRS was probably because of the association of both variables with herd size. The PRRS arterivirus can reduce the number of circulating lymphocytes and neutrophils, as well as lymph node cell populations (Christianson and Joo 1994), therefore it may still have a direct influence on herd immunity and susceptibility to other diseases, such as PE.

Introducing infected stock from external sources, particularly replacement breeding stock, may result in the introduction of new infections. It is an important epidemiological risk factor for the transmission of *Serpulina hyodysenteriae* between herds (Harris 1984). My survey suggested an important link between PE and nucleus herds. Stratified analysis indicated that herd size was probably confounding the relationship between nucleus herds

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and PE but only six nucleus herds were analysed in the survey. Analysis of a greater number of nucleus herds may clarify this relationship further. Nevertheless, five out of six nucleus herds had experienced PE and this link was further supported by the independent relationship found between PE outbreaks and acquisition of replacement breeding boars from nucleus herds. Source of replacement breeding stock has previously been associated with an increased risk of PE. Ward and Winkelman (1990) stated that acute PE tended to occur in closed herds, while Rowland and Lawson (1992) reported an incidence of 0.25% in gilts and boars on UK testing stations. A recent increase in the number of acute PE cases in high health herds supports these findings (Boeckman 1995).

In establishing their nucleus herds, breeding companies aim to prevent entry of infectious agents into the nucleus to protect both their clients' herd health interests and their own reputations. Primary specific-pathogen-free pig production, isolated farrowing and medicated or segregated early weaning are all practices which have led to the production of immunologically naive pigs, which are susceptible to a wide range of pathogens (Dee 1997), many of which are strictly monitored in specific disease control programmes, for example atrophic rhinitis and enzootic pneumonia (Alexander and Harris 1992). It has been proposed, particularly in viral diseases, such as transmissible gastro-enteritis, that new animals may represent susceptible stock which encourage re-emergence of subclinical disease already present in a herd (Siegel et al 1991). Replacement gilts are often bought in on a regular basis, possibly preventing immunological stability in the breeding herd. Although it is tempting to suggest that boars from nucleus herds encourage re-emergence of subclinical PE because they are immunologically naive, it is highly unlikely that the introduction of one new susceptible animal would be responsible for such an outbreak. On the contrary, it is more likely that the introduction of an otherwise healthy boar, which is excreting *Lawsonia intracellularis* in its faeces, may be the source of new infections.

My results indicate that PE may be present in some nucleus herds, possibly at a subclinical level. If so, then replacement animals from these herds could be responsible for transferring *L. intracellularis* infection to new herds. Robertson et al (1992) found that the source of replacement breeding stock and purchase of growers could affect the occurrence of swine dysentery and concluded that *S. hyodysenteriae* could be introduced into a clean herd via carrier pigs. Therefore, replacement of breeding stock by a single source of hysterectomy-derived pigs, monitored for *S. hyodysenteriae*, may reduce the occurrence of swine dysentery. In PE, the stress of movement and change of environment

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might trigger excretion for a sufficiently long period of time to allow spread to other animals in the herd. This is potentially an important epidemiological hypothesis which merits further investigation. Confirmation that such a transmission pathway is taking place would probably require long-term monitoring of young stock and boars leaving the herd, preferably incorporating PCR.

The immediate environment of the pig, including bedding, composition of flooring and ventilation are all possible determinants of disease in pigs, ranging from foot lesions to behavioural problems. Management operations which increase pig-to-faeces contact can also influence the transmission of faecally-spread enteric diseases. Apart from source of breeding stock, the other most important epidemiological findings in my survey were the associations between PE and floor type, faecal waste removal methods and movement of pigs, factors which could potentially play a major role in pig-to-faeces contact. The hypothesis that slatted or meshed flooring is a risk factor for PE is surprising, but interesting, since this type of flooring has been designed to try to minimise the amount of time spent cleaning and should reduce the level of faecal material on a pen floor. Nevertheless, the success of these floors requires adequate cleaning between batches of pigs. It is possible that farmers over-rely on the efficiency of interrupted flooring, while those who house pigs on solid floors or straw are more aware of the need for better hygiene, using mechanical or automatic means of cleaning, rather than a slurry system, to remove waste faeces. The success of slatted or meshed flooring also relies on good design. Too wide a slat may act almost like a solid floor, allowing accumulated faecal material to lie on the surface. Similarly, if the spaces provided by a mesh floor are too narrow in diameter, they may not allow faeces of a more solid consistency to pass through into the pit below. The high correlation between slatted/meshed flooring and nipple drinkers or slurry systems observed in the survey results reflects the general design of a pig unit. Stratification helped to clarify to a certain extent that flooring was the main risk factor rather than nipple drinkers. The risk associated with interrupted flooring appeared to be more significant in the weaner accommodation than in the growers or finishers, where there was a greater likelihood of confounding by slurry systems and nipple drinkers. While the high correlation which existed among these design features may have obscured their relationships, it seems that floor type is important, at least in the younger age-groups, where they are perhaps first being exposed to *L. intracellularis*. It is also possible that poorly designed slatted or meshed flooring places increased stress on weaners during a

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period when they are already physiologically stressed by movement, weaning and changes in nutrition.

The association between increased pig-to-faeces contact and occurrence of PE was further supported by the significantly higher proportion of PE negative herds using all-in all-out methods on a house basis for their growers. In the general management of pigs, emphasis is often placed on routine strict hygiene in the farrowing crates and early weaner accommodation. Older pigs are often not treated in the same way, either due to lack of time and manpower or because a continuous flow system makes it impossible to carry out thorough cleaning procedures. This survey suggested that the all-in all-out movement of pigs only helped to reduce PE if it was conducted on a house basis and not just a pen basis. The main reason for this is likely to be that thorough disinfection, incorporating power washing, fumigation and resting of pens is only feasible when all the pigs have been cleared from the building and when building design allows it.

Recent controlled challenge trials have demonstrated the efficacy of certain antimicrobials in the treatment of PE (McOrist et al 1996, McOrist et al 1997). Prior to this, treatment was based on circumstantial evidence or previous experience. Reports of successful field therapy were often confusing, largely because it is difficult to ascertain the exact treatment protocols which have been used against diseases, such as PE, at the farm level. In my survey, it was only possible to attribute statistical significance to feed medication in the growers, although some trends were observed in the other age-groups. PE negative herds were significantly more likely to have never medicated their growers' feed, further illustrating that herds which had recorded PE were more likely to require treatment. The widespread incorporation of chlortetracycline is not surprising, as it is an inexpensive drug, easy to incorporate and specifically recommended in the treatment of common respiratory conditions, such as *Mycoplasma hyopneumoniae* infection (enzootic pneumonia) and pneumonic pasteurellosis (Andrews et al 1988), as well as PE.

In the grower accommodation a significantly higher proportion of PE positive herds ($p<0.05$) reported "occasional" medication with chlortetracycline, suggesting that it was being used to treat infections as they arose and perhaps that the treatment had not been administered for a sufficient period of time or at the correct dose rate. Holyoake and Cutler (1995) reported a relationship between the withdrawal of the antibiotics dimetridazole and olaquindox and the occurrence of acute PE outbreaks three to five weeks later. They concluded that, while antibiotics can protect pigs from infection, they

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may prevent an immune response, rendering pigs susceptible to infection when antibiotics are withdrawn. Furthermore, they surmised that this three to five week period represented the incubation period of *L. intracellularis* and should be considered when designing medication regimes. There is no published information on the efficacy of these particular antibiotics against PE.

Although there are potentially a large number of organisations from which to obtain data, including farm addresses, lack of co-operation can be a problem in planning surveys such as this. It is important to make the objectives of a study clear to those who may be helping and to justify costs involved (Thrusfield 1995). If collection of data represents a breach of confidentiality, it may be impossible to secure information, regardless of available funds. In this study, the difficulties encountered in trying to obtain addresses led indirectly to a variation in the response rate between regions and may also have affected the validation results. In Scotland, where I had access to a large number of farm addresses, the follow-up reminder card produced a very good response rate (almost 70%). For most of the units in England, I did not have access to addresses, so was unable to send reminders. This was reflected in its lower response rate. Ideally, every unit in the UK would be questioned, and particular measures incorporated to ensure a 100% response rate. Obviously, surveys like that are impractical and expensive, defeating the purpose of this choice of study model, which is to facilitate survey organisation, avoid interviewer bias and permit respondents to reply anonymously if they wish, potentially increasing the response rate (Thrusfield 1995). While random sampling of a proportion of the total number of farms would lessen the expense to some extent, a source of addresses for all the farms in the UK would still be required and, since data protection has encouraged the adoption of a confidentiality policy by many companies and associations, this method is becoming harder to implement.

My attempt at survey validation was similarly affected. In the general census for 1995, the herd size frequency distributions in England/Wales and Scotland were very similar, as they were between England and Scotland in my survey results. When herd size was compared between the census and survey, I found a lower proportion of smaller herds and a correspondingly higher proportion of medium/large herds in the survey than there were in the census. This was observed for England and Scotland separately, and subsequently for Britain as a whole.

This survey is, therefore, representative of the medium to large enterprises in Britain. The reasons for this difference may be related to the sampling selection procedure. My source of addresses in Scotland was a feed mill, not an overall census. This would tend to exclude smaller herds, which are not likely to purchase their feed from such a source, so the survey population was not wholly representative of the general pig population. Over 40% of herds in the census had under 10 sows. These would be more accurately described as small holdings rather than pig production units. The veterinary surgeons involved in questionnaire distribution are less likely to visit such small holdings, at least on a routine basis, which could explain their under-representation in the survey population sample. Since the surveyed risk of PE appeared to be higher in larger herds, the smaller herds apparently not included in my survey may not have contributed to the absolute number of PE outbreaks, overall. This is further supported by the finding that herd size was still a highly significant risk factor, even when herds with under 50 sows were excluded from the analysis. Because larger herds (>50 sows per unit) are responsible for producing most of the UK pig meat, it could be argued that the survey was more relevant to the pig industry. Other general problems can occur in questionnaire surveys. Herds which have the disease of interest may be more likely to respond. Larger herds may have better trained stockmen and employ routine veterinary supervision, making them more aware of herd disease status. This could also explain the association between herd size and PE, PRRS and enzootic pneumonia. Many of these problems could have led to an overestimate of PE prevalence in Great Britain, but this was unavoidable.

Despite these difficulties, this questionnaire survey helped to highlight specific areas, particularly relating to source of replacement stock and environmental management, which would be worth more in-depth investigation and consideration in attempts to control PE.

CHAPTER FIVE

AN ASSESSMENT OF DIRECT COSTS OF PORCINE PROLIFERATIVE ENTEROPATHY TO THE BRITISH PIG INDUSTRY

INTRODUCTION

During the last two decades, significant advances have been made in pig medicine and management, especially in relation to diseases normally associated with high mortality. In the United Kingdom, selective culling has been used to eradicate diseases like swine fever and porcine foot and mouth disease (Mengeling 1992), while vaccines against parvovirus (Mengeling 1992), Aujesky's disease (Kluge et al 1992) and, more recently, enzootic pneumonia, have contributed to eradication or significant reduction in the incidence of these conditions. Improved management has been an important additional factor in these improvements, despite increasing intensification.

Control programmes conducted by leading commercial pig breeders in Europe and North America have increased the availability of pigs free of specific pathogens, such as *Serpulina hyodysenteriae* and *Actinobacillus pleuropneumonia* (Alexander and Harris 1992). These programmes have placed hysterectomy-derived piglets into segregated early weaning systems. This style of separation of age-groups onto different sites is also becoming increasingly popular on commercial production units.

While these measures have proven to be well suited to many infectious diseases, particularly of viral aetiology, there appears to be a number of disease conditions which are either able to thrive under these stringent control regimes, or which have merely taken advantage of the reduced competition from eradicated diseases. Proliferative enteropathy (PE) is an example of these "emerging diseases", which also include oesophago-gastric ulceration and porcine polyserositis and arthritis (PPA or Glässer's disease) caused by *Haemophilus parasuis*. In its acute form, PE is capable of causing sudden deaths in valuable breeding stock, but is also responsible for suppressing the production efficiency defined by daily weight gains and feed conversion ratio in younger pigs. Previous studies have indicated that the performance of weaned pigs can be seriously hampered by the presence of lesions typical of chronic PE. Gogolewski et al (1991) found that the daily liveweight gain in pigs affected with chronic PE ranged from 17 to 98% of the mean daily liveweight gain in their unaffected counterparts. Reports of losses to the Australian pig industry have varied from 30 to 141 dollars per sow for PIA and PHE, respectively (Holyoake et al 1996, Cutler and Gardner 1988), while the overall cost to the United States pig industry has been estimated at 20 million dollars (Mapother et al 1987). The costs of a disease can be thought of as a combination of the losses which it incurs, e.g. livestock mortality or infertility, and the extra expenditure which subsequent management of the

disease outbreak may require, such as medication or veterinary expertise. The problems associated with estimating such costs are numerous. Firstly, it may be difficult to attach a numerical value to a finisher pig prior to its achieving marketable weight. Secondly, although direct performance can be more easily accounted for in terms of daily liveweight gain or feed intake, it is harder to estimate other costs, such as veterinary attention and advice. Thirdly, the process of attributing loss is further complicated by other factors which can be easily overlooked. For instance, diseased animals may have reduced appetites, leading to a potential reduction in feed costs (McInerney et al 1992). It has also been argued that increased production caused by reduction in costs can lead to reduced market prices. Since these reduced market prices actually decrease direct income, they represent a loss to the producer. Therefore, for the producer to benefit from disease reducing and cost-cutting measures, the increased production achieved must be great enough to counteract the possible subsequent drop in market value (Crooks et al 1994). This chapter describes how the direct financial losses due to porcine proliferative enteropathy were estimated by analysing performance data obtained from experimental challenge trials. These trials used pure cultures of the aetiological agent of PE, *Lawsonia intracellularis*, as challenge inocula, allowing improved comparability with control data and eliminating variations due to concurrent diseases and field conditions.

MATERIALS AND METHODS

Production Challenge Trials

In three separate trials, various parameters were measured to assess and compare production between groups of pigs dosed with challenge inocula of *Lawsonia intracellularis* or control buffer solution. All the pigs were derived from the same Large White/Landrace/Duroc genetic background, to enhance consistency between the trials. Pigs were weaned between 21 and 24 days of age, without creep feed, weighed, individually identified and housed in indoor pens. They were allocated to groups of four to eight pigs, based on stratified weight, to minimise the possibility of initial bias at the start of the trial, *i.e.* the initial pig weights were ranked, then distributed randomly to one of each of the groups in descending order of weight. The pigs were fed the same proprietary feed as a wheat and barley based meal with soybean added and water ad libitum. Pigs were dosed two to six days after weaning. The feed consisted of

Trial protocols

In each trial, group A pigs were dosed with between 3.4 and 5.0 x 10^8 *Lawsonia intracellularis* organisms and group B pigs were dosed with sucrose-potassium-glutamate buffer only, using methods previously described (McOrist et al 1993). The number of organisms given to the challenged pigs was expected to produce lesions of proliferative enteropathy. Each pig was weighed prior to dosing and on a weekly basis until the end of the trial. Health status was monitored daily. Group feed intake, based on the total amount offered and refused was determined weekly. Trials 1, 2 and 3 were terminated three, four and seven weeks after dosing. Pigs were euthanased and weighed prior to necropsy. The presence of lesions indicative of proliferative enteropathy and the presence of *L. intracellularis* were investigated using standard diagnostic techniques (McOrist et al 1993). The trial protocols are summarised in Table 5.1.

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TABLE 5.1 Trial protocols

Trial Number	*L. intracellularis* Challenge	Number of Pigs	Duration of Trial (weeks)
1 A	Yes	7	3
B	No	7	3
2 A	Yes	8	4
B	No	7	4
3 A	Yes	5	7
B	No	4	7

Performance parameters

The performance parameters measured were the average daily liveweight gain (ADG), calculated by dividing the mean weekly weight gain for each group by 7, the group feed intake per day and the feed conversion ratio (FCR) for each group. This was calculated as the kg of feed consumed/kg of weight gain.

Estimation of Loss

Feed costs

The probable feed costs incurred by the presence of PE was determined. This was based on the differences in feed conversion ratio between groups A and B within each of the trials. The target weight gain required from weaning to marketing was assumed to be 90 kg (Anonymous 1995b). Multiplying the target weight gain by the projected feed conversion efficiency gave the amount of feed which would have been consumed prior to marketing. This was in turn multiplied by £ 0.175, the assumed current cost of one kg of British pig feed (Anonymous 1995b, Anonymous 1996).

The projected feed conversion efficiency was calculated by presuming that the recorded change would continue for half of the post-weaning period only. This allowed

proportional adjustment of the feed conversion efficiency value for British pigs which, in the current climate, has been estimated at 3.0 (Anonymous 1995b).

Average daily gains and days to slaughter

The costs arising from the changes in average daily weight gains were estimated by assuming that the changes observed would only persist for, again, half of the post-weaning period. This then permitted adjustment of the number of days to slaughter, *i.e.* the number of days required for weaned pigs to reach market weight in the United Kingdom, currently estimated at 150 days (Anonymous 1995b). The cost of one unit of breeding pig space in the United Kingdom is approximately £ 400 to £ 650 sterling (Anonymous 1995b). This includes breeding, management, labour and financial costs and is important in the estimation of direct costs, because it is affected by an increase in the number of days to slaughter. If one sow produces 20 piglets per year, then the cost of the growing pig is £20 to £33. This cost can then be divided by the number of pigs available to that unit of space per year, *i.e.* the number of batches of pigs per year, plus 10 days per batch for cleaning on an all-in all-out basis.

Mortality

Although mortality is not a major symptom of PE, it does occur in the acute form, and should be included in attempts to identify factors contributing to loss. Each year, approximately 2% of pigs close to slaughter die in the United Kingdom (Anonymous 1995b). This amounts to 290,000 pigs per year. It has been surmised, in survey-based studies, that acute PE causes between 2 and 5 per cent of this mortality (Duran 1994, Christensen et al 1995), or 5,800 to 14,500 pigs per year.

Survey

A British survey of the prevalence of proliferative enteropathy was reported in Chapter 4 and the results used to allow the estimated loss components derived for each pig to be applied to the pig industry as a whole. The questionnaire was designed and distributed to 569 British production units, which is approximately 3% of the total 20,000 British farms (Chapter 4). Farmers were asked if PIA had been diagnosed on their unit in the previous three years, with possible replies of "yes", "no" and "don't know".

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RESULTS

Performance data from trials

Table 5.2 outlines the results from the three trials. There were marked reductions in the average weight gains of all the challenged pigs compared to the control pigs in each of the trials (range 9 to 21%). In addition, there was an associated reduction in feed conversion efficiency in PE affected pigs compared to control pigs (range 6 to 20%).

Table 5.3 shows the likely costs of the extra feed which would be required to finish these pigs and the costs of inefficient utilisation of the housing and management facilities available, caused by the increased number of days to slaughter. The likely increased cost per kg of feed ranged from £1 to £5 and the cost of the reduced breeding space available ranged from £0.3 to £0.9 per pig. Combining these costs gave a total of £1.30 to £5.90 per pig.

Example Using Results from Trial 1

Days to slaughter

Challenge pigs average daily gain (ADG) =   248g/d (21% lower than the ADG for
                                                    the control group in this trial)

To allow for the assumption that this reduced growth rate only lasts for half of the growth period, the percentage reduction is halved = 10.5%.

If the pigs were growing to target, they would reach slaughterweight in 150 days.

By growing at 10.5% of target rate, they would then reach slaughterweight at 110.5% of the target period = 165.75 days (166 days).

Cost of breeding space

The number of days to slaughter is extended by 10 days, to allow for pen cleaning and resting = 176 days.

Dividing 365 days by 176 gives the number of "turn arounds" which are possible at the performance level represented by this group of pigs = 2.07.

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The cost of the pig breeding space (£400) is divided by the number of weaned pigs per space (20) and the number of batches per year (2.07) = £9.70. The decrease in efficiency of space utilisation compared to the control pigs cost is £0.90 per pig.

Feed costs

Adjustment of the target FCR (3.0) is achieved by multiplying it by half the percentage decrease in feed conversion efficiency observed in the challenged pigs, again assuming that the FCR will be decreased to this degree for only half the growing period. This value is multiplied by the cost per kg of feed and the 90 kg weight gain required to market the pig.

Therefore, a 10% decrease in target FCR = 10% of 3.0 = 0.3. Adjusted FCR = 3.3

The likely feed cost to market is 3.3 x £0.175 x 90 = £52.00 per pig.
The feed costs for the control pigs were £47.00, therefore the difference between control and challenge pigs is £5-00 per pig, representing the direct costs attributed to the increased feed requirements.
The total direct costs resulting from the increased feed and space requirements = £5.90 per pig.

Survey

Of 569 questionnaires distributed in the UK, 319 were returned (56% response rate). 51 of these units no longer kept pigs. Of the 268 units which did, 83 had had PE diagnosed in the previous three years, giving a three-year prevalence of 31%. Therefore, the annual number of slaughter pigs at risk of developing proliferative enteropathy is estimated at 1.5 million *i.e.* (31% ÷ 3) x 14.5 million slaughter pigs. The within-herd prevalence of PE is reportedly between 5 and 40% of growing pigs (Pointon 1989), leading to an estimate of between 75,000 and 600,000 pigs affected each year with proliferative enteropathy in the UK.

Multiplying the estimated number of pigs affected by chronic PE by the cost per pig gives a total direct cost of between £442,000 and £3.5 million, depending on the degree of effect upon production.

Mortality

The total number of pigs which die each year from the acute form of PE is estimated to be between 5,800 and 14,500. The current market value (February 1996) of a pig at slaughter is £130, so the total financial loss due to acute PE may be between £0.75 million and £1.9 million.

When the costs due to mortality and the direct costs from reduced production are combined, the total direct loss due to proliferative enteropathy in the UK may range from £1.2 million to £5.4 million.

Calculations for trials 2 and 3 showed that direct costs attributed to PE were £1.30 and £5.90 per pig, respectively. With an estimated 75,000 to 600,000 pigs affected per year, the overall direct cost of PE ranged from £97,500 to £780,000 and £442,000 to £3.5 million, respectively. The total costs when mortality losses were included ranged from £850,000 to £2.7 million in trial 2 and £1.2 million to £5.4 million in trial 3.

Therefore, taking all three trials into consideration, the total direct costs of proliferative enteropathy in Britain may range from £0.85 million to £5.4 million per year, depending on the extent of the effect of disease on performance efficiency.

Although some of these costs may have been incurred anyway, without the presence of PE, leading to a reduction in this cost, there are also certain indirect costs which cannot be directly accounted for, which could have increased this final figure.

TABLE 5.2 Daily liveweight gains and feed conversion ratios

Trial number and challenge status	Mean start, finish weight (kg)	Average daily gain (kg/day) during study[b]	ADFI[c] (kg)	FCR during study[d]
1 Yes	5.9 (0.3)[a], 11.1 (0.7)	0.248 ±0.048 (21%)	0.51	2.0 (20%)
No	6.3 (0.3), 12.9 (0.6)	0.314 ±0.058	0.57	1.6
2 Yes	5.3 (0.4), 12.3 (1.81)	0.250 ±0.089 (9%)	0.41	1.6 (6%)
No	5.5 (0.77), 13.2 (1.68)	0.275 ±0.123	0.40	1.5
3 Yes	7.0 (0.39), 12.9 (1.3)	0.168 ±0.06 (21.5%)	0.58	3.4 (20%)
No	7.5 (0.22), 14.5 (2.3)	0.214 ±0.072	0.58	2.7

Notes:

a The values in parenthesis is the standard error.

b The value in parenthesis is the percentage decrease in mean daily liveweight gain of challenged pigs compared to control pigs.

c Average daily feed intake

d FCR = feed conversion ratio, defined as the number of kg of feed consumed per kg of weight gain in each group. The value in parenthesis is the percentage deterioration in feed conversion efficiency of the challenged pigs compared to the control pigs.

TABLE 5.3 Feed costs and ultilisation of pig space

Trial number and challenge status	Days to slaughter[a]	Likely feed cost to market[b]	Annual number of batches and likely cost per pig space[c]
1 Yes	166	£52	2.07 - £9.7
No	150	£47	2.28 - £8.8
2 Yes	157	£48	2.19 - £9.1
No	150	£47	2.28 - £8.8
3 Yes	166	£52	2.07 - £9.7
No	150	£47	2.28 - £8.8

Notes:

[a] this is the mean number of days taken by each group to gain the 90 kg required to reach slaughterweight. The target period of 150 days is adjusted for the challenge groups by multiplying it by half of the percentage decrease in mean daily weight gain for the respective groups, compared to their control cohorts. See example below.

[b] this is the likely feed cost per pig, calculated as the current cost of feed (£0.175 per kg) x likely feed conversion ratio (3.0 x 0.5 percentage reduction in efficiency for each group) x 90 kg weight gain required to reach slaughter. See example.

[c] the annual number of batches is determined by dividing 365 by the number of days to reach market weight, at the likely rate of gain in each group, plus 10 days for cleaning. The cost of a breeding space in the UK (£400-650) is then divided by the number of piglets weaned per space and the number of batches per space year to give a likely cost for the facilities per pig in each group. See example.

DISCUSSION

Proliferative enteropathy is one of the major emerging diseases influencing the productivity of growing pigs. Continued clarification of relevant epidemiological aspects will undoubtedly lead to proposals for control programmes which are more specific to PE. Control measures can often be expensive, however, and the agricultural industry is frequently required to justify the steps it takes to stem the effects of animal disease. For these reasons, economic analysis of a particular disease often goes hand-in-hand with its epidemiology. In the past, it was very easy to justify spending on the prevention and eradication of diseases like foot-and-mouth disease and Rinderpest, where the high losses were more than outweighed by the control costs. PE fits into a new category of less dramatic diseases which can be quite insidious in course, or which are more elaborate in nature, making control more awkward and time-consuming (Ellis and James 1979).

The main purpose of economic analysis is to identify the optimum level of output in relation to the expenditure required to achieve it. The optimum control regime will be attained where costs are at the lowest level possible *i.e.* avoidable costs are equal to zero (McInerney et al 1992).

Previous investigations into the economics of animal diseases have also concentrated on productivity comparisons, particularly with respect to changes in average daily gain between animals with and without the disease of interest (Powers and Harris 1973). This type of study has been criticised for being unrealistic because it concentrates on the individual producer and is incapable of evaluating the economic effects on the entire industry (McInerney et al 1992). Nevertheless, it does represent a worthwhile contribution towards estimation of the effects of animal disease. Furthermore, the pig producer represents an important and integral part of the agri-business economy. Changes in the various production components can be used to assess the extent to which the producer fails to achieve targets, such as days to slaughter. The monetary value calculated from these changes represents the direct cost of the short-falls to the producer.

The pig farmer is likely to be the first affected by increasing disease costs. Although the effects will eventually filter through to the food-processing sector and retail industry, they are better equipped to cope with changes in economic climate, absorbing increased costs by passing the expense on to the consumer. As a result, a reduction in sales does not necessarily mean that revenue will be reduced (Crooks et al 1994).

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This study is the first to investigate the cost of PE in Great Britain and several assumptions were made in the calculations involved, but these were extracted both from current industry costs and from the knowledge of PE which has accumulated to date. The final estimate is in line with the variability encountered in previously reported costs to the USA and Australia which were $20 million per annum (Mapother et al 1987) and $15 to $141 per sow per year (Cutler and Gardner 1988, Holyoake et al 1996), respectively.

It is difficult to directly compare the figures obtained in this study with these previous reports, however, partly due to differences in data collection. This study used trial based data which, although a more accurate method for evaluating direct losses, could differ somewhat from field data.

The relatively small numbers of pigs used in each trial precluded statistical evaluation of the performance figures. Due to the availability of a larger number of pigs, field studies may be more realistic from a statistical standpoint, perhaps allowing a more authentic assessment of the financial burden incurred by PE. However, they may also introduce increased variability arising from different management systems, source of pigs and the presence of other diseases. Other endemic infections and disease conditions could interfere with any one of the production components measured but it is impractical to completely control for the occurrence of such possibilities. For these reasons, and despite their drawbacks, experimental trials represent the most accurate method for establishing the influence of disease on certain production elements.

I could not determine a reliable method to calculate a number of other factors which would affect the final true financial loss to the producer. For example, possible additional costs include those caused by increased variation in both carcass quality and kill-out weights. The rate of lean meat deposition is greatest in the 40-120 kg liveweight range (Stahly 1994) and can be affected by a number of factors, including endocrine function, genetic selection and level of feed intake (Boyd and Beerman 1992). Since chronic PE occurs in pigs of six to 20 weeks of age, it is likely to affect the lean:fat ratio in the final carcass. While reduced average daily weight gains and feed efficiencies are likely consequences of chronic PE, reduced back-fat is also a probable outcome, since increased energy intakes (above an optimum level) tend to increase fat deposition, particularly later in the growth phase. Reduction of fat content would serve to increase the percentage of lean-meat per kg of carcass, which may counterbalance some of the costs incurred by increased days to slaughter etc. Holyoake et al (1996) were able to allow for this

compensation using an AUSPIG decision support system for simulating the economic impact of PE in Australia. As there is currently no official back-fat premium scheme operating in the UK, I was unable to quantify these effects.

The variation in liveweight and quality of animals is also a valid concern, even if they are not sold for slaughter. Poor quality or weak replacement breeding stock may have a detrimental effect on the breeding programme. Growth depression may also affect the reproductive efficiency in first time breeding gilts. If infection with *L. intracellularis* is detrimental to the growth rate of the gilt, conception rate may also be reduced, although it may be difficult to ascertain if this particular phenomenon occurs. It would certainly be difficult to quantify its effect on production economy.

Further costs not measurable in this study were the advisory and veterinary costs of animal disease, including the state veterinary service and other support organisations, such as those offering advice on nutritional and disease control matters. While it should be relatively straightforward to account for the effect of therapeutic drugs on a production unit's finances, the use of antimicrobials in production diseases can actually have a beneficial effect over and above their therapeutic value. In experimental trials, preventative use of tiamulin increased the average daily liveweight gain of grower pigs challenged with *Lawsonia intracellularis* by 15% when compared to non-infected control cohort pigs (McOrist et al 1996b). Accurate assessment of therapeutic costs, particularly for tiamulin, would need to allow for this financial saving caused by the drug's growth promoting effect.

Evaluation of public costs has been ignored by many investigations into economic impact of disease on agriculture, probably due to the inherent difficulties which are encountered and often easily overlooked. These wider costs may include reduced product quality, detrimental effects on international trade relations and public investment into the state veterinary service. They are further complicated by the close relationship between the different classes of livestock products, including beef and poultry, once they reach the retail level of production. Consumers tend to view the different meat products as substitutes for each other and their final choice is influenced by their income, product price and even current welfare or health issues (Crooks et al 1994). There is also a general consensus that mortality actually helps to maintain meat prices, by reducing the market supply. Crooks et al (1994) used computer models to show that a 1% decline in hog mortality over a five-year period in the USA had major ramifications for price, production

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and consumption not only for pork and other pig meat products, but also for beef and poultry. They concluded that the employment of any new measures aimed at increased production would serve to reduce market prices. Such measures would only be of subsequent benefit to the producer if the reduction in market prices was less than the reduced costs brought about by the adoption of the new control system. These models can quickly become redundant in the face of changes in global pork supply and consumption due to swine fever or foot and mouth disease outbreaks, leading to an increase in both price and supply. The increasing use of computer models, however, may help in future assessments of the wider effects of disease and a simulation has already been used to examine the effect of PE on an "average" farm in Australia, although this concentrated on productivity and profitability at the farm level (Holyoake et al 1996).

A further criticism of most economic analyses is that they are often conducted at a single point in time, which does not allow for the appraisal of changes over time. Supply and demand is not a static phenomenon and neither are many of the costs used in this study, for example animal feed costs and pigmeat prices.

It is also vital to recognise in animal disease studies such as these, that the figure given for direct costs is not necessarily the amount which would be saved if the disease was completely controlled or eradicated. Ideally, economic analyses should aim to provide guidance on the most appropriate and cost effective control procedures. Realistically, these should be based on avoidable costs, rather than absolute costs (McInerney et al 1992). There comes a point in the control of disease where no further gain will be obtained from continued attempts at improvement, *i.e.* no matter what measures are introduced or how much money is invested, losses will never be reduced below a certain level. Therefore, it may not be economically rational to eradicate disease completely.

These figures may not quantify the true avoidable costs in this case, because expenditure on normal farms and on those suffering from proliferative enteropathy may not reflect the apparent differences observed between the groups in this study. For instance, although a farm may not be experiencing major production losses associated with PE, the apparent financial gains obtained from good pig performance may be reduced by the control measures required to maintain the situation, such as a high rate of antibiotic incorporation. Nevertheless, the new emerging production diseases appear to have a severe impact on the profitability of affected farms and are worthy of attempted control.

CHAPTER SIX

FARM SAMPLING SURVEY INCORPORATING POLYMERASE CHAIN REACTION

INTRODUCTION

The relationship between *Lawsonia intracellularis* and its host is extremely close, as has been consistently demonstrated by the obligate intracellular nature of the organism and its ability to enter and establish a habitat in epithelial cells of the intestine of the pig (Rowland et al 1973, McOrist et al 1996b). While the use of molecular biology and cell culture techniques have allowed further transmission studies and strain comparisons (Knittel et al 1996a), many of the epidemiological features of *L. intracellularis* infection in traditional farrow-to-finish units and on modern separate-site farms are still unclear. The progress of epidemiological investigations in PE has been slow. The within-herd prevalence of other fastidious organisms, such as *Mycobacterium paratuberculosis* infection in cattle (Goodger et al 1996), has been successfully studied using serological techniques. This is not yet an option for PE, because difficulties have been encountered in validation of tests such as an enzyme-linked immunosorbent assay (ELISA) aimed at *L. intracellularis* (Holyoake et al 1996).

The polymerase chain reaction (PCR) test has been developed for detection of the *L. intracellularis* genome in the faeces of pigs experimentally challenged with *L. intracellularis* (Jones et al 1993c). However, although experimental use of the PCR on faeces samples is valuable and necessary for assessment of the test, the within-herd prevalences and patterns of transmission of *L. intracellularis* on farms can only be realistically investigated by application of the PCR in field circumstances. This would allow closer scrutiny of age-groups which do not normally develop overt clinical signs of chronic PE (porcine intestinal adenomatosis), such as nursing mothers and their litters. Additionally, animals could be followed through all the stages of production, without the need to sacrifice individuals for confirmation of PE at post-mortem examination.

Experimental detection of *L. intracellularis*-specific PCR product in faeces has indicated that individual pigs are capable of excreting the bacteria for at least 10 weeks after infection (Chapter 3), illustrating the strong relationship between organism and host. In the absence of other data, various epidemiological theories can be proposed. It is possible that transfer of infection into the weaner and grower accommodation occurs if pigs are mixed together. Also infected growers could still be infected and excreting *L. intracellularis* as replacement breeding gilts, thereby becoming a source of infection for their offspring, or a means of propagating infection through the herd. Gilts and sows have

been strongly implicated as source animals for group A rotavirus infections in neonatal piglets, as rotavirus antigen has been found in the faeces of sows and gilts before, during and after farrowing (Gelberg et al 1991). The presence of rotavirus in their litters' faeces further supports this hypothesis for the transmission of infection. Piglets do ingest their mother's faeces, which may be a normal mechanism for maintaining iron levels, helping to prevent anaemia to which young indoor piglets are susceptible (Sansom and Gleed 1981). It is not unreasonable to suggest that this is one possible method for the transmission of *L. intracellularis*, therefore I developed a sampling survey to investigate the role of the breeding gilt in the epidemiology of PE. In addition, the follow-up study of their litters over time enabled determination of within-herd prevalence in different age-groups, using the PCR to detect *L. intracellularis* in faeces samples.

MATERIALS AND METHODS

Five breeder/finisher swine production units were selected for the farm sampling survey, based on their proliferative enteropathy status and ease of access for collection. All were located in central/eastern Scotland. Two of the farms had been diagnosed positive for PE, based on necropsy and histopathological lesions in the month prior to sampling. One farm had a history of intermittent incidents of PE and suffered an acute outbreak of PE after sampling had begun, confirmed by the presence of microscopic lesions in affected pigs. Of the other two farms, one had a past history of disease, although none in the last two years, while the other had never been diagnosed PE positive. These latter two farms were treated as control negative units for the purposes of the sampling survey. One unit mixed its own pig feed from wheat and barley on the farm, while the other four used a commercially prepared wheat/barley/soybean meal.

Unit Description

Large unit-chronic infection

This farm had approximately 600 breeding sows. PE had been diagnosed in April 1996 by post-mortem examination at the local veterinary investigation centre (V.I. Bush, Jill Thompson personal communication). Typical clinical signs of chronic PE, including diarrhoea, weight loss and reduced weight gains, had been noted and, apart from enzootic pneumonia and porcine reproductive and respiratory syndrome, no other diseases had been reported. Replacement breeding stock were obtained from a multiplier herd, although additional breeding gilts were bred on-site. Piglets were weaned at three weeks of age, when they were placed in groups of 30 to 40 in partially slatted pens for approximately four weeks (nursery accommodation). They were then moved into the main weaner accommodation, which comprised three different buildings. Alternatively, they were moved directly into the main weaner accommodation at weaning. This was dictated by available space. Once in the main weaner accommodation, the pigs were moved in the same batches at regular intervals through the pens until finished. There was no identification system for the young stock after weaning. However, at my request, and where time permitted, three or four random piglets from each litter were identified by

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notching the ears. All young stock were housed on partially slatted flooring. Boars were kept separately in boar pens with solid floors and straw, while dry sows were housed in partially slatted accommodation in crates. Weaners, growers and finishers were moved in batches, but only on a pen basis, and not by house. Although finisher pigs were never medicated, weaners and growers were regularly medicated with zinc oxide. Antibiotics were not used regularly. However, during the trial, several batches of pigs in the weaner accommodation were treated with tylosin in feed (Tylan, Elanco Animal Health, Basingstoke). Waste faeces was removed into pits running into a slurry system, emptied twice a year. The sampling commenced in May 1996.

Small unit - chronic infection

This was a 160-sow unit, which had been diagnosed PE positive in April 1996 by post-mortem examination at the veterinary investigation centre. The clinical signs were weight gain variations and diarrhoea in the grower pigs and diarrhoea in the weaners, all indicative of chronic infection. Replacement gilts were bought in from a multiplier unit, while a nucleus herd was used as a source of replacement boars. Piglets were weaned at 21 days of age and moved to partially meshed weaner "kennels", where they were usually mixed with one other litter. They remained here for four weeks. At seven weeks of age they were moved to larger "kennels" in the same building. This was the only stage on this unit where all-in all-out by pen was practised. Elsewhere, flow was continuous. Grower and finisher pigs were housed on solid floors with straw. After sampling had begun in May 1996, the weaners were medicated with tiamulin (Tiamutin, Leo Animal Health, Princes Risborough) in the water two days before and three to four days after an intended move, usually at seven weeks of age and again at 11 weeks of age. This was aimed specifically at treatment of PE. Faecal waste was removed via a pit and slurry system in the weaner accommodation and manually in the growers and finishers.

Acutely infected unit

This unit housed between 150 and 200 sows. All replacement breeding stock were bred on site. Piglets were weaned at four weeks of age, then housed in flat decks with fully meshed flooring. After four weeks in this accommodation, they were moved to grower pens, where they were either housed on partial slats or a solid floor with straw bedding. Pigs were moved as litters wherever possible, although some mixing did occur to allow

poor pigs to remain for longer periods in the weaner flat decks. However, all-in all-out was not practised on a house basis. Faecal waste was removed either manually or via a slurry system. This farm had not had an outbreak of PE in the previous year, but an acute episode occurred in August 1996, during a particularly hot spell of weather. The diagnosis was based on the clinical signs of sudden deaths and haemorrhagic diarrhoea, consisting of a black, tarry scour, and confirmed by post-mortem examination at the veterinary investigation centre. At the time of diagnosis, 14 gilts were treated parenterally with tiamulin (Tiamutin, Leo Animal Health, Princes Risborough), followed by whole house treatment with in-water tiamulin for three days, when all the breeding gilts and boars were treated. Treatment was continued in the form of chlortetracycline (Aureomycin, Willows Francis Veterinary, Crawley, West Sussex) in the water for five days and this was repeated two weeks later in mid-September. As a result, all the stock in the breeder gilt and boar accommodation were medicated intermittently over a four week period immediately after the outbreak. This coincided with part of the sampling survey, as the outbreak occurred approximately four months after the first samples were collected, in May 1996.

Negative farm - split site

This farm was split onto two sites. All the breeding stock were housed on one unit, which was the main unit studied. The growers were moved on a weekly basis to the finisher unit. 130 sows were housed in the breeding unit and all replacements were obtained from a multiplier herd. Approximately three to five days after farrowing, each sow was moved with her piglets out of her farrowing crate to a solid floor "kennel". Piglets were weaned at 21 days of age, when they were moved to weaner "kennels" with partially meshed floors. They remained there for five weeks and then were moved to the grower/finisher unit at eight weeks of age, where they were housed on partially slatted floors. All-in all-out was practised on a pen only basis in the weaner and grower accommodation and two litters were often mixed together. PE had been diagnosed in the finisher unit at post-mortem examination by the attending veterinary surgeon, and the growers were treated with chlortetracycline (Aureomycin) as a result. However, it had never been diagnosed in the breeding unit and, as this was the focus for sampling, this farm was treated as a negative unit. Waste faeces was removed manually on the breeding unit and with a slurry system on the finisher unit. Sampling commenced in April 1996.

Negative farm-single site

This was a 100-sow unit. PE had not been diagnosed and there were no clinical signs to indicate its presence, apart from occasional diarrhoea in post-weaning pigs. All replacement breeding stock were homebred and piglets were weaned at 21 days of age. They were housed as litters wherever possible on partially meshed flooring. At eight weeks of age, they were moved to partially meshed grower pens in a separate building and at 12 to 14 weeks of age, they were housed in finisher accommodation, consisting of partially slatted flooring. All-in all-out was not practised by house. Weaners were treated regularly for diarrhoea with chlortetracycline and growers were treated regularly for pneumonia with tylosin (Tylan). Sampling commenced in May 1996.

Sampling Protocol

Faeces samples were taken on rectal swabs. On each farm, all the replacement breeding gilts were sampled over a four month period at least four weeks prior to farrowing, although most were sampled at mating. Repeat samples were taken either at or within the first five days of farrowing. Each gilt's offspring were sampled at three weeks of age, when approximately 50% of each litter was sampled. They were sampled again four to six weeks after weaning, usually at eight weeks of age. On the smaller chronically infected unit, the acutely infected unit and the split site negative unit, litters were still adequately separated and identified at the eight week old stage. However, on the larger, chronically infected unit and the single site negative unit, litters at eight weeks of age were often mixed together, with no method of individual identification. At my request, and for the entire period of the survey, these two farms only mixed the litters under investigation with each other and not with any litters originating from sows or gilts which were not included in the survey. Where applicable, additional growers and breeding boars were also sampled. Table 6.1 summarises the number of samples taken from each farm in each age-group.

TABLE 6.1 Farm sampling survey protocol

Farm*	Gilts prefarrow	Gilts postfarrow	Litter	Weaners	Growers	Boars
A	101	55	48	8 groups[a]	-	3
B	15	12	12	9 litters	10[b]	-
C	23[c]	18[d]	16[e]	15 litters[e]	10[b]	3
D	37	29	29	28	6 litters	-
E	29	21	18	-	50 samples[f]	-

* Farm A = large chronic   Farm C = acutely infected

  Farm B = small chronic   Farm D = split site negative unit

  Farm E = single site negative unit

[a]   Eight groups of weaners represented all the litters: average of six litters per group.

[b]   Rectal swabs were collected from 10 separate pigs.

[c]   Three gilts aborted, one gave birth to a stillborn litter and one gilt died during the acute PE outbreak. A further two did not hold to service and three could only be sampled at the pre-farrowing stage.

[d]   To compensate for those gilts lost to the survey during the acute outbreak, five were included which were sampled at farrowing only and their litters were followed through to the weaner stages.

[e]   Two litters were not sampled at the three week stage, but were sampled as weaners. Three litters were sampled at three weeks of age but were not sampled as weaners. A total of 13 gilts and their offspring were followed through the entire period from mating to post-weaning.

[f]   On farm E, representative animals from each litter were sampled after they entered the grower accommodation.

Sample Processing

Faecal swabs were stored frozen at -70°C after transport times from each farm, ranging from 20 minutes to two hours. Swabs from the gilts, boars, weaners and growers were prepared individually for PCR assay while, for the litter samples, each set of swabs were doubled up, providing two to three samples per litter, representing approximately four to six piglets per litter. Samples were prepared using the DN*Ace* Clinipure Purification System. Each swab was thawed and placed in 500µl of 0.1M Tris-EDTA (TE) buffer, pH 8.0, in a 1.5ml eppendorf microcentrifuge tube. This was agitated and vortexed briefly, then centrifuged at 500 g in a microcentrifuge for 2 min, to remove solid debris from the sample. The supernatant was removed to a fresh eppendorf tube and incubated at 100°C for 10 min in a hotblock then centrifuged at 10,000 g for 20 sec to form a pellet. This pellet was washed twice in TE buffer, centrifuged at 10,000 g for 20 sec and incubated in 500µl guanidine thiocyanate lysis buffer at 20°C for 1h. After this incubation period, 15µl of silica carrier suspension was added to the lysis buffer/pellet suspension and briefly vortexed. This was placed on ice for 5 min, centrifuged for 20 sec at 10,000 g, and the lysis buffer supernatant discarded. The pellet was washed twice in 500µl of ethanol wash buffer and the suspension centrifuged at 10,000 g for 20 sec. After complete removal of the wash buffer by aspiration, the pellet was incubated at 60°C for 5 min. The dried pellet was resuspended in 30µl of elution buffer and the suspension was again incubated at 60°C for 5 min. The supernatant eluent was removed to a fresh tube after a final centrifugation for 20 seconds at 10,000 g. For PCR, a 5µl aliquot of the DNA eluent was incorporated into a 50µl reaction mix containing 8µl of dNTPs (200µM each), 2.5µl (50ng) primer A and 2.5µl (70ng) primer B (Jones et al 1993c), 5µl PCR buffer, 23.5µl of water, 1.5µl (1.5mM) $MgCl_2$ and 2 units of *Taq* polymerase in a 2µl volume. Each reaction was placed in a thermal cycler and subjected to the following cycling profile: one initial cycle at 95°C for 5 min, 55°C for 45 sec and 72°C for 45 sec, followed by 40 cycles each consisting of 95°C for 45 sec, 55°C for 45 sec and 72°C for 45 sec. The final cycle consisted of 95°C for 45 sec, 55°C for 45 sec and 72°C for 10 min. Each set of reactions always included a positive control of DNA derived from cultured organisms of *L. intracellularis* and a negative control consisting of a water template. Where possible, a faeces sample artificially infected with *L. intracellularis* co-cultured as described

previously (Lawson et al 1993) was included in the whole extraction protocol as an extra positive control sample.

The 319-bp positive PCR product was detected using electrophoresis on a 1% agarose gel (Appendix A). 10µl of each reaction product was mixed with 2µl of gel loading buffer and added to individual wells on the gel. The gel was stained with ethidium bromide, viewed with an ultraviolet light and visible bands compared to DNA molecular weight standards. The sensitivity of the PCR was approximately 5×10^5 organisms per gram of faeces, determined by artificially infecting normal pig faeces with ten fold dilutions of a known concentration of *L. intracellularis*, extracting the DNA and subjecting it to the PCR, as described in Chapter 2. The specificity of the PCR primers used in this assay was confirmed by a failure to generate a band of the expected size after subjecting *D. desulfuricans*-derived DNA to identical PCR parameters (Chapter 2).

RESULTS

Larger unit-chronic infection

No *L. intracellularis*-specific PCR product was detected in any of the faeces samples from the gilts before or after farrowing, or from the boars tested. Similarly, all the piglets sampled were negative at three weeks of age. However, positive PCR product was detected in two separate pens of pigs in the weaner accommodation. Each of these pens housed approximately 30 pigs and were in the same building where the initial post-mortem diagnosis of PE had been made nine months earlier. In one pen, 33% of the samples taken were positive, while in the second pen, 28% of the samples were positive (this is illustrated as a mean value of 30% in Figure 6.1). Some of those pigs were hairier than normal and there was diarrhoea present on the floor of the pens. None of those pigs had received any medication. Eleven samples collected from pigs in the second separate building used for housing weaners were all negative.

Smaller unit-chronic infection

None of the gilts or boars tested were faeces positive on PCR. None of the litters tested were PCR positive at three weeks of age. Since the normal practice was to house two litters together in one pen between three and 11 weeks of age, positive samples could only be matched to one of two litters and more specific identification was not possible. However, four "kennels" were used to house seven litters, as one litter was not mixed. PCR positive faeces samples were obtained from two of the pens accommodating two litters each. If only one litter in each of these pens was infected, this would represent at least two out of seven (28%) litters. In the absence of a more accurate method of litter identification, this is the most realistic estimate of within-age-group prevalence. In the first of these two pens, one out of four (25%) samples tested was positive and in the second pen, one out of three samples tested was positive (33%). Again, this is illustrated as a mean value in Figure 6.1. Clinical signs were not obvious in these weaners, although there were occasional loose faeces. Throughout the sampling period, the growers continued to have intermittent diarrhoea. There was weight variation in those pigs in the grower sheds, but this was difficult to attribute directly to the presence of PE, since the continuous flow system inevitably led to a mixture of age-groups in the same barn.

However, 20% of the faeces samples collected from these growers at the time of post-mortem diagnosis in April 1996 were positive. At the end of the sampling period in January 1997, intermittent diarrhoea was still occurring in the grower accommodation. These results are illustrated in Figure 6.1.

Acutely infected unit

Sampling of mating gilts began in May 1996 and the first litters tested were born in early September. As a result of an acute outbreak of PE, which began in the second half of August, one sow, two gilts and two boars died. Several other breeding animals developed a black, tarry diarrhoea typical of acute PE. Numerous gilts and boars were anorexic and lethargic, and there were several abortions in the month following the start of the outbreak. In early September, diarrhoea was reported in pigs of three to six months of age in the grower/finisher accommodation, although no diagnosis was attempted. *L. intracellularis*-specific PCR product was detected in two out of 23 (8%) gilts which were sampled prior to farrowing. One of these gilts was due to farrow four weeks later. Her faeces were negative on PCR at farrowing. This gilt's litter was negative at both three weeks and eight weeks of age in the weaner accommodation. The second gilt was PCR positive eight weeks prior to farrowing but she aborted during the acute outbreak so her litter could not be examined. Samples were collected from three boars. Two of these were PCR positive. The third sample, although negative, came from a lethargic, anorexic boar which had developed a severe haemorrhagic diarrhoea. Faeces samples were collected from 16 litters at three weeks of age and 15 of these litters were resampled at eight weeks of age. None of their faeces samples were PCR positive at either time. Ten samples were collected from 10 individual pigs at 14 weeks of age in the grower accommodation. Two were very soft in consistency and one of these two samples was positive for *L. intracellularis*-specific PCR product. These were collected approximately five months after the acute outbreak. The positive sample originated from a litter born eight weeks after the outbreak. However, their dam had been PCR negative when tested at farrowing. These results are illustrated on Figures 6.1 and 6.2.

Negative units

No *L. intracellularis*-specific PCR product was detected in any of the samples tested on the two negative farms. On the split-site negative farm, all the samples which were

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collected were processed for PCR. Extra swabs representative of seven litters in the grower accommodation were also collected from the second site, where PE had previously been diagnosed. On the single site negative unit all the gilt samples collected before and after farrowing were tested, and all the litter samples were tested. However, although there was an identification system consisting of ear tattooing, weaner pigs were mixed to such an extent that the accuracy of sample collection was markedly reduced.

FIGURE 6.1 Sampling survey results on three units with acute or chronic proliferative enteropathy
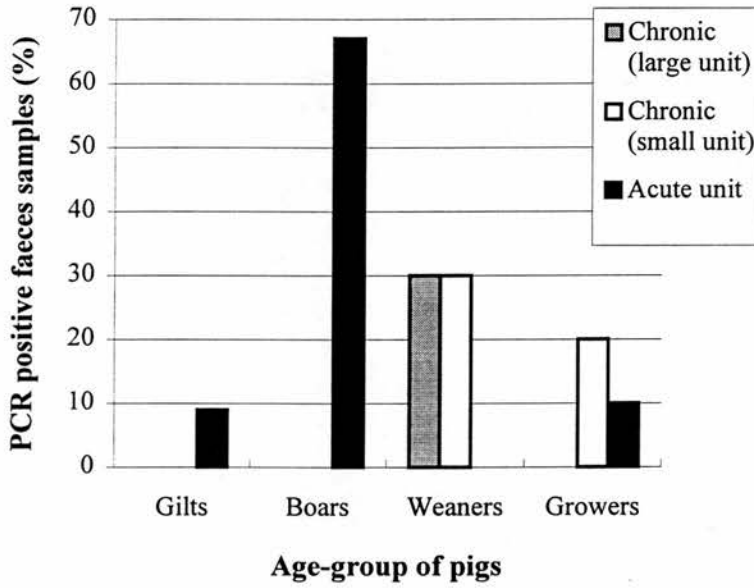
Note:

No PCR positive faeces samples were found on either of the two negative farms

FIGURE 6.2

Opposite is a photograph of an agarose gel showing the PCR results from the acutely infected unit in my field study.

Lane 1: PCR product from positive control infected faeces

Lane 2: PCR product from grower pig at 14 weeks of age

Lane 3: PCR product from gilt (ID 583) faeces four weeks prior to farrowing

Lane 4: PCR product from boar faeces

Lane 5: PCR product from second gilt (ID 362) faeces eight weeks prior to farrowing

Lane 6: PCR with water substituted for template DNA (negative control)

Lane 7: PCR product from positive control DNA extracted from co-cultured

     Lawsonia intracellularis

Lane 8: *Hae*III digest of ΦX174 DNA marker

Electrophoresed on a 1.5% agarose gel.

bp
1,353
1,078
872
603
310

1   2   3   4   5   6   7   8

DISCUSSION

On-farm Epidemiology

This is the first PCR-based epidemiological field study of proliferative enteropathy to be performed in Great Britain. The PCR described and validated in Chapter 2 was used to detect *Lawsonia intracellularis* DNA in pig faeces, thus demonstrating the presence of infection on commercial pig units naturally infected with acute or chronic proliferative enteropathy. PE had previously been confirmed on these units by the presence of typical microscopic lesions in the intestinal mucosa. Infection was not detected in the single-site negative herd which had never been diagnosed PE positive at post-mortem, nor on either of the units on the split-site negative herd. Although PE had been diagnosed on the latter unit in the previous three years, this had been based on gross pathology, without microscopic confirmation, possibly being a false positive diagnosis.

The PCR findings demonstrated the importance of older weaners and/or growers as excretors of the organism on positive farms. On each of the two chronically infected units, 30% of samples tested from affected litters were PCR positive indicating that, within a group of infected pigs, as many as one third may be excreting *L. intracellularis* at any particular time. On the smaller chronically infected unit and the acutely infected unit, between 10 and 20% of grower pigs tested were also excreting *L. intracellularis*. This agrees with other sampling surveys incorporating the PCR assay (Lanza et al 1996) and with previous reports based on ileum examinations (Pointon 1989). A similar farm sampling survey in Spain reported that 88% of the PCR positive pigs were in the grower/finisher age-group (Lanza et al 1996), providing further verification that young growing stock is the optimal age-group for PCR screening of faeces from suspect farms.

In my hands, the PCR was capable of identifying diseased animals, and there was also a connection between diarrhoeic animals and positive PCR results, also reported by a recent sampling survey in Denmark (Møller et al 1996). This Danish survey found that 40% of faeces samples from growers on units with diarrhoea were PCR positive, while only 5% of samples from healthy pigs were PCR positive. Although diarrhoea is not considered pathognomonic for PE, these findings indicate how important the disease is economically, as most diarrhoeic pigs are likely to be below their optimum level of performance. It is

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also an important point to consider when selecting animals on a suspect unit, *i.e.* choosing diarrhoeic animals may maximise the chances of detecting infection.

Overall, there was a dearth of evidence to suggest that gilts might be responsible for directly infecting their young in the neonatal phase of production. All the three-week old litters tested on all five farms were PCR negative. The majority of litters were tested at three weeks of age. Previous reports have indicated that the incubation period is approximately two or three weeks (Jones et al 1993d, McOrist et al 1994a), while the challenge work in Chapter 3 suggested an incubation period of 13 days, depending on initial infectious dose. If infection had taken place in the first week of life, some level of excretion might have been expected at the time of sampling in this study. The negative findings may be attributed to a number of causes. Firstly, if the gilts were not passing high levels of *L. intracellularis* in their faeces, then the initial infectious dose to each piglet may have been comparatively low. Consequently, it may have taken some time for the bacteria to multiply to a level where excreted organisms reached detectable numbers. Secondly, medication may have reduced the number of organisms in gilts' faeces to the extent that insufficient organisms were ingested by their offspring to produce a detectable level of shedding. This is an unlikely hypothesis since neither of the chronically infected herds medicated their gilts around farrowing and, despite detectable levels of infection in the weaners and growers on these farms, there was still no detectable infection in their three-week old litters. Thirdly, common to other infectious diseases, such as post-weaning diarrhoea caused by *E. coli* (Taylor 1995), the suckling litters could have gained a high degree of colostral immunity to PE which subsequently declined at weaning. It is also significant that the crypts of the distal small intestine elongate and the villi decrease in height at weaning, thus presenting a larger proportion of immature enterocytes (Hampson 1986). As this is the cell type favoured by *L. intracellularis* organisms, anatomical alteration in the intestinal crypts may explain the apparent lack of infection in young litters. Lastly, it is possible that piglets are truly negative in the first three weeks of life, only contracting infection from growers and finishers after weaning.

The outbreak on the acutely infected farm provided an opportunity to examine the immediate after-effects of an acute episode in a few animals, as a number of the gilts in the study had been among the breeding stock affected in the outbreak. This particular incident also presented some difficulties which hampered the study. Livestock losses incurred by the infection included both abortions and stillbirths. One of the abortions

occurred in a PCR positive gilt, obviously curtailing the follow-up of her offspring. The second gilt had been PCR positive four weeks prior to farrowing, but she was then included in a treatment regime. This may explain why her faeces were PCR negative at farrowing and her litter's faeces samples were negative at three and eight weeks of age. In addition, the mother of the litter from which the positive sample in the grower accommodation originated was not confirmed PCR positive, therefore it was not possible to establish direct transmission of infection between gilt and offspring in this family. Given the sensitivity of the PCR (Chapter 2), the negative PCR result from this gilt does not rule out the possibility that she was excreting *L. intracellularis* at farrowing, perhaps at a low or intermittent level. Nevertheless, these findings all suggest that direct transmission of *L. intracellularis* from mother to young is unlikely.

Indirect transmission of many infectious pathogens can occur, usually via the pigs' environment, stockmen, clothing, implements, water, feed or vectors, such as rodents or birds. These modes of transfer would require survival of organisms outside the host for a sufficient period of time prior to infection of a new host. Alternatively, excretion of a large number of viable organisms may help to ensure subsequent intake of enough organisms to induce disease in a susceptible animal, even if a proportion of these organisms die in the interim period. Based on the sensitivity of the PCR (Chapter 2), levels of excretion do appear to be high, so environmental contamination with infected pig faeces may be sufficient to ensure persistence of infection in the environment.

Previously, Roberts et al (1977) reported that PE can persist on a unit for several years, based on their clinical observation of protracted poor performance and productivity, together with post-mortem confirmation of disease. In my study, the PCR clearly demonstrated the presence of *L. intracellularis* on two chronically infected farms for 10 months after the initial post-mortem diagnosis had been reported. Persistence was further demonstrated by the pattern of infection observed in the acutely infected unit, where two separate age-groups of pig were excreting *L. intracellularis* within a five-month period, accompanied by the typical clinical signs of both forms of PE. This suggests that *L. intracellularis* has adapted well to the pig rearing environment, possibly through contamination of the environment by carrier pigs and efficient survival mechanisms outside the host. A high degree of excretion of organisms, together with housing of pigs in close proximity to each other, may help to promote establishment of new infections. A

constant supply of naive animals is also likely to be integral to the persistence of PE, as it is to the epidemiology of many infectious diseases (Hurnik 1997).

Individual animals can excrete organisms for at least 10 weeks post-challenge (Chapter 3), so it is feasible that infected growers may still excrete organisms as breeding gilts or infected finishers may infect susceptible weaners, thus establishing a circle of infection in the herd revolving around environmental contamination. If this is the case, direct transmission from gilt to young may not be required. On the acutely infected unit, the positive gilts were not tested as growers, so I could not be certain if the outbreak was a new infection in these gilts or if it was a re-emergence of existing subclinical infection which they had perhaps contracted as young growing stock. Similarly, it was impossible to know if the infection in the grower pens had originated from the acute outbreak in the breeding stock, although the time-scale would suggest this. In the absence of evidence to suggest otherwise, it appears that transmission was indirect, possibly via stockmen and their clothing or inadequate cleaning and biosecurity. For example, although footbaths were provided, many of them were polluted and poorly maintained.

Infection can gain entry onto a herd in a number of ways, ranging from air-borne transfer of infectious particles, such as the foot and mouth disease virus (Mengeling 1992), to mechanical vectors (Thrusfield 1995). Some other intracellular organisms, such as the majority of the *Rickettsia* species, use an arthropod vector to transfer them to new hosts (Murray et al 1994). Source of replacement breeding stock is also considered a major route for transfer of infectious agents, including *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* (Alexander and Harris 1992). Once established in a herd, *L. intracellularis* seems to be capable of prolonged survival, but the initial source of infection is still unclear. During the outbreak on the acutely infected unit, two of three breeding boars tested were significant excretors of *L. intracellularis*. Although the third boar was negative, it was passing profuse, haemorrhagic diarrhoea. It is possible that failure to detect *L. intracellularis* DNA could have been due to PCR inhibition, compounded by the presence of blood in the diarrhoea, which reportedly contains its own PCR inhibitors, consisting of proteins, heavy metals and haem (Panaccio and Lew 1991). These boars represent an important source of infection. They regularly have contact with sows and gilts and, although they are unlikely to gain access to weaner and grower accommodation, they have access to walkways and passages. On many smaller units, weaners and growers may gain access to the same walkways during movement between

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pens and houses, allowing possible contact with infected boar faeces. The role of other animals present on pig farms, such as rodents, was not investigated in this study.

Despite the fact that the PCR is able to detect *L. Intracellularis* organisms in the field and is useful in investigating transmission patterns, it does have certain limitations. Low assay sensitivity is a major drawback for current epidemiological studies into PE. If long-term excretors exist in the field, as they appear to experimentally (Chapter 3), it is possible that they excrete the organism at lower levels, out of the PCR detection range. Similarly, animals recovering from PE may be shedding lower numbers of *L. intracellularis*. Antimicrobial therapy reduces shedding levels and excretion periods in other pig enteric infections, such as *Salmonella choleraesuis* (Jacks et al 1981). It is also likely that appropriate antimicrobial therapy also reduces the faecal burden of *L. intracellularis* in treated pigs, minimising the chances of detecting PE. In addition, low within-herd prevalence of any infectious disease dictates that a high number of samples are taken, and preferably on more than one occasion. The PCR also relies on the presence of excreted organisms to diagnose PE, so in the event of intermittent shedding, the timing of sample collection may also be important. Biphasic excretion patterns have been reported in coccidiosis (Harleman and Meyer 1985) and rotavirus (Fu and Hampson 1989) infections, and the intermittency of shedding observed in swine dysentery (Harris and Lysons 1992) indicates that such fluctuations may be fairly common, possibly influencing the results obtained from prevalence studies, such that positive farms may be underestimated.

CHAPTER SEVEN

SURVIVAL OF *LAWSONIA INTRACELLULARIS* IN SIMULATED
ENVIRONMENTAL CONDITIONS

INTRODUCTION

Ultimately, the control of PE will depend on the reduction of infection on farms by measures including factors such as stocking density, mixing of stock and type of housing, along with a reduction of pathogen numbers by programmes incorporating the use of antibiotics and disinfectants. Because there may be relatively long-term excretion of *L. intracellularis* in the faeces of infected pigs, which could carry infection between groups of pigs, particular attention to the use of effective disinfectants is required. The disinfectant study described in this chapter was partly prompted by the lack of data on disinfectant efficiency. There was also no information on the survival of *L. intracellularis* in air, both features of the organism being of great relevance to discussion of appropriate cleaning regimes, including resting periods for pig facilities, and for the assessment of possible carry-over of infection between groups. While *L. intracellularis* is an intracellular organism, dependent on viable host cells for its multiplication, and hence may be expected to have a short survival time outside the host, it is also related to the *Desulfovibrio* species, a group of extracellular, environmental bacteria (Gebhart et al 1993). Therefore, particular attention was paid to methods capable of evaluating the viability of *L. intracellularis* in its extracellular state, as this could indicate the presence of possible infectious agents persisting in faeces.

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MATERIALS AND METHODS

Bacteria

Four strains of *Lawsonia intracellularis* were used: three, 1482/89 (NCTC 12656), 916/91 (NCTC 12657) and LR189/5/83, had been isolated from acute proliferative enteropathy lesions in separate five month-old British pigs, the fourth, 51/89 had been isolated from a chronic proliferative lesion in a British pig at 10 weeks of age. The strains were isolated and maintained in co-culture in the rat intestinal epithelial cell line IEC-18 (ATCC CRL 1589), using methods described previously (Lawson et al 1993). These strains had initially been grown after the inoculation of intestinal material onto cultured cells. Passage of the bacteria was performed by the treatment of infected cells with potassium chloride and rupture of the treated cells by passage through a needle. The released organisms were used to inoculate fresh cells. Each isolate had been passaged between seven and 16 times after initial isolation. During this isolation process, each strain was tested for freedom from *Chlamydia* and *Mycoplasma* species and other bacterial contaminants by standard culture methods and immunologic methods using commercial reagents.

Cell Culture and Monitoring of Infection

Co-culture of *L. intracellularis* in cell culture monolayers grown on glass coverslips (13mm diameter) in small glass bottles has been described previously (Lawson et al 1993). Briefly, a suspension of *L. intracellularis* in Dulbecco's modified Eagle's medium (DMEM), with 7% fetal calf serum (FCS), was added to monolayers of IEC-18 cells which had been grown for 24 hours. The co-culture was incubated for five days in a microaerobic atmosphere at 37°C. Assessment of the numbers of *L. intracellularis* within the cells on each coverslip, with or without the test procedure (see below), was made by counting the number of infected cells on each coverslip after an indirect immunoperoxidase stain incorporating the monoclonal antibody IG4, which is specific to *L. intracellularis* (McOrist et al 1987).

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Exposure to Disinfectants

Bacterial suspension in disinfectant

Selection of test disinfectants was made after consultation with specialist pig practitioners in East Yorkshire, UK and Iowa, USA. The disinfectants tested and their mode of action are outlined in Table 7.1 (see Appendix C also).

TABLE 7.1

Disinfectant	Trade Name	Mode of Action
Potassium peroxomonosulphate	Virkon	Powerful oxidising agent
Sodium hypochlorite	Domestos	Chlorine releasing - denatures proteins by oxidising peptide links
Phenolic combination	Battle's Black	Targets cell membrane and inactivates enzymes in the cytoplasm - leakage of cell contents
Iodophor	Pevidine	Interferes with bacterial oxidative phosphorylation
H_2O_2/peracetic acid	Sorgene	Oxidation and denaturation of bacterial proteins and lipids
Chlorhexidine and cetrimide mixture	Savlon	Impairs bacterial membrane permeability by binding to phospholipids and proteins

Separate one ml suspensions of *L. intracellularis* in DMEM were added to separate one ml solutions of two dilutions of each disinfectant in small plastic tubes, with one control solution of 50:50 vol/vol DMEM/water prepared instead of disinfectant. The concentrations used are detailed in Table 7.2. Each disinfectant was prepared in sterile, distilled water, according to the manufacturers' instructions. Each tube was incubated at 20°C for 30 min without agitation, then centrifuged at 10,000 g for 5 min. Each test and control bacterial pellet was washed in sterile 0.1M phosphate buffered saline, pH 7.6 (PBS), then resuspended in 1.5ml DMEM with 7% FCS added.

Test and control bacterial suspensions were used as inocula to infect triplicate monolayers which were evacuated and incubated at 37°C in microaerobic conditions, as described

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above. After the five day culture, the coverslips were harvested, acetone fixed and immunostained for counting. Disinfectants, like antimicrobials, can have both bactericidal and bacteristatic effects. Unlike antimicrobials, only their ability to completely eradicate infection is of interest (Maris 1995). In our study, a disinfectant was only considered effective if it succeeded in reducing the number of organisms obtained in the control assay by 99%.

Exposure to Air

Survival of L. intracellularis in medium in air

Suspensions of *L. intracellularis*, strain LR189/5/83 1 SN/7, were harvested from fresh five day co-cultures, adjusted to a suspension of approximately 10^4 organisms and one ml portions were added to four separate groups of monolayers which had been grown on coverslips for 24 hours.

Group A: control positive coverslips. The bacteria were incubated microaerobically at 37°C for three hours, one, two, three, five or seven days separately in co-cultures.

Group B: after addition of the bacteria to the cells, co-cultures were incubated in air at 5°C for the same times as for group A.

Group C: after addition of the bacteria to the cells, co-cultures were incubated in air at 20°C, for the same times as for group A.

Group D: after addition of the bacteria to the cells, co-cultures were incubated in air at 37°C, for the same times as for group A.

Once infected, co-cultures in groups B, C and D were maintained in DMEM and 7% fetal bovine serum at their respective temperatures. After completion of each timed culture, duplicate coverslips for each group and time were harvested from the culture bottles, acetone fixed and immunostained for bacterial counts.

Survival of cell-free L. intracellularis in air at different temperatures

In separate experiments, *L. intracellularis* inocula were exposed to different temperatures, prior to infection of cell culture monolayers. Firstly, a 1ml volume of *L. intracellularis*

(strain 1482/89 K18) inocula was adjusted to a suspension of 10^4 organisms/ml in DMEM, with 7% FCS added, then incubated in air at 5°C or 37°C in a plastic vessel. After zero, one, three, six, 24 or 32 hours, 1.5ml aliquots of the suspension at each temperature were added to fresh cell monolayers, in triplicate. The monolayers were evacuated and incubated microaerobically at 37°C for five days, then the coverslips were harvested, acetone fixed and immunostained.

Secondly, a 1ml inocula of strain LR189/5/83 1 SN/8 was diluted in DMEM with 7% FCS added to a concentration of 10^4 organisms/ml, and divided into three aliquots. Each aliquot was exposed to 5°C, 20°C and 37°C for three hours, 24 hours, 48 hours, four, six or eight days after preparation. Samples of each were used to inoculate standard 24h IEC-18 cell culture monolayers in triplicate. Three control cell cultures were infected in parallel with non air-exposed *L. intracellularis*. All infected co-cultures were incubated at 37°C in microaerobic conditions for five days, after which they were harvested and immunostained for counting.

RESULTS

Exposure to Disinfectants

The efficacy of various disinfectants against suspensions of *L. intracellularis*, indicated by attempted culture of the organism after exposure, is summarised in Table 7.2. In summary, the sodium hypochlorite mixture was the most effective disinfectant when used at the manufacturer's recommendation against strain 51/89. Although it caused monolayer damage by rupturing the IEC-18 cells during testing of strain 916/91, when diluted 1:1000, it successfully reduced the bacterial count to less than 0.2% of the control count. Dodecyl ammonium bromide was highly effective against both strains at the recommended concentration but, like many of the other formulations tested at 1/1000 of the recommended concentration, there was no effect on bacterial multiplication. In fact, there was an increase in bacterial count in the monolayers tested with all the disinfectants at this level of dilution, except for sodium hypochlorite.

Survival in Air

Bacterial survival in medium in air

The results of incubating *L. intracellularis* (strain LR189/5/83) in medium above monolayers in air at 5°C, 20°C and 37°C, are illustrated in Figure 7.1, where the bacterial counts obtained are compared to the control culture counts. The control co-cultures achieved the expected growth curve, while there was no growth at all from those co-cultures incubated at 5°C and 20°C. *L. intracellularis* co-cultures which had been incubated at 37°C did survive, but only to a minimal extent.

Survival of cell-free L. intracellularis in air

The growth curves of *L. intracellularis* strains 1482 and LR189/5/83 after cell-free incubation in air at varying temperatures, prior to inoculation of culture monolayers, are shown in Figures 7.2. and 7.3, respectively. Although these strains were examined over different time periods, both graphs suggest that exposure of organisms to lower temperatures leads to increased viability when compared to organisms exposed to higher temperatures. This is in contrast to the growth curves in Figure 7.1, which indicates a slightly better survival rate at 37°C.

Notes for Table 7.2:

[a] values shown as mean number of cells heavily infected with *L. intracellularis* (>30 per cell) in co-cultures five days after addition of bacteria to cells. Proportion of test compound count to control count is shown as a percentage in brackets.

[b] manufacturer's recommended concentration for use.

[c] fraction of manufacturer's recommended concentration.

[d] monolayer damage. Sodium hypochlorite, H_2O_2/peracetic acid and phenol all damaged the monolayer cells, when used at the recommended concentrations, to the extent that no assessment of their efficacy could be made for strain 916/91. A similar problem was encountered in the assessment of potassium peroxomonosulphate and the H_2O_2/peracetic acid mixture against strain 51/89.

- not tested.

TABLE 7.2 Co-culture of *Lawsonia intracellularis* strain 916/91 or 51/89 following bacterial suspension in disinfectant or control solution.

Disinfectant	Concentration	Strain 916/91	S.E.M.	Strain 51/89 SN7	S.E.M.
Control (No disinfectant)	Medium only	508[a]	113	1163	228
Potassium peroxomonosulphate	1%[b]	57 (11)	44	0 (0) [d]	-
	1:1000[c]	1320 (260)	455	1472 (126)	100.6
Sodium hypochlorite	100%[b]	0 (0) [d]	-	0 (0)	0
	1:1000[c]	<1 (<0.2)	0.6	791 (68)	112
Phenolic mixture	0.33%[b]	0 (0)[d]	-	205 (18)	50
	1:1000[c]	1029 (202)	89	2143 (184)	386
Iodine	100%[b]	5 (1)	5	<1 (<0.09)	0.33
	1:1000[c]	910 (179)	224	2328 (200)	255
H_2O_2/peracetic acid	0.5%[b]	0 (0)[d]	-	0 (0)[d]	-
	1:1000[c]	931 (183)	351	1630 (139)	182
Dodecyl ammonium bromide	3.3%[b]	0 (0)	0	0 (0)	0
	1:1000[c]	508 (100)	212	1957 (168)	352

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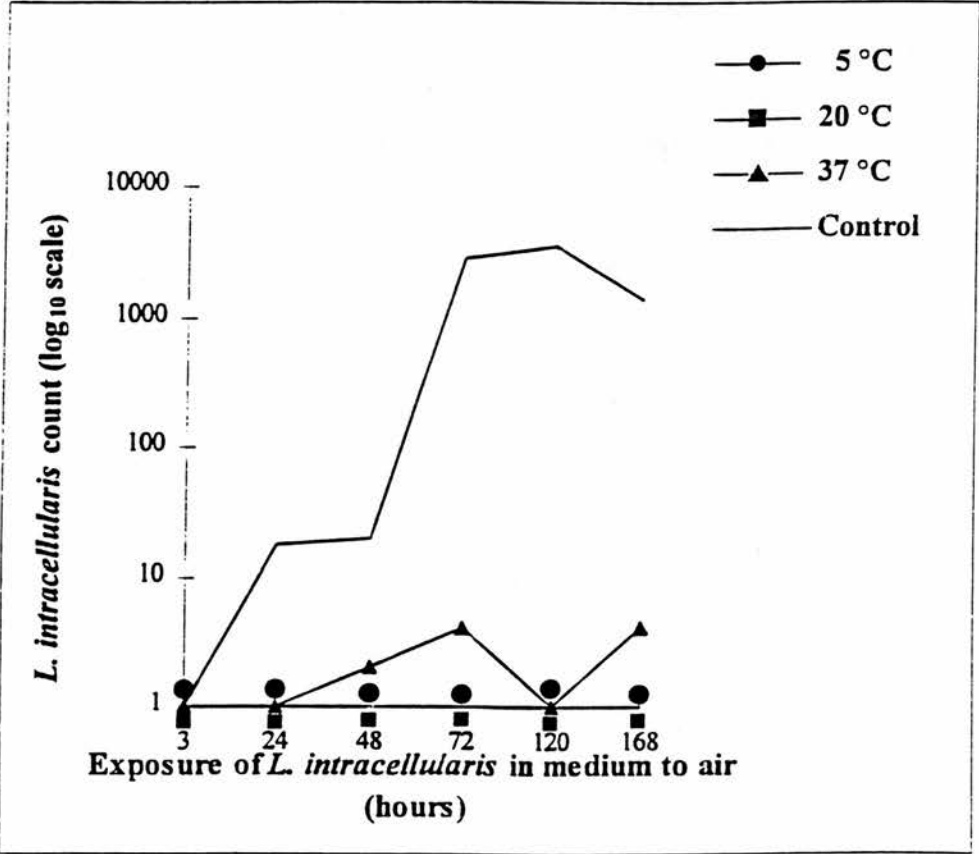**FIGURE 7.1** Survival of *Lawsonia intracellularis* in medium in air

FIGURE 7.2 Survival of *Lawsonia intracellularis* strain 1482/89 after exposure to different temperatures prior to inoculation of cell culture monolayers
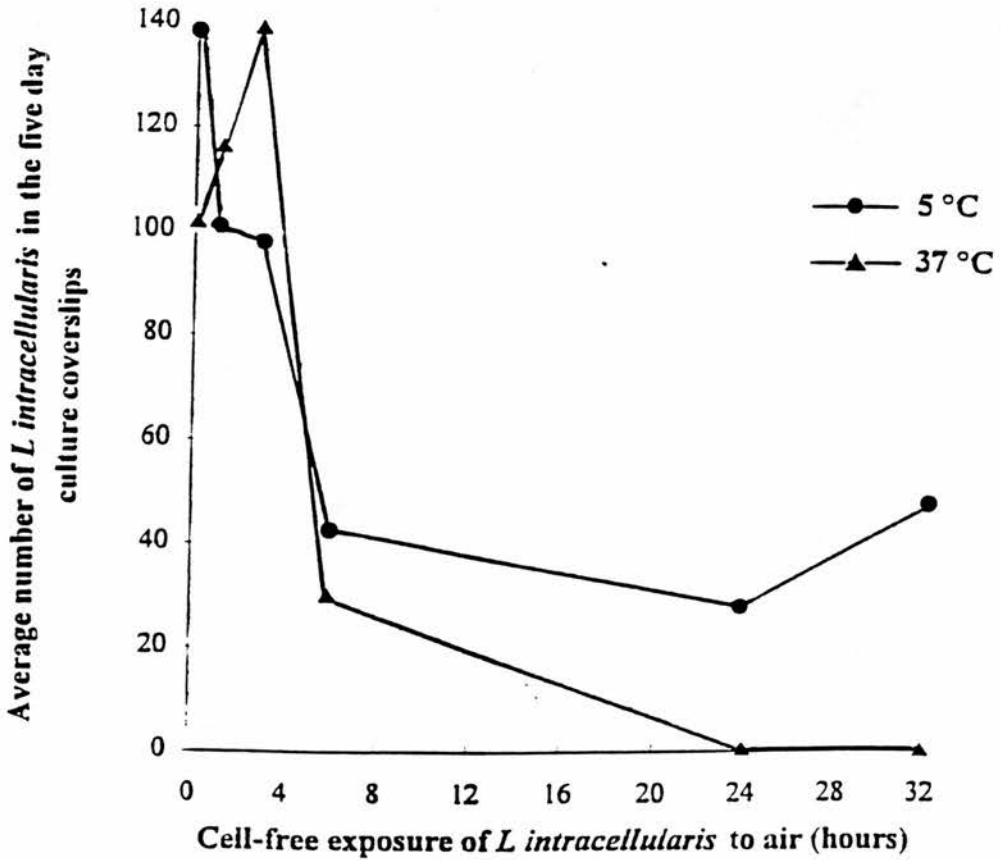
FIGURE 7.3 Survival of *Lawsonia intracellularis* strain LR189/5/83 after exposure to different temperatures prior to inoculation of cell culture monolayers
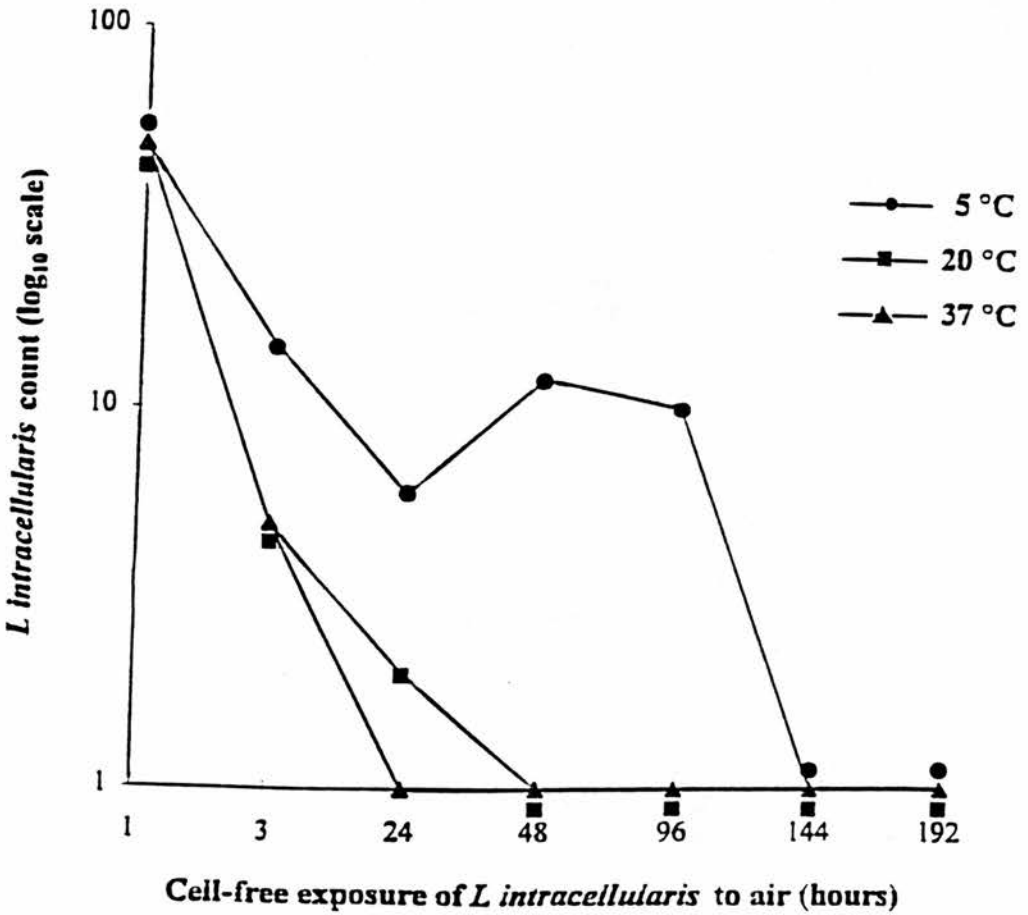
DISCUSSION

If environmental contamination and indirect transmission of infectious organisms are important aspects of PE, then bacterial survival outside the host must contribute to the epidemiology. In this study, *L. intracellularis* organisms were capable of survival in air and certain disinfectants for a considerable period of time, especially in relation to likely cleaning regimes currently in use in the pig industry. *L. intracellularis* is an obligate intracellular organism and cannot survive for long in the extracellular state. Subsequent propagation of infection within a pig herd relies on exposure of susceptible pigs to the organism, followed by generation of the bacteria in the crypt epithelial cells of the terminal ileum. Excretion in their faeces leads to further environmental contamination.

Efficiency of disinfection is important in the control of infectious diseases, particularly those which are spread via the environment. It can be affected by a number of factors, including temperature, pH, moisture levels and dilution of disinfectant and the period of application of the disinfectant. The presence of organic materials such as faeces and dirt, the density of infective animals and the infectivity of the excreted organisms (Fotheringham 1995) may also influence disinfectant efficacy. These factors make standardisation of field trials complex, particularly when applied to accommodation, another highly variable factor.

In this particular study, we found that the most effective disinfectant reagents were sodium hypochlorite and the quaternary ammonium formulation. Although potassium peroxomonosulphate may have been effective against strain 51/89, it damaged the monolayer cells and failed to kill strain 916/91 to a satisfactory degree when diluted to a 1% suspension. The potassium peroxomonosulphate tested was "Virkon", which has been designed as an antiviral compound, although it does have reported activity against spore-forming bacteria, such as *Bacillus subtilis* at 1% dilution (Coates 1996). However, against tougher organisms like *Mycobacteria tuberculosis*, it has very poor efficacy, even when used at 2% (Broadley et al 1993).

This study examined the effect of each disinfectant on the survival of *L. intracellularis* in the absence of organic load. Many efficacy trials for disinfectants have incorporated models which simulate organic load through the addition of clinical substances like blood or serum (Holton et al 1994, Coates 1996). The cell culture monolayer was an integral part of our study design, since it is the only method available for quantification of *L.*

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intracellularis organisms. As it is extremely vulnerable to contamination by organisms ubiquitous in pig faeces, such as coliforms or *Chlamydia* species (Szeredi et al 1996), the model could not include assessment of disinfectants in the presence of faeces. This is important in relation to the two most efficient disinfectants in this study, as the activity of both the chlorhexidine/cetrimide quaternary ammonium mixture and hypochlorite is affected by organic material, such as faeces (Linton et al 1987). The inability of the cell culture monolayer to evaluate the disinfectants in the presence of faecal material was an important weakness of the model. However, even in the presence of faeces, sodium hypochlorite is highly effective against *Serpulina hyodysenteriae,* the causative agent of swine dysentery, with no organisms recovered even after exposure periods as short as five minutes (Chia and Taylor 1978). It also has reported efficacy against other Gram-negative infections of pigs, such as *Actinobacillus pleuropneumoniae*, again even in the presence of organic load (Gutiérrez et al 1995). Nevertheless, thorough cleaning to remove dirt and faeces prior to disinfection with substances like chlorhexidine, quaternary ammonium compounds and hypochlorite solutions, is still necessary to allow optimum activity of these agents.

Some of the disinfectants induced monolayer damage. As a result, no attempt could be made to evaluate their activity. One particular example was the hydrogen peroxide/peracetic acid formulation which, although completely ineffective at 1:1000 dilution, caused extreme cellular damage at normal concentrations. A similar combination has been very effective against various *Mycobacteria* species and *Cryptosporidium parvum* (Holton et al 1994), even in the presence of organic material, so it could be surmised that it would be effective against *L. intracellularis* if used at the correct concentration. This could be true of many of the disinfectants which caused monolayer damage at the recommended concentrations. Although 1:1000 dilution seemed to produce an ineffective mixture in many cases, it was also necessary to avoid monolayer damage. It is highly unlikely that the concentration of disinfectant used in the field would be as low as this. Bacterial resistance to disinfectants, whether acquired or intrinsic, is a recognised occurrence, but it is not believed to parallel antimicrobial resistance (Linton et al 1987). *L. intracellularis* is a Gram-negative organism, therefore it possesses an outer membrane which could function to decrease bacterial permeability to external substances, such as disinfectants. Similarly, the outer membrane of Gram-negative organisms contains porin protein which selectively allows nutrients into the cell, while impeding the flow of larger

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molecules. In the gastrointestinal tract, the presence of such a barrier may provide protection from the toxic effects of fatty acids and bile salts. This barrier may confer a certain amount of intrinsic resistance to some disinfectants, such as phenols (see Table 7.2). Additionally, the shedding of intestinal epithelial cells, concurrent with excretion of *L. intracellularis,* may provide added protection to the bacteria.

It is difficult to make broad comparisons for disinfectant activity against various organisms. Testing methods vary widely from suspension assays, similar to that used in our study, to carrier tests on a selection of different surfaces. Porcine tissue models may more accurately assess antiseptics (Woolwine and Gerberding 1995). While the suspension method may not be the most stringent technique for evaluating these compounds, it did provide a convenient procedure suitable for preliminary screening. Carrier methods may be more accurate, but they must be strictly controlled. Furthermore, they are incompatible with organisms like *L. intracellularis*, as their design would require extracellular testing of an intracellular organism.

The apparent ability of *L. intracellularis* to survive in air for long periods of time was surprising, considering both its fastidious nature and its seeming inability to adopt any of the mechanisms exhibited by other intracellular organisms, such as the production of bacterial endospores by *Coxiella burnetii* (Scott and Williams 1990) which confer increased resistance to desiccation, sunlight, increased temperature and chemical disinfectants. There is no indication that *L. intracellularis* can change form like the *Chlamydia* species, which alter their structure to become elementary bodies capable of independent survival outside the host (Andersen 1993). In the first survival-in-air model, incorporating inoculated monolayers incubated in air at 5°C, 20°C and 37°C for increasing time periods, the aim was to reproduce conditions in the field, where *L. intracellularis* may be excreted within cells shed from the intestinal mucosa. The complete lack of growth at 5°C and 20°C is likely to have been due to a combination of temperature and atmospheric conditions on the IEC-18 cells themselves, which may have influenced their ability to support growth. The optimum conditions for maintenance of *L. intracellularis* within cells is 37°C in a microaerobic atmosphere (Lawson et al 1993). The recovery of a small number of organisms from monolayers maintained at 37°C, would further support this supposition.

In the second model, *L. intracellularis* had increased survival at 5°C compared to 37°C (Figure 7.2) while, in the third model, it survived for up to six days in cell-free medium,

maintained at 5°C (Figure 7.3). Incubation at 20°C and 37°C markedly reduced survival time, no organisms survived longer than 48 or 24 hours, respectively. The extracellular survival of *L. intracellularis* in air at 5°C is highly relevant, since temperatures such as these do occur on farms during British winters, particularly in passage-ways and outside holding pens, for example. It is also likely that this hardiness will be enhanced by faecal material. The causative agent of swine dysentery, *Serpulina hyodysenteriae*, has shown similar survival patterns in the laboratory. Like *L. intracellularis*, it does not respond well to higher ambient temperatures, lasting only 24 hours at 37°C, but can survive up to 48 days at 0 to 5°C within dysenteric faeces. The survival time of *S. hyodysenteriae* was lengthened to 61 days by dilution of infected faeces in tapwater (Chia and Taylor 1978). Olson (1995) found that *S. hyodysenteriae* could survive for five to six days in an aerobic effluent lagoon, in quantities capable of causing disease in sentinel swine. While this study was undertaken in warmer summer months, where decreased survival might be expected, it does indicate that laboratory data should only be used as an approximate guide to field conditions.

CHAPTER EIGHT

GENERAL DISCUSSION

Introduction

Proliferative enteropathy is a complex disease and many of its epidemiological aspects are perplexing. It is becoming an important concern of the international pig industry, because of its significant economic effect and its ability to survive, persist and re-emerge, despite new approaches in herd health management.

Historically, the eradication and elimination of major and ubiquitous pathogens has helped to free niches for other virulent organisms by reducing the competition which had previously restrained them. Despite rigorous control measures instigated in minimal disease and SPF herds, including all-in all-out and strict hygiene, PE is an increasing problem (Boeckman 1995). *Haemophilus parasuis*, the aetiological agent of porcine polyserositis and arthritis, is also in this category of "emerging diseases". Like *Lawsonia intracellularis*, *H. parasuis* is a fastidious organism with very specific culture requirements and is often difficult to isolate from diseased animals (Rapp-Gabrielson et al 1997). In the past, *H. parasuis* has been associated with occasional outbreaks in nursery piglets but its incidence is now increasing, often linked with the porcine reproductive and respiratory virus, another relatively new pig pathogen (Dee 1997).

The main conclusions drawn from my epidemiological investigations partly hinge on the polymerase chain reaction assay for diagnosis of *L. intracellularis* infection in faeces samples. The epidemiological features suggested in the PCR-based challenge and field studies could be combined with many of the questionnaire survey findings to propose some specific control measures for PE.

PCR as a Diagnostic Assay for Proliferative Enteropathy

The PCR has contributed to my initial attempts to elucidate possible excretion patterns in pigs, both experimentally and amongst commercial herds in the field. Its limited sensitivity is common to the PCR-based diagnosis of many faecally-carried infections and is probably due to inadequate removal of inhibitors, rather than an inability to concentrate or purify target DNA. The sensitivity of the PCR for detection of *L. intracellularis* in the challenge studies (Chapter 3) and field studies (Chapter 6) was approximately 5×10^5 organisms per gram of faeces, which was comparable with similar PCR assays used for the detection of other enteric infections. Stacy-Phipps et al (1995) developed a multiplex

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PCR to detect 10^5 enterotoxigenic *E. coli* per gram of faeces, while Widjojoatmodjo et al (1992) reported a PCR sensitivity of 10^6 colony forming units, for detection of *Salmonellae*. Biological metabolites such as haem derivatives and polysaccharides are recognised as inhibitors of the PCR assay (Monteiro et al 1997). Faeces samples also contain a large population of micro-organisms which may lead to a higher concentration of non-target DNA than would normally be present in less contaminated samples, such as blood. This may be significant because non-target DNA is also capable of inhibiting the PCR (Stacy-Phipps et al 1995) by interfering with primer-to-template binding in the amplification reaction.

In my farm sampling survey, there was a tendency for PCR positive faeces samples to be diarrhoeic (Chapter 6), a pattern which has also been reported for other enteric infections. Stacy-Phipps et al (1995) observed this phenomenon in human *E. coli* enteritis and suggested that diarrhoeic faeces had a shorter intestinal transit time, which may minimise the level of PCR inhibitors such as dietary material, bilirubin, urobilinogen and anaerobic bacteria in the final faeces sample. The association between diarrhoeic faeces and successful detection of *L. intracellularis* organisms by PCR may also be due to the presence of a greater concentration of target organisms in the faeces of diarrhoeic pigs. Based on reports of excretion levels for other infectious enteric diseases of pigs, including salmonellosis, where a higher challenge dose produced a higher excretion level (Gray et al 1996), it could be concluded that more severe clinical signs, such as diarrhoea, are associated with an increased faecal burden of *L. intracellularis* in PE. More *L. intracellularis* in the faeces of severely affected pigs would suggest the presence of severely infected hyperplastic lesions in the intestinal mucosa. Both McOrist et al (1993) and Knittel et al (1996b) reported a dose effect in pigs challenged with cultured *L. intracellularis*. Higher challenge doses led to higher percentages of pigs with typical pathological features, clinical signs (including diarrhoea) and PCR positive faeces. While proliferating epithelial cells and an associated increase in mucosal wall thickness may reduce the intestine's capacity to absorb proteins and fluids, the underlying causes of the diarrhoea in PE have not been clarified. Potential toxins have yet to be identified. Most of the body's water absorption takes place in the colon, however, which can also be affected by the proliferative changes observed in PE. Whatever the pathogenic mechanisms involved, if there is a greater risk of transmission of *L. intracellularis* from

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diarrhoeic faeces, then management factors which limit its subsequent contact with pigs are important epidemiologically.

Epidemiological Features and Control of Proliferative Enteropathy

Excretion of L. intracellularis

The duration times of infection and excretion can be critical in the transmission of infectious agents. In PE, a long excretion period may increase the level of *L. intracellularis* in the environment and maximise the number of susceptible pigs which could come into contact with a shedding pig. It may also help to ensure spread of infection through the different stages of production. The challenge work (Chapter 3) indicated that the excretion time of *L. intracellularis* could be protracted in individual pigs. It has generally been assumed that, since the incubation period is around 14 to 21 days and since clinical signs of infection are observed in weaners from six to 16 weeks of age (Rowland and Lawson 1992), young pigs become infected shortly after weaning, develop disease and excrete organisms in sufficient quantity to infect susceptible pigs via the faecal-oral route (McOrist and Lawson 1989a). Since it has been shown that young piglets of seven or 15 days of age are capable of becoming infected experimentally with *L. intracellularis* (McOrist and Lawson 1989b, McOrist et al 1994b), then long-term excretion of organisms would allow transmission of the infection after the piglets are weaned. It was evident from the challenge work that infected pigs could become persistent excretors for at least 10 weeks following infection. Such a long shedding period would also mean that infected pigs could still be contaminating their environment when they reach the grower accommodation. At this stage, groups of pigs are also more likely to be mixed together, depending on the management system, thus adding momentum to the build up of infection. Long-term excretion would explain why infection appeared to have become persistent in two chronically infected herds in my field study (Chapter 6) and is likely to be a critical factor in the prevention and control of PE.

Although other infectious diseases are often considered to be persistent as a result of carriage in tonsils, lymph nodes, spleen or blood, long-term excretion from the gastro-intestinal tract can be as effective in maintaining an infection. Low-grade excretors occur

in swine dysentery and are considered carriers of *S. hyodysenteriae*, despite the fact that these organisms have never been isolated in tissues outwith the large intestine and its lamina propria (Kinyon et al 1980). Gray et al (1996) reported that the challenge dose of *Salmonella choleraesuis* organisms governed both the severity of clinical signs observed, including the presence and extent of diarrhoea and the duration of excretion. Pigs given a high dose (10^9 cfu/ml) of *S. choleraesuis* shed organisms for 15 weeks post challenge and were tissue positive at six and 15 weeks post challenge. Persistent shedding in these pigs, *i.e.* the creation of a carrier state, appeared to be dose-dependent, as pigs given a lower dose only shed organisms for nine weeks post challenge. A low challenge dose (less than 10^3 cfu/ml) failed to induce either clinical signs or faecal shedding. They also found that faecal shedding became intermittent from five to seven weeks after challenge, concluding that negative faecal culture was not a fool-proof method for assessment of herd or individual animal status. Similarly, in the PE challenge trial (Chapter 3), the magnitude of the initial infectious dose appeared to affect both the length of the excretion time of *L. intracellularis* and the likelihood of each challenged pig becoming an excretor. If dose also affects the pattern of shedding over time, then lower doses may produce intermittent shedding. This is important for confirmation of PE in suspect herds, since low levels of excretion might easily be missed by PCR.

Environmental contamination

Indirect transmission of infectious disease requires a vehicle to transport organisms from one host to the next. It is unlikely that *L. intracellularis* can multiply outside the protective environment of the epithelial cell (Gebhart et al 1993). Therefore, the success of indirect transmission will rely on the excretion of organisms in sufficient quantities to ensure survival and recycling of infection in susceptible hosts. The preliminary results of Chapter 6 suggest that *L. intracellularis* is more robust than anticipated, providing certain environmental conditions are met. Also, the sensitivity of the PCR in the field study indicates that the number of organisms excreted in the faeces of diseased pigs can be high. Combined with a long excretion period, these features would point to environmental contamination by and indirect transmission of *L. intracellularis*. This may help to explain why two different age-groups were infected within a short period of time in the field study.

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Direct transmission of *L. intracellularis* is likely to play a large part in horizontal transmission between weaners and growers but the aim of the farm sampling survey (Chapter 6) was to determine if asymptomatic gilts could be infecting their piglets in the farrowing house in a similar way. Although infection was present in the weaner and/or grower stages on three units, no *L. intracellularis* organisms were detected in the faeces of any of the gilts or their litters on two units. On the acutely infected farm, the PCR demonstrated the presence of *L. intracellularis* in two pregnant gilts, but only during the acute outbreak. Although it is possible that some gilts may have been excreting at a lower level, it seems unlikely that they were a direct source of organisms for their piglets. Similar epidemiological observations have been described for porcine cryptosporidiosis. Quílez et al (1996) reported that *Cryptosporidium parvum* was more prevalent in weaners than finishers. Infected animals ranged from one to six months of age and oocysts were not found in suckling piglets or adults. In coccidiosis, although there seems to be a very low prevalence (<5%) of sows excreting *Isospora suis*, piglets are capable of excreting oocysts from four days of age (Lindsay et al 1997). Sources of *Cryptosporidium* and *I. suis* infections for piglets remain unidentified. In cryptosporidiosis, the most likely transmission route is through contamination of the environment by infected growing stock, while *Isospora* is believed to spread via the contaminated farrowing crate and its immediate surroundings (Lindsay et al 1997). Similar to *C. parvum* and *I. suis*, indirect transfer of organisms between pigs seems to be an important route for *L. intracellularis*.

Control of environmental contamination

Based on the apparent importance of indirect transmission, control of PE would appear to rely, at least in part, on reducing environmental contamination. All-in all-out housing of pigs minimises contact between litters and allows completion of comprehensive cleaning procedures between batches of pigs. Many minimal disease herds already conduct such procedures, however, and it seems that more stringent cleaning measures may be required to deal with emerging diseases. In chronically infected grower pigs, which are actively shedding into the environment, the cycle of infection must be broken. To do this effectively requires thorough cleaning in the absence of excreting pigs. Nursery depopulation is a possible option for the control of chronic PE, where it is likely that environmental contamination by older pigs is the cause of infection in younger piglets. It may not be a valid alternative in herds affected by acute PE, since environmental

contamination could still occur from infected breeding adults. Nursery depopulation has been implemented in an effort to eradicate the PRRS virus. Dee and Joo (1994) developed this method to prevent the transfer of infection from older growing stock to newly weaned piglets in herds infected with the virus because all-in all-out systems had not been effective in controlling the disease. Transplacental infection of the foetus can occur in PRRS, and is common in late gestation (Christianson and Joo 1994). In the event of infection *in utero*, attempts to reduce environmental and horizontal transmission are not likely to be completely effective. Therefore, in the control of PRRS, nursery depopulation is only successful in the presence of an immunologically stable population of breeding animals, where the activity of the virus is reduced. The crux of PRRS control appears to be an intense cleaning and disinfection schedule once the immune status of the sows and gilts has been established by serological testing. In view of the significant role which environmental contamination appears to play in PE, the following points may be of value in reducing the environmental burden of *L. intracellularis*:

- Complete depopulation of the nursery
- Wash three times within a one week period with power washer, preferably at 95°C
- Thoroughly disinfect
- Wash out and disinfect slurry pits
- Leave housing empty (down-time)
- Wean pigs off-site and do not reintroduce to the main herd
- Perform strict biosecurity *e.g.* undiluted disinfectant in footbaths

On the whole, measures such as these were successful in eradicating the PRRS virus and improving overall performance. A down-time of fourteen days was incorporated, but this would probably not be necessary for *L. intracellularis*. Based on the survival-in-air studies, a down-time of one week would seem to be sufficient. Particular attention was paid to disinfection of slurry pits in the attempts to eradicate PRRS, since this virus has also been found in faeces. The use of slurry pits in the growing stock accommodation was associated with an increased risk of PE in the postal survey (Chapter 4). Meticulous inclusion of these in the cleaning protocol would be prudent. Depopulation and cleaning are also best performed in the summer months, when the ambient temperature is higher

and likely to reduce bacterial viability. This is recommended practice for a number of pig pathogens, including *S. hyodysenteriae* and PRRS virus.

Pig housing

Control of environmental contamination is an empirical and logical factor in the control of other enteric diseases of pigs, such as colibacillosis, salmonellosis, TGE and spirochaetosis. Nevertheless, in many instances there are other epidemiological factors which require specific control, depending on the disease. The questionnaire survey results indicated that certain management procedures which cause a susceptible pig to come into contact with infected faeces are potential risk factors for the development of PE in a herd. To summarise, although the movement of pigs on a pen basis was not a significant risk factor as such, the all-in all-out housing and movement of grower pigs on a house basis was a significantly protective measure. Slatted and/or meshed flooring was strongly linked to episodes of PE, which was not anticipated. Generally, solid flooring has been associated with an increased risk of enteric disease. Xiao et al (1994) reported an increased *Cryptosporidium* infection rate in nursing piglets and *Giardia* infection in weanlings on a farm with porous concrete flooring, compared to the rate in a cohort farm with slatted or meshed flooring. They also detected *Giardia* infection, but only in sows on the farm which used porous solid flooring. However, *C. parvum* infection, in particular, presents a different clinical picture to PE. Quílez et al (1996) reported in a Spanish survey that, although 24% of weaners and 5.6% of fattening pigs infected with *C. parvum* had diarrhoea, it was not statistically associated with the infection. On the contrary, infection rates were higher in non-diarrhoeic than diarrhoeic pigs in both the weaners and fatteners. Furthermore, the correlation between the excretion of oocysts and diarrhoea showed that infection was asymptomatic in over 90% of pigs. Therefore, specific epidemiological aspects may vary between diseases, depending on whether infectious organisms are transmitted via diarrhoeic or non-diarrhoeic faeces.

In our postal survey, straw bedding featured as a protective factor in the weaner and finisher accommodation (p<0.001 and p<0.05, respectively). Heard (1969) discussed the use of perforated flooring to encourage the removal of faecal material from pens for the control of porcine salmonellosis. He noted that deep straw acted as an extreme form of perforated flooring, allowing rapid passage of faeces and urine. Since it could be argued that it is the diarrhoeic faecal material which harbours the greatest burden of organisms in

PE, straw bedding may provide more protection against infection by allowing fluid faeces to filter through, minimising pig to faeces contact. It is also likely that, in a well-managed deep straw bedding system, clean straw will be placed on top of contaminated straw on a regular basis, with pigs in direct contact with a cleaner surface.

There have been two other recent surveys reported from Spain and the USA. Although they also studied risk factors in combination with PCR on faeces samples, each survey studied less than 75 units (Lanza et al 1996, Bane et al 1997). The American survey found that solid flooring systems, continuous pig flow and lack of hygiene were not associated with PE. The Spanish survey did not identify any risk factors relating to management or housing of pigs.

Source of infection

Knowledge of the health status of herds supplying replacement breeding stock is vital in controlling many pig diseases and active measures are often taken to ensure that such stock are at least specific-pathogen-free. The possible role of nucleus herds was identified in my questionnaire survey as a potential source of infection. Jackson (1980) reported that acute PE was the cause of death in over 70% of boars on four pig testing stations in the UK but there are no other records implicating nucleus herds as a potential source of infection. In my survey, five out of six nucleus herds were PE positive and procurement of replacement boars from such herds was a risk factor for the other types of commercial unit. In addition to their convincing role in the postal survey, boars also featured as positive excretors in my farm sampling survey. This is important epidemiologically, as infected breeding boars may have frequent opportunities to infect both gilts and sows at mating. They could also contaminate passage-ways and mating pens, contributing to the propagation of infection during outbreaks and between outbreaks.

The control of swine dysentery has involved schemes aiming to police herds in their choice of replacement breeding stock. Members of swine dysentery schemes were only allowed to introduce new animals from other qualifying herds, or using hysterectomy/hysterotomy methods, artificial insemination or embryo transfer (Goodwin and Whittlestone 1984). Although successful, such schemes relied on regular veterinary inspections and submission of faeces or intestinal samples to approved laboratories if there

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was any suspicion of disease. One scheme probably owed its success to the prohibition of a designated number of antimicrobials for routine treatment or as growth promoters, including tiamulin and tylosin (Goodwin and Whittlestone 1984). Certain antimicrobials are able to mask the signs of swine dysentery and can invalidate diagnostic tests (Harris and Lysons 1992). It is likely that a control programme based on PCR-monitoring of potential excretors for *L. intracellularis* would have to follow similar guidelines, as both tiamulin and tylosin are probably effective in the treatment and prevention of PE (McOrist et al 1996b, McOrist et al 1997b). In addition, monitoring of replacement breeding stock for the presence of *L. intracellularis* in faeces may require an assay capable of detecting low numbers of organisms. An alternative option would be to purchase replacement stock from a non-medicated herd with no history of PE.

Quarantine and acclimatisation are methods employed in the control of PRRS. Dee et al (1997) recommended that any incoming stock, including replacement breeding gilts, should be isolated in a separate airspace for a set period - as long as 60 days. However, Bane et al (1997) reported that isolation of new pigs had no effect on the occurrence of PE, emphasising that PE may be very difficult to control by such methods, since its onset is often chronic in nature. Although it has been used to control PRRS, it is slightly easier to establish the PRRS status of a herd, due to the availability of an immunofluorescence assay for detection of serum antibodies in exposed animals (Yoon et al 1992). Interpretation of anti-PRRS titres allows an estimation of the stage of infection in a herd and facilitates the design of control protocols which are more suited to individual farm requirements. Determination of overall herd immune status is not an option in the control of PE as there are no blood tests currently available.

Strategic medication

Medicated early weaning (MEW) is one form of strategic medication designed to minimise infection in young piglets and generally requires removal of piglets from their mother at around five days of age. The sow is medicated from five days before to five days after farrowing and the piglets until almost three weeks of age. Medicated early weaning has been implemented in the successful control of TGE and swine dysentery (Alexander and Harris 1992) and *S. hyodysenteriae* has been eradicated from individual units by medicating all pigs for three weeks with antimicrobials known to eliminate the organism (Harris 1984). This has only been worthwhile when other factors involved in the

epidemiology have been taken into account, such as the role of rodents or slurry pits. In coccidiosis, sows are not believed to be a significant source of oocysts for their young as attempts to control the infection in piglets by medicating their mothers with amprolium around farrowing have met with variable success (Ernst et al 1985, Robinson and Morin 1982). Nevertheless, the farrowing area is still considered to be the main source of infection and improvements in performance are believed to have been coincidental with more stringent sanitation procedures. *Haemophilus parasuis* has been controlled to some extent by medication, although this is now becoming less effective (Dee 1997). There is currently no evidence to imply that the mother is a significant source of *L. intracellularis*. The possible effects of sow medication are not known because there has been no specific testing in this area. In Chapter 6, however, medication of male and female breeding adults appeared to decrease the subsequent level of infection in the weaners and growers, compared to the other PE positive units in the study. Strategic medication for PE may be useful in treating affected grower pigs or for preventing it during times of stress, such as movement and mixing, but attempts to eradicate the disease may not be practical in the presence of an unidentified source of infection, *i.e.* reduction may be followed by re-infection.

Porcine and other carrier animals

Without a proper detection or monitoring system, disease may enter a herd via healthy shedding animals, hence unchecked breeding boars may play a significant role. Although *L. intracellularis* was not detected in any of the tissues from group 1 and 2 pigs (Chapter 3) at necropsy between three and 13 weeks post challenge, organisms were still detected in the faeces for a period up to 10 weeks post challenge. A more sensitive diagnostic test may have been able to demonstrate a longer excretion period. If such long-term excretors occur naturally, they could be true or subclinical carrier pigs for PE. Diagnostic reliability would be a key factor in identification of potential carriers on farms but there are still certain diagnostic barriers which hamper epidemiological investigations. Apart from one pig in group 2 (Chapter 3), which failed to gain weight over the period of the trial, it was difficult to differentiate "excretors" from "non-excretors" on the basis of clinical signs, which emphasises the problems associated with detecting individual diseased animals in a large group of pigs. Similar problems have helped prevent eradication of porcine salmonellosis. *Salmonella* spp. can establish in macrophages with a weak cellular immune

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response leading to the creation of healthy, carrier pigs which are difficult to identify (Schwartz 1991). This has made both elimination of the carrier state and eradication of the organism impossible. These difficulties point to the need for multiple sampling on a unit potentially infected with enteric disease, to maximise the chances of selecting a pig which is shedding organisms at a sufficient level to allow detection. With regard to PE, in particular, this presents further obstacles since the PCR is already an expensive assay, even without the added requirement for repeat or multiple testing.

Since rats, mice, dogs, cats and birds feature as carriers, or even hosts, for other pig diseases such as swine dysentery, toxoplasmosis and salmonellosis, this area may be worth specific investigation. There has been no published evidence to implicate non-porcine carriers in the epidemiology of PE. Nevertheless, the list of species reported to have been infected with *L. intracellularis* is growing all the time and now includes the deer, ostrich and emu (Cooper et al 1997, Lemarchand et al 1997), at least underlining the wide host range which *L. intracellularis*, or closely related organisms, seems capable of infecting.

Immunity, stress and disease status

Protracted excretion of *L. intracellularis* by growing pigs and contamination of their environment does not explain why there is thought to be a rising number of acute outbreaks. All-in all-out and disinfection by site, strict biosecurity and quarantine of in-coming stock are among control measures currently in operation. Similar problems have been encountered in the control of *Haemophilus parasuis* infection, where the disease has most commonly occurred in high health pigs (Dee 1997). In one report, immunisation of finisher stock failed to prevent the development of clinical signs upon placement in new, disinfected accommodation (Templeton 1997). Isolation of in-coming replacement breeding stock for 30 days prior to their introduction into a new herd did not prevent an outbreak of *H. parasuis* in the breeding sows three weeks later. It is possible that current management methods which have aimed to improve health status may have actually reduced the immunity of young growing stock to some diseases. Different levels of immunity, together with strain variation and initial infectious dose, may produce different responses to infection, ranging from complete clearance of organisms to a subclinical carriage state. Carriers could flare up at a later time, given the right conditions, such as stress brought on by mixing, other diseases, movement, or extreme temperature fluctuations. Bane et al (1997) found that recent commingling of pigs (in the previous 30

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days) was significantly associated with PE (p<0.05), while recent herd repopulation and the use of new buildings were only marginally significant. They speculated that the introduction of immunologically naive replacement breeding stock, together with an associated reduction in antibiotic usage, might explain these risks. This could also explain the recent upsurge in the acute form of PE in high health herds. Alternatively, commingling of pigs may lead to higher stress levels, in turn reducing immunity and triggering excretion in asymptomatic carriers. Increased stress levels are sometimes caused by intercurrent disease. Dee (1997) suggested that *H. parasuis* might be associated with the appearance of the new PRRS viruses which cause multisystemic disease and immune cell depletion in pigs (Rossow et al 1995). Experimentally, these viruses appear to have a putative role in the pathogenesis of porcine polyserositis and arthritis (Solano et al 1997) and *Streptococcus suis* infection (Galina et al 1994) but there has been conflicting opinion in this area. In my study, the association between PRRS and PE appeared to be confounded by herd size but it is possible that an independent relationship still exists between the two diseases, which could not be established by my survey. For example, the dynamics of the two infections and the relationship between them may differ in larger herds, especially if one or both infections become endemic. This is more likely in a larger herd, where subpopulations of non-exposed animals can occur, leading to fresh cycling of disease at a later date. There is insufficient evidence to preclude the possibility that many recent outbreaks of PE in high health herds could have been due to the existence of previously undetected carrier pigs, since there have been no specific control or monitoring programmes for PE.

Future Work

Improved diagnostic tests

The limitations of the PCR for diagnosis of PE justify further work aimed at improving ante-mortem diagnostic methods. Improved PCR sensitivity would require some major adaptations. Pre-enrichment has been used to improve the sensitivity of a PCR for diagnosis of salmonellosis (Stone et al 1994), but would not be suitable for *L. intracellularis* as culture on cell-free medium is not possible and the cell culture system is incompatible with clinical samples. Nested PCR is also an excellent way to increase the sensitivity. It does, however, require that each extracted DNA sample is processed twice,

doubling the cost of each amplification assay. Nested PCR also necessitates even more scrupulous attention to the separation of DNA extraction and preparation areas from post-PCR areas, to avoid the contamination of samples with PCR product. Because the sensitivity of the assay is heightened by this nested technique, the risk of producing false positive results is much greater.

Possibly the most practical alternatives for improvement of the *L. intracellularis*-specific PCR are immunomagnetic separation and "booster" PCR. The inclusion of magnetic beads coated with the *L. intracellularis*-specific monoclonal antibody IG4 (McOrist et al 1987), for example, may help to separate the organisms prior to DNA extraction and this could potentially increase test sensitivity. However, immunomagnetic separation has been criticised for being both laborious and expensive (Stone et al 1994). "Booster" PCR has already been described for detection of *E. coli* chromosomal segments in faeces samples. Saulnier and Andremont (1992) increased the primer concentration at the end of the first round of amplifications and exposed the same reaction mix to further cycles, using a slightly different temperature profile. If an increase in PCR sensitivity is likely to be accompanied by an increase in costs, then the test would probably not be viable in the veterinary sphere. The expense of the assay is a major drawback for PCR-based diagnosis of PE and is compounded by the fact that reliable diagnosis seems to require a large number of samples.

An immunofluorescence assay has recently been described by Knittel et al (personal communication). It was used to detect serum IgG against *L. intracellularis* in 90% of pigs experimentally challenged with cultured *L. intracellularis*. Concomitant PCR was carried out on faeces samples from the same pigs, but only detected shedding in 43%. This assay appears to have overcome previous problems of variability and poor specificity (Lawson et al 1988, Holyoake et al 1994) by incorporating pure cultured *L. intracellularis* organisms as antigen in the assay. Its increased sensitivity suggests that refined blood assays may be a promising, cheaper and less cumbersome alternative to the PCR.

Vaccination

Vaccination has played a large part in the control of colibacillosis in piglets, mainly by immunisation of the mother prior to farrowing (Hall 1989). The control of other emerging diseases, such as PRRS, has relied to a certain extent on vaccination of naive pigs prior to their introduction onto an infected herd (Dee 1997). In the USA, a modified live virus

vaccine has been produced commercially to induce immunity to the PRRS virus in young growing pigs. This vaccine has also been used to attempt to stabilise the immunity of the breeding population prior to nursery depopulation in the control of PRRS. It does have disadvantages. Its efficacy for heterologous protection is uncertain and it confuses subsequent serological diagnosis due to an inability to distinguish between natural antibodies and vaccine (Dee et al 1996). Control of *H. parasuis* infection by vaccination is also becoming less reliable, due to antigenic variation. There are reportedly as many as 15 different serovars of *H. parasuis* (Kielstein and Rapp-Gabrielson 1992) and experimental work has shown that cross protection does not necessarily occur between all heterologous strains (Rapp-Gabrielson et al 1997). Similar difficulties have impeded vaccination-based control of swine dysentery. In Australia, vaccination of experimentally challenged pigs with an inactivated whole cell vaccine conferred increased protection compared to non-vaccinated control pigs, but this protection was variable and incomplete, with three of six vaccinated animals developing signs of swine dysentery up to four weeks after challenge (Hampson et al 1993). In another trial, Olson et al (1994) found that inactivated, adjuvanted vaccine exacerbated the onset, clinical and pathological signs in those affected, although there were fewer deaths in the vaccinated pigs.

Development of a vaccine against *L. intracellularis* may be feasible in the future. Currently, the cell culture system is not capable of producing the quantity of organisms which would be required for a vaccination trial. In addition, there is very little known regarding the development of natural immunity which, together with potential strain variation, could affect the efficacy of vaccination. Despite these problems, attempts to stimulate the immunity, especially in naive replacement pigs, may be the next logical step in the control of PE.

APPENDICES

APPENDIX A

DNA EXTRACTION AND PURIFICATION KITS

Geneclean II Purification Kit Protocol

1. Excise gel slice of desired band size from gel and weigh

2. Place in a 1.5ml eppendorf tube and add 3 volumes of NaI stock solution

3. Place the tube in a 50°C waterbath for 5 min, to allow complete liquefaction of the gel

4. Add 5μl of 10% glacial acetic acid solution for each 1ml of NaI/gel slice solution

5. Add 5μl of glassmilk (silica matrix in water), mix and place on ice for 5 min

6. Pellet the mixture by centrifuging at 10000 g for 5 sec then aspirate the NaI supernatant

7. Wash the pellet 3 times in 400μl of New Wash (concentrated ethanol solution)

8. Elute the DNA from the silica into 10μl of TE buffer or water by incubation at 50°C for 5 min

9. Centrifuge at 10000 g for 5 sec, and remove the eluted DNA to a fresh tube

10. A second elution step can be performed to increase the final yield of DNA

Wizard Purification Kit protocol

1. Transfer aqueous of PCR product to a clean tube

2. Aliquot 100µl of direct purification buffer to a new tube and 30-300µl of PCR reaction

3. Vortex briefly

4. Add 1ml of resin and vortex briefly 3 times over 1 min

5. Prepare one Wizard column by attaching it to the end of a syringe. Remove the plunger of the syringe and set aside

6. Pipette the resin and PCR product mix into the syringe barrel and reattach the plunger

7. Depress the plunger to gently and slowly force the slurry into the column

8. Detach the syringe from the column and remove the plunger. Reattach the barrel to the minicolumn and pipette 2ml of 80% isopropanol into the syringe

9. Reattach the plunger and wash the column with the isopropanol

10. Centrifuge the column at 10000 g for 20 sec to dry the resin

11. Transfer to a new tube and apply 50µl of water to the column and wait for 1 min

12. Centrifuge for 20 sec at 10000 g to elute the purified DNA

QIAGEN PCR Purification Kit protocol

1. Extract the PCR reaction with 1 volume of chloroform

2. Transfer the aqueous supernatant to a fresh tube and add 1ml of buffer QPT

3. Equilibrate a QIAGEN tip-5 with 1ml of buffer QPT

4. Apply the sample onto the QIAGEN tip-5

5. Wash the tip 3 times with 1ml washes of buffer QB and force out the remaining solution

6. Elute the single and double stranded DNA with 800μl of buffer QF then reinitiate flow with a short push using a pipettor

7. Force out the remaining solution

8. Precipitate the DNA with 0.8 volumes of isopropanol and 0.3M sodium acetate, pH 4.8

9. Incubate for 10 min on ice

10. Centrifuge at 10000 g for 30 min at 4°C and remove the isopropanol

11. Wash the DNA pellet once with 80% ethanol then dry

12. Redissolve in 20 to 50μl of buffer

STAINS

Haematoxylin and Eosin Staining (using Shandon Linistain GLX)

1. Dewax paraffin sections with xylene

2. Rehydrate sections with graded alcohols

3. Rinse in tap water and treat with Lugol's iodine

4. Rinse again, bleach in 5% sodium thiosulphate and wash in water

5. Stain with haematoxylin and wash in water

6. Wash in 1% acid alcohol, tap water and Scott's tap water substitute

7. Wash in tap water

8. Stain slides with 1% aqueous eosin for 1 to 2 min

9. Dehydrate slides in methylated spirits and absolute alcohol

10. Clear slides in xylene and mount

Indirect Immunofluorescence Assay for Detection of *Lawsonia intracellularis* in Fixed

Tissue Sections

1. Cut sections 4μm thick onto vectabonded slides and incubate overnight at 37°C

2. Dewax and bring to water

3. Mount in Shandon sequenza coverplates

4. Trypsinize in 0.1% w/v trypsin with 0.1% $CaCl_2$ in distilled water (pH 7.8)

5. Wash 3 times in 0.1M PBS, pH7.3

6. Add ~ 100μl of mouse monoclonal antibody IG4 diluted 1:200 in PBS

7. Incubate for at 37°C for 1 to 2h

8. Wash 3 times in PBS

9. Add ~ 100μl of rabbit anti-mouse FITC diluted 1:10 - 1:20 in PBS

10. Incubate for at 37°C for 30 min

11. Wash 3 times in PBS

12. Mount in Shandon Immumount and examine under UV light microscope

Young's Modification of the Warthin-Starry Silver Stain

Buffer   Acetate buffer pH 3.8 (critical pH)

              0.2M sodium acetate (1.5ml)

              0.2M acetic acid (15mls)

              Distilled water (500mls)

Solution A   1% silver nitrate in buffer pH 3.8

Solution B   0.2g silver nitrate in 9ml buffer

              0.3g quinol in 8mls buffer

              3g gelatin in 45mls buffer

Method

1.   Bring paraffin sections to water and rinse in distilled water

2.   Rinse sections in buffer

3.   Place slides in 1% silver nitrate in buffer at 60°C for 1h

4.   Rinse in buffer preheated to 60°C

5.   Pour off the buffer and add developing solution B

6.   Mix constantly until the solution becomes golden yellow

7.   Wash the slides in warm running water and mount in neutral mountant

A coplin jar of buffer is also incubated at 60°C and the slides placed in this after impregnation and prior to adding solution B. The silver nitrate and the quinol are placed in clean plastic universals and incubated along with the slides. The gelatin is placed in a

suitable volumetric flask and also incubated with the above at 60°C for 1h. All three are mixed <u>immediately prior to use</u> once the slides have been put into the buffer wash (step 5).

Immunoperoxidase staining (IPX) for *Lawsonia intracellularis* in infected IEC-18 cell monolayers

Materials

Buffer: 0.08% Tween (80)

0.1% bovine serum albumin in 0.1M PBS, pH 7.3

DAB: DAB 10mg

20ml PBS

Filter and add 40μl of H_2O_2 before use

Method

1. Rinse monolayer in 0.1M PBS

2. Block endogenous peroxidase twice in PBS/azide for 15 min

3. Rinse twice in PBS for 10 min each time

4. Rinse in buffer for 10 min

5. Add primary antibody (IG4) diluted 1:200 and incubate at 37°C for 30 min

6. Rinse in PBS then twice in buffer for 10 min each time

7. Add horseradish peroxidase conjugate (sheep anti-mouse IgG) diluted 1:25 and incubate for 1h at 20°C

8. Rinse in buffer for 10 min then rinse in PBS for 10 min

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9.   Place in DAB solution for 5 min

10. Rinse in distilled water

11. Wash in tap water

12. Counterstain with haematoxylin on Shandon Linistain GLX

Agarose Gel Electrophoresis

Materials

1 to 2 g of agarose (for 1 to 2% gel)

100ml of 1 x TAE buffer, pH 8.0 for gel

Approximately 2 litres of 1 x TAE buffer, pH 8.0 for submergence of gel

0.5µg ethidium bromide/ml of gel

Method

1.   Pour buffer into a Pyrex beaker or flask

2.   Add agarose and mix to dissolve

3.   Microwave at full power for approximately 2 min or until mixture starts to boil

4.   Remove from microwave to mix

5.   Return to medium power until agarose is completely dissolved

6.   Cool to 55 to 60°C and add ethidium bromide

7.   Once sufficiently cooled, pour gel into mould and leave to set for 30 to 60 min

8.   Pour 2 litres of TAE buffer over the gel prior to loading the samples

9.   Mix 10µl of sample with 2µl of gel loading buffer and attach to voltmeter

10. Run at approximately 5 volts per centimetre of gel for 1h

Nondenaturing Polyacrylamide Gel Electrophoresis

Materials

Denaturing acrylamide gel solution

10 x TBE, pH 8.0

10% ammonium persulfate

TEMED (N,N,N',N' - tetramethylethylenediamine)

Method

1. Wash the two glass plates and spacers in warm water and detergent, rinse in tap water, deionized water then ethanol

2. Dry and treat one surface of each plate with a silicone solution to prevent the gel from sticking too tightly to the plates and to minimise subsequent tearing of the gel

3. Lay the larger of the two plates down and arrange the spacers on each side to run parallel with the edges

4. Keep spacers in place with petroleum jelly and place the smaller plate on top

5. Bind the side and bottom edges tightly with gel-sealing tape to ensure a watertight seal

6. Add 750µl of ammonium persulfate (10%) and 40µl of TEMED to the polyacrylamide gel solution

7. Pull the mixture into a 60ml syringe and pour immediately into the space between the two glass plates of the sequencing apparatus

8. Hold the plates at a 45° angle to fascilitate even spreading of the gel, then lower to just above the horizontal and maintained at this low angle

9. Place a sharkstooth comb into the gel solution, flat side down and positioned 2 to 3mm below the top of the short plate, carefully avoiding bubbles

Preparing gel for sample loading

1. Remove the tape and clean off any extra acrylamide

2. Remove the combs and clean

3. Fill the bottom reservoir of the tank with 1 x TBE buffer to submerge the gel plates in

2 to 3cm of buffer

4. Place the gel sandwich in the sequencing electrophoresis apparatus and clamp the plates in place

5. Pour 1 x TBE buffer in the top reservoir to about 3cm above the top of the gel

6. Rinse the top of the gel with buffer to remove fragments of loose gel

7. Reinsert the comb ensuring that the teeth form wells in the gel

8. Preheat the gel by running at 45 volts per centimetre for 30 min

9. Load the samples onto the sequencing gel and run at 1500V (55mA) for 3h, at which point samples are reloaded in new lanes and the gel run for an additional 2h

Gel drying

1. Drain the buffer

2. Remove the sandwich and rinse in water to cool the plates

3. Lay the sandwich with the short plate up on paper towels and gently pry the plates apart with the aid of a spatula (the gel should stick to the bottom plate)

4. Once the plates are separated, rinse the gel with dionised water and remove the excess fluid 5.Turn the gel onto Whatman 3MM paper, wrap in Saran Wrap and placed in a gel dryer overnight

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Tris-EDTA buffer (TE), pH 8.0

1. Add 10mM solution of Tris to 1mM EDTA

2. Adjust the pH to 8.0 using HCl

TAE buffer (50x stock solution)

1. Mix 242g of Tris with 100ml of 0.5 M sodium EDTA solution

2. Adjust pH to 8.0 using acetic acid (57mls required)

3. Add water to make 1 litre

4. Autoclave

5 x Tris-borate buffer (TBE)

54g Tris base

27.g boric acid

20ml 0.5M EDTA (pH 8.0)

10 X phosphate buffered saline (PBS), pH 7.3

Materials and method

1. Add 400g NaCl, 10g KCl, 10g KH_2PO_4 (Anhydrous) and 57.5g Na_2HPO_4 (Anhydrous) to 2 litres double deionised water and stir

2. Make up to 5 litres with double deionised water

3. Stir to dissolve and autoclave

4. Dilute 1:10 with deionised water to give 0.1M PBS, pH 7.3

10% Sodium dodecyl sulphate (SDS)

Dissolve SDS in distilled water at a rate of 10% weight/volume

5M NaCl

Dissolve 29.2g of NaCl in water and adjust final volume to 100mls

CTAB/NaCl solution

1. Dissolve 4.1g NaCl in 80mls water

2. Add 10g of CTAB slowly while heating and stirring

3. Adjust final volume to 100ml

This will prepare a 0.7M NaCl solution containing 10% weight/volume CTAB

CTAB = Hexadecyl trimethyl ammonium bromide

_Hae_III digest of ΦX174

1. Dilute 20µg of _Hae_III digested ΦX174DNA in 145µl of marker buffer (100mM Tris Cl pH 8.0 and 1mM EDTA)

2. Add 40µl of gel loading buffer

3. Store 5µl aliquots at -20°C

SOB Medium

Materials

2.0% Tryptone

0.5% Yeast extract

10.0mM NaCl

2.5mM KCl

10.0mM $MgSO_4$

Method (to make 1 litre)

1. Dissolve 20g Tryptone, 5g of Yeast extract and 0.5g NaCl in 950ml deionised water

2. Prepare a 250mM KCl solution by dissolving 1.86g of KCl in 100ml of deionised water. Add 10ml of this stock KCl solution to the solution in step 1

3. Adjust pH to 7.0 with 5M NaOH, then bring the volume to 980ml with deionised water

4. Prepare a 1M solution of $MgCl_2$ by dissolving 20.33g in 100ml of deionised water

5. Autoclave both solutions

6. Cool to 55°C. Add 10ml of 1M filter sterilised $MgCl_2$ and 10ml 1M $MgSO_4$ prior to use

SOC Medium

Add 10ml of 2M filter sterilised glucose solution (36g of glucose in final volume of 100ml deionised water) to 1 litre of SOB medium.

Lysis buffer

120g guanidine thiocyanate

111.2ml 0.1M Tris buffer (pH 6.4)

8.8 ml 0.5M EDTA (pH 8.0)

Triton x 100

Tween lysis buffer

50mM Tris pH 8.5

1mM EDTA

0.5% Tween 20

Diatomaceous earth preparation (DE)

1g DE

50µl HCl (concentrated)

Wash buffer

120g guanidine thiocyanate

100ml 0.1M Tris pH 6.4

LB Agar (per litre)

Materials

10g of NaCl

10g of Tryptone

5mls sterile water

5g of Yeast extract

20g of agar

Method

1. Add the tryptone, yeast extract and NaCl to 950ml of deionised water

2. Adjust pH to 7.0 with 5N NaOH and bring the final volume to 1l

3. Allow to cool and add antibiotic if needed

4. Autoclave and pour into petri dishes (about 25ml/100mm plate)

LB - Ampicillin Agar

Method

1. Autoclave 1 litre of LB agar

2. Cool to 55°C

3. Add 50 to 100mg of filter-sterilised ampicillin depending on the desired final concentration

4. Pour into petri dishes (25ml/100mm plate)

LB - Ampicillin-Methicillin Agar

Method

1. Autoclave 1 litre of LB agar

2. Cool to 55°C

3. Add 20mg of filter-sterilised ampicillin

4. Add 80mg of filter-sterilised methicillin and pour into petri dishes (25ml/plate)

Statistical methods performed by EpiInfo

1. Category variables

To evaluate the relative risk (RR) for a categorical exposure factor, the data is summarised as outlined in Table B1 below.

Table B1

	Disease	No disease	Total
Exposed	a	b	e
Non-exposed	c	d	f
Total	g	h	n

Relative risk (RR): this is the ratio of the incidence of disease in the exposed compared to the ratio of disease in the non-exposed: $a/e \div c/f$. The further the RR is from 1, the greater is its strength of association with the disease. A relative risk of less than 1 suggests a protective factor.

The significance of any association is calculated using the chi-squared (χ^2) test for contingency tables. This test compares the observed data in each of the four categories of the table with the expected values if a particular exposure factor has no effect. The χ^2 is obtained by calculating (observed-expected)^2 / expected for each of the four categories in the table and taking the sum of these values:

$$\chi^2 = \Sigma(O\text{-}E)^2 \div E$$

(O=observed category values and E=expected category values)

By comparing the result with the χ^2 distribution, the probability of an observed difference arising by chance alone can be calculated. EpiInfo calculates the χ^2 test, incorporating a continuity correction, called the Yates' continuity correction:

$$\chi^2 = \Sigma(|O-E|-\tfrac{1}{2})^2 \div E$$

2. Continuous variables.

EpiInfo will calculate the mean, mode, median and standard deviation for continuous variables. For evaluation of the significance of the difference between the mean values of two populations (e.g. diseased and non-diseased) it will establish firstly by analysis of variance (ANOVA) if the two populations have a similar variance. If the data are also normally distributed, then the ANOVA results can be used. If not, or if the variance is different between the two populations, then EpiInfo will perform the Kruskal-Wallis test. This is a non-parametric test which is equivalent to the Mann-Whitney or Wilcoxon two-sample test when only applied to two samples.

3. Confounding variables.

A confounding variable is a factor which correlates with both the disease under study and a proposed causal factor being considered in the analysis. It is the only form of bias which can be adjusted for in the analysis, but requires prior recognition of possible confounding factors.

Stratified analysis in EpiInfo adjusts for this phenomenon by applying the Mantel-Haenszel procedure. This produces a summary relative risk which is weighted to take into account the different relative risks from each stratum produced. The Mantel-Haenszel chi-square is calculated by EpiInfo. A p-value is produced to establish the level of significance which can be attributed solely to one risk factor, taking any confounding by another factor into account.

Cover letter for questionnaire survey

3rd Oct 1995

Dear Sir/Madam,

I am a veterinary surgeon at the Royal (Dick) School of Veterinary Studies in Edinburgh, carrying out a survey on Porcine Intestinal Adenomatosis (P.I.A.).

P.I.A. is a worldwide disease of pigs which causes thickening of the intestines and diarrhoea, resulting in poor weight gains in weaners. As a result, the number of days to slaughter increase. It is currently thought to be present in up to 30% of pig herds in the United Kingdom.

I would be grateful if you would fill in the enclosed questionnaire. Even if you have never had any problems with this disease and can only answer one or two questions, your reply is still important to us.

Please return the completed questionnaire as soon as possible, using the stamped, addressed
envelope provided. We hope to publish our conclusions in one of the pig farming periodicals, although all results from the survey will be confidential and anonymous.

If you have any queries, please contact me at the above address.

Thankyou very much for your time and cooperation.

Yours faithfully,

UNIVERSITY OF EDINBURGH

A SURVEY OF PORCINE INTESTINAL ADENOMATOSIS (P.I.A.) IN PIGS

SECTION ONE:- P.I.A.

1. Do you currently keep pigs?   Yes ☐

   (Please tick one box)   No ☐

   **If yes, please continue with the questionnaire. If no, thank you for your help.
   Please return the questionnaire in the envelope provided.**

2. Have you had P.I.A. diagnosed on your   Yes ☐
   farm in the last three years?
                                                   No ☐
   (Please tick one box)
                                                   Don't know ☐

**IF YOU ANSWERED YES TO QUESTION 2 IN THIS SECTION, PLEASE GO TO QUESTION 3.
IF YOU ANSWERED NO, PLEASE GO STRAIGHT TO QUESTION 6 ON PAGE 2.**

3 If yes, who diagnosed it?   Veterinary surgeon ☐

   (Please tick one box)   Self ☐

                Government Animal Health Service or V.I. Centre ☐

                                Other, please state...

4. How was it diagnosed?   Post-mortem examination ☐

   (Please tick one box)   Symptoms in live pigs ☐

                                                       Don't know ☐

                                Other, please state...

☐☐☐

5. Which of the following clinical signs did you see?

   (Please tick one or more boxes)

diarrhoea	☐
bloody diarrhoea	☐
sudden death	☐
weight loss	☐
reduced weight gains	☐
inappetance	☐

6. Have you ever had any of the following conditions on your farm?

   (Please tick the appropriate boxes for each disease.)

DISEASE	YES	NO	DON'T KNOW
SWINE DYSENTERY			
MANGE			
BLUE EAR DISEASE			
ENZOOTIC PNEUMONIA			
COCCIDIOSIS			
ERYSIPELAS			
PARVOVIRUS			

SECTION TWO:-FARM DETAILS

1. Which of the following best describes your enterprise?

   (Please tick one or more boxes)

Nucleus	☐
Multiplier	☐
Breeder/Weaner	☐
Breeder/finisher	☐
Finisher only	☐
Indoor	☐
Outdoor	☐

   Other, please state..

2. How many sows do you have?   ....

   (Please write the number on the dotted line.)

3. Where do your replacement gilts come from?

   (Please tick one or more boxes)

None stocked	☐
Homebred	☐
Multiplier herd	☐
Nucleus herd	☐

   Other, please state..................................

4. Where do your replacement boars come from?

   (Please tick one or more boxes)

None stocked	☐
Nucleus herd	☐
Multiplier herd	☐
Homebred	☐

   Other, please state...

SECTION THREE:- BIRTH-FINISHING PIGS

Preweaning

1. At what age do you wean your piglets?

   (Please tick one box)

21 days or less	☐
22-28 days	☐
29-35 days	☐
36-42 days	☐
43 days or more	☐

2. a) Do you offer creep feed prior to weaning?

   (Please tick one box)

Yes	☐
No	☐

   b) If so, is it medicated?

   (Please tick one box)

Yes	☐
No	☐

   c) If so, please state the drug(s) used...

Weaning-Finishing

3. Floor Type.

(Please indicate all floor types used on the farm in the last 3 years)

	WEANERS	GROWERS	FINISHERS
SOLID FLOOR			
PARTIALLY MESHED			
PARTIALLY SLATTED			
FULLY MESHED			
FULLY SLATTED			
SHAVINGS			
STRAW BEDDING			

4. Pig Movement and Mixing

a.) Do you routinely practise all in-all out by pen?

(Please tick one box for each group of pigs.)

Weaners	Yes ☐	No ☐
Growers	Yes ☐	No ☐
Finishers	Yes ☐	No ☐

b.) Do you routinely practise all in-all out by house?

(Please tick one box for each group of pigs.)

Weaners	Yes ☐	No ☐
Growers	Yes ☐	No ☐
Finishers	Yes ☐	No ☐

c.) What is the usual number of times groups of pigs are mixed as:

(Please write the number of times in the boxes)

WEANERS ☐

GROWERS ☐

FINISHERS? ☐

5. Water

How is water given to your pigs?

(Please tick which applies to each group of pigs. More than one box may be ticked for each group.)

	WEANERS	GROWERS	FINISHERS
INDIVIDUAL TROUGH			
GROUP TROUGH			
NIPPLE DRINKER			
OTHER, PLEASE STATE			

6. Feed

How is feed given to your pigs?

(Please tick the boxes which apply to each group of pigs.)

	WEANERS	GROWERS	FINISHERS
TROUGH			
HOPPER			
FLOOR			
OTHER, PLEASE STATE			

7. Do you medicate your pig feed?

(Please tick one box for each group of pigs as appropriate, and state the drug(s) used)

	WEANERS	GROWERS	FINISHERS
NEVER			
OCCASIONALLY			
REGULARLY			
ALL THE TIME			
PLEASE STATE WHICH DRUG(S) IS/ARE USED			

8. How is pig dung removed?

(Please tick which method(s) is/are used for each group of pigs.)

	WEANERS	GROWERS	FINISHERS
MANUALLY			
AUTOMATIC SCRAPER			
SLURRY SYSTEM			
OTHER, PLEASE STATE			

9. If a slurry system is used, how many times a month is it emptied?

(Please write the number of times in the box next to each group of pigs.)

WEANERS ☐

GROWERS ☐

FINISHERS ☐

No slurry system used ☐

SECTION FOUR:- SOWS AND GILTS

1. Do you now, or have you in the last three years
   medicated your <u>sows</u>' feed?

   (Please tick one box)

   Yes ☐

   No ☐

   If yes, please state the drug(s) you use...

2. Do you now, or have you in the last three years
   medicated your <u>gilts</u>' feed.

   (Please tick one box)

   Yes ☐

   No ☐

   If yes, please state the drug(s) you use ....

3. How do the sows and gilts receive their water?

   (Please tick one or more boxes)

	SOWS	GILTS
NIPPLE DRINKER		
INDIVIDUAL AUTOMATIC DRINKER		
TROUGH		
OTHER, PLEASE STATE		

4. Do you now, or have you in the last three years
    medicated your <u>sows</u>' water?   Yes   ☐

                                                         No   ☐
    (Please tick one box)

    If so, please state the drug(s) you use...

5. Do you now, or have you in the last three years
    medicated your <u>gilts</u>' water?   Yes   ☐

                                                         No   ☐
    (Please tick one box)

    If so, please state the drug(s) you use...

6. Would you be prepared to be involved further in this study?

    If so, please write your name and address below.

    Name   ....

    Address ....

                 ....

                 ....

THANK YOU FOR YOUR TIME FILLING IN THIS QUESTIONNAIRE

PLEASE RETURN IT IN THE STAMPED ADDRESSED ENVELOPE PROVIDED.

APPENDIX C

MATERIALS

$\alpha^{35}S$	(Amersham)
Acetic acid (glacial) AnalaR	(BDH)
Acrylamide 100g	(Promega)
Agarose (electrophoresis grade)	(Sigma)
Ammonium persulfate 25g	(Promega)
Ampicillin	(Sigma)
Apa	(Promega)
Battle's Black disinfectant	(Battle, Hayward and Bower Ltd.)
Baytril 5% injection	(Bayer)
Biotaq	(Bioline)
Bovine serum albumin	(Sigma)
Chloroform/isoamyl alcohol (24:1)	(Sigma)
Columbia agar base	(Oxoid)
CTAB (Hexadecyl trimethyl NH_4 Br)	(Sigma)
DAB	(Sigma)
Deoxyadenosine triphosphate (dATP)	(Pharmacia)
Deoxycytodine triphosphate (dCTP)	(Pharmacia)
Deoxyguanosine triphosphate (dGTP)	(Pharmacia)
Deoxyuridine triphosphate (dUTP)	(Pharmacia)
Diatomaceous earth	(Sigma)

DN*Ace* Clinipure Purification System	(Bioline)
Domestos	(Centaur Services)
Dri-block DB-3H	(Techne)
*EcoR*I	(BRL)
EDTA	(Sigma)
EpiInfo version 6	(Centers for Disease Control and
Prevention,	Atlanta, Georgia, USA)
Eppendorf microcentrifuge tubes	(Alpha Laboratories)
(0.5, 1.0ml)	
Eppendorf tips (1-100µl, 100-1000µl)	(Alpha Laboratories)
Ethanol AnalaR	(BDH)
Ethidium bromide 10mg/ml	(Sigma)
Falcon 2059 polypropylene tubes	(Becton Dickinson)
Flow tissue culture slide	(Flowgen)
fmol sequencing kit	(Promega)
Gel loading buffer	(Sigma)
GenecleanII purification	(BIO 101 Inc., CA, USA)
Guanidine thiocyanate	(Sigma)
*Hae*III digest (ΦX174)	(Sigma)
Hybaid Recovery Amplification Kit	(Hybaid)
Hydrogen peroxidase	(FSA Laboratories, Fisons)
Hydrochloric acid AnalaR	(BDH)
IPTG 1g (Isopropylthiogalactopyranoside)	(Stratagene)
Isopropanol AnalaR	(BDH)

Kodak X-Omat film	(Sigma)
Magnesium Chloride	(BRL Life Technologies)
Methicillin	(ICN)
MSE Microcentrifuge	(Lehes)
Mineral oil	(Sigma)
Monoclonal antibody IG4	(SAPU)
*Not*I	(Promega)
Orthoboric acid AnalaR (Boric acid)	(BDH)
PCR Buffer	(BRL Life Technologies)
pCR-Script Amp SK (+) Cloning Kit	(Stratagene)
Petroleum jelly	(Boots Chemist plc)
Pevidine	(C-Vet Livestock Products)
pGEM-T Vector System	(Promega)
Phenol chloroform isoamyl alcohol (25:24:1)	(Sigma)
Primers (*L. intracellularis*-specific)	(Oswel Genetics)
Proteinase K 20mg/ml	(Sigma)
Qiagen tip-5	(Qiagen)
Qiaprep plasmid extraction kit	(Qiagen)
Sac	(Promega)
Saran wrap	(Genetic Research Instrumentation Ltd.)
Savlon Veterinary Antiseptic Concentrate	(Mallinckrodt Veterinary Ltd.)
Sealant tape	(Philip Harris Scientific)
Sequencing gel apparatus	(Pharmacia)

Sheep anti-mouse affinity-purified IgG   (Sigma)

fluoroscein isothiocyanate conjugate

Silicone   (Philip Harris Scientific)

Sodium chloride   (BDH)

Sodium dodecyl sulphate   (Sigma)

Sodium hydroxide AnalaR   (BDH)

Sorgene   (Sorex Ltd.)

Sterile Tissue Culture Water   (Sigma)

Submarine gel electrophoresis apparatus   (Pharmacia GNA-200)

TA Cloning System   (Invitrogen)

Taq polymerase   (BRL Life Technologies)

TEMED 50ml   (Promega)

Thermal cycler PHC3   (Techne)

Tiamutin 2% premix   (Leo)

Triton X100   (Sigma)

Trizma base   (Sigma)

Tryptone   (Oxoid)

Tween 20   (Sigma)

Tween 80   (Sigma)

Virkon   (Antec International)

Voltmeter   (Biorad)

Whatman 3MM paper   (Phillip Harris)

Wizard DNA purification columns   (Promega)

X-Gal 250mg   (Stratagene)

(bromochloroindoylgalactopyranoside)

Yeast extract   (Oxoid)

ADDRESSES

Alpha Laboratories Ltd. Parham Drive, Eastleigh, Hants. SO50 4NU, UK

Amersham International plc. Amersham Place, Little Chalfont, Bucks. HP7 9NA, UK

Antec International Sudbury, Suffolk CO10 6XD, UK

Battle, Hayward and Bower Ltd Allenby Road Industrial Estate, Lincoln, UK

Bayer plc. Animal Health Business Group, Eastern Way, Bury St. Edmunds, Suffolk IP32 7AH, UK

BDH Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leics. LE17 4XN, UK

Becton Dickinson & Co. 2 Bridgewater Lane, Lincoln Park, New Jersey 07035, USA

Boots Chemist plc. Nottingham, UK

BRL Life Technologies 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK

C-Vet Livestock Productions Leyland, UK

Centaur Services Castle Cary, Somerset, UK

Fisons Scientific Equipment Bishop Meadow Road, Loughborough, Leics. LE11 0RG, UK

Genetic Research Instrumentation Ltd. Gene House, Dunmow Road, Felsted, Dunmow, Essex CM6 3LD.

ICN Biomedicals Ltd. Unit 18, Thame Park Business Centre, Wenman Road, Thame, Oxfordshire OX9 3XA, UK

Invitrogen BV DeSchelp 12, 9351 NV Leek, The Netherlands

Lehes Laboratory Utrustning and Service Stockholm, Sweden

Leo Laboratories Ltd. Longwick Road, Princes Risborough, Bucks. HP27 9RR, UK

Mallinckrodt Veterinary Ltd. Uxbridge, Middlesex, UB9 6LS

Oswel Genetics Oswel DNA Service, Laboratory 5005, Medical and Biological Science Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton SO16 7PX, UK

Oxoid Wade Road, Basingstoke, Hants., RG24 0PW

Pharmacia Biotech 23 Grosvenor Road, St. Albans, Herts. AL1 3AW, UK

Phillip Harris Scientific 2 North Avenue, Clydebank Business Park, Clydebank, Glasgow G81 2DR, UK

Promega Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK

Quiagen Ltd. Boundary Court, Gatwick Road, Crawley, West Sussex RH10 2AX, UK

Scottish Antibody Production Unit (S.A.P.U.) Law Hospital, Carluke, Lanarkshire, UK

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PUBLICATIONS ARISING FROM WORK IN THIS THESIS

McOrist S., Smith S.H., Shearn M.F.H., Carr M.M. and Miller D.J.S. (1996) Treatment and prevention of porcine proliferative enteropathy with oral tiamulin. Vet. Rec. 139:615-618.

Smith S.H. and McOrist S. (1997) Development of persistent intestinal infection and excretion of *Lawsonia intracellularis* by piglets. Res. Vet. Sci. 62:6-10.

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Papers and Articles

Treatment and prevention of porcine proliferative enteropathy with oral tiamulin

S. McOrist, S. H. Smith, M. F. H. Shearn, M. M. Carr, D. J. S. Miller

The effect of an oral treatment or prevention programme, incorporating the antibiotic tiamulin, on the development of proliferative enteropathy in experimentally challenged pigs was studied. Twenty weaner pigs were challenged orally with a virulent inoculum of *Lawsonia intracellularis* strain LR189/5/83, a British isolate of the causative agent of porcine proliferative enteropathy, and seven control pigs were dosed with a buffer solution. Seven of the 20 challenged pigs were left untreated; they gained less weight than the controls and three of them developed mild to moderate diarrhoea two weeks after the challenge. All seven developed lesions, six visible grossly, of proliferative enteropathy, and numerous intracellular *L intracellularis* were detected in sections of the intestines examined three weeks after the challenge. To test a 'prevention' dosing strategy for tiamulin, six of the challenged pigs were dosed orally with 50 ppm tiamulin, incorporated in a 2 per cent stabilised premix, given from two days before the challenge until they were euthanased. To test a 'treatment' strategy, the remaining group of seven challenged pigs were dosed orally with 150 ppm tiamulin given in the premix from seven days after challenge until they were euthanased. All the control pigs and the 13 pigs treated with tiamulin, either before or after challenge, remained clinically normal and had no specific lesions of proliferative enteropathy in sections of the intestines examined post mortem.

PROLIFERATIVE enteropathy (ileitis) is a common disease which affects pigs maintained under various management systems worldwide. It is particularly noticeable in herds of high health status, and occurs in acute and chronic forms. In pigs six to 16 weeks old, the chronic form of the disease is evident as a reduction of growth rate and a failure to thrive. Estimates of the reductions in weight gain and feed conversion efficiency are generally between 20 and 30 per cent (Gogolewski and others 1991, Mackinnon 1993). Older pigs with the acute form develop bloody diarrhoea and die suddenly. Previous attempts to formulate advice on possible treatments were hampered by the lack of data on the causative agent and its antibiotic susceptibility, and on the likely responses of the host to treatment. Furthermore, previous models of infection with proliferative enteropathy were based on inocula consisting of homogenised intestinal mucosa derived from naturally affected animals (Roberts and others 1977, Mapother and others 1987). In some studies, corticosteroids were given to the challenged pigs to increase the infectivity of the inoculum (Mapother and others 1987). No specific treatment trials have been reported using that type of model. The selection of medications for the treatment and control of the disease has therefore relied on empirical clinical data without the use of a satisfactory model of infection in the target species, incorporating controls and dose titrations.

Recent work has established that the causative agent is *Lawsonia intracellularis*, an obligate intracellular bacterium (McOrist and others 1993, 1994, 1995a). The cell culture of this organism requires specialised techniques, including microaerobic conditions and cell lysis passage techniques. In spite of these difficulties, data on the likely minimum inhibitory and bacteriocidal concentrations of several antibiotics for *L intracellularis* have been reported (McOrist and Gebhart 1995, McOrist and others 1995b). The preparation of large quantities of organisms for challenge trials is difficult and time consuming, but conventional pigs are highly susceptible to infection (McOrist and others 1993). Germ-free pigs are resistant to colonisation (McOrist and others 1993). Recent studies have reproduced the chronic form of the disease in recently weaned pigs, which have shown some reductions in weight gain. It is difficult to reproduce the acute form of the disease owing to problems in procuring susceptible pigs four to five months old.

The pleuromulin antibiotic tiamulin is widely used in the pig industry as in-feed medication for the prevention and treatment of diseases caused by bacteria sensitive to its action. For example, it is widely used to prevent swine dysentery caused by the anaerobe *Serpulina hyodysenteriae*. Tiamulin acts by binding to bacterial ribosomes and blocking protein synthesis. The minimum inhibitory and bacteriocidal concentrations of tiamulin for *L intracellularis* have been estimated in vitro (McOrist and Gebhart 1995, McOrist and others 1995b).

Materials and methods

Bacteria

L intracellularis strain LR189/5/83.1 was isolated by co-culture in the rat enterocyte cell line IEC-18 (American Type Culture Collection CRL 1589) using the methods described by Lawson and others (1993). This strain was originally isolated from the intestinal lesions of acute proliferative enteropathy in a five-month-old British pig. Following its initial growth after the inoculation of porcine-derived intestinal material onto cultured cells, the bacterial strain was released from the cells by cell lysis and passaged on to fresh cells. After four passages, the bacteria were stored frozen at −70°C.

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During its isolation the cultured strain had been tested for freedom from *Chlamydia* and *Mycoplasma* species and other bacterial contaminants by cultural methods and standard immunological methods, using commercial reagents (Lawson and others 1993). For this trial, frozen bacteria were resuscitated for co-culture in the enterocyte cell line and expanded by weekly passage into large cell culture flasks. Previous animal challenge trials had indicated that at least 10^6 organisms, from the lowest possible passage number, were required in an oral inoculum to ensure that most challenged pigs developed disease (McOrist and others 1993). After nine passages, the bacteria were finally harvested fresh on the day of challenge, both from within the supernatant portion of the cultures and from the infected cells collected from the flasks. Bacteria for the inoculation of pigs were suspended in sucrose-potassium glutamate buffer containing 5 per cent fetal calf serum, a preservative for intracellular bacteria (Lawson and others 1993). The inoculum was kept chilled before the oral challenge. Portions of the inoculum were retained for culture of *L intracellularis* (see below). Also, 1 ml of the inoculum was distributed onto two blood agar plates and two brucella broths (Oxoid) and incubated in an atmosphere of 7 per cent oxygen, 10 per cent carbon dioxide and 83 per cent nitrogen for seven days at 37°C.

Number and infectivity of bacteria

The number of *L intracellularis* bacteria in the inoculum was assessed by three different methods. First, cytospin smears of the supernatant portion of the inoculum were stained directly for *L intracellularis* by a modified Ziehl-Neelsen stain and examined by light microscopy. Secondly, 1 ml of a 1/80 dilution (in medium) of 1 ml of the supernatant portion of the inoculum was added to each of four IEC-18 cell cultures grown on 13 mm diameter glass coverslips in small cell culture bottles. The inoculated cells were incubated by the standard methods for five days and immunostained as described below. Thirdly, as the flask cell cultures were being inoculated with cell lysate containing *L intracellularis* one week before final harvesting, two separate cultures of IEC-18 cells grown on the coverslips (area 1·33 cm^2) in small cell culture bottles were inoculated and incubated in parallel for five days as a monitor of the flask cultures. After harvesting and washing, the coverslip co-cultures were immunostained for *L intracellularis* by an indirect immunoperoxidase method incorporating monoclonal antibody (mAb) IG4 specific to *L intracellularis* (McOrist and others 1987). The number of heavily infected cells (>30 bacteria per cell, estimated average 50 per cell) was counted on each coverslip. A heavily infected cell was assumed to have arisen from the multiplication of one bacterium entering the cell. Calculations incorporating the dilutions and areas of the cultures gave an estimate of the number of viable organisms present in the experimental inoculum (Table 2).

Pigs

The pigs were housed at the Institute of Animal Health, Compton, after having been procured at weaning (24 days old) from a local source of a commercial synthetic breed of hybrid landrace/large white. Twenty-seven pigs were split into four groups on a stratified weight basis, and were placed into barrier housing. The pigs in groups 1 and 4 were fed antibiotic-free feed and those in groups 2 and 3 were given feed containing the test antibiotic at the nominal doses (Table 1). The tiamulin had been obtained as a proprietary preparation of Tiamutin 2 per cent Premix (Sandoz) from Leo Laboratories. The pigs were examined clinically every day and weighed weekly.

Trial protocol

The trial protocol is summarised in Table 1. The pigs in groups 1, 2 and 3 were each challenged orally with 10 ml of the fresh inoculum, given via a stomach tube attached to a syringe.

TABLE 1: Group allocations and protocol

Group	Pigs	Challenge with *L intracellularis*	Antibiotic	Strategy	Nominal dose* (ppm)	(mg/kg bodyweight/ day)
1	1-7	Yes	None	–	–	–
2	2-13	Yes	Tiamulin	Prevention	50	2·5
3	14-20	Yes	Tiamulin	Treatment	150	7·5
4	21-27	No	None	–	–	–

* Actual doses on analysed feed samples were within 5 per cent of the nominal doses
ppm Parts per million in the feed

Group 2 pigs were given feed containing premix with oral medication, starting two days before the oral challenge, and continuing throughout the trial, a 'prevention' strategy. The nominal dose of oral medication was 50 ppm tiamulin (approximately 2·5 mg/kg bodyweight per day).

Group 3 pigs were given feed containing premix with oral medication, starting seven days after the challenge and continuing throughout the remainder of the trial, a 'treatment' strategy. The nominal dose of oral medication was 150 ppm tiamulin (approximately 7·5 mg/kg bodyweight per day).

The pigs in groups 1, 2 and 3 were challenged once with the inoculum, all the doses being given in one afternoon, group after group, with group 1 being dosed last. The pigs of group 4 were dosed orally with 10 ml of sucrose-potassium glutamate preservative alone, and the pigs of groups 1 and 4 were given unmedicated feed throughout. None of the pigs was given any other form of medication. All the pigs were necropsied three weeks after the challenge or control dosing.

Necropsy

The pigs were euthanased with a lethal injection of barbiturate. A thorough examination was made of each pig and samples of the proximal ileum, mid ileum, terminal ileum, proximal colon, caecum, spiral colon, ileocaecal lymph node and tonsil were collected from each of them. These tissues were fixed immediately by immersion in 10 per cent buffered formalin and processed routinely for histological examination. Separate sections of each tissue were stained with haematoxylin and eosin for light microscopical examination, and by the indirect immunoperoxidase stain incorporating mAb IG4 for *L intracellularis* (McOrist and others 1987).

TABLE 2: *Lawsonia intracellularis* LR 189/5/83 challenge inoculum

Inoculum sample and analysis	*L intracellularis* visualised	Count of heavily infected cells
Supernatant portion Cytospin smear, modified Ziehl-Neelsen	+++	(100%)
1/80 dilution, coverslip culture	+++	244 269 214 187
	Mean	229
Cell lysate portion Parallel coverslip culture	+++	100,000

Calculation of numbers of *L intracellularis* in inoculum

Supernatant portion: 229 (mean number of heavily infected cells)
x 50 (average bacteria per cell)
x 80 (dilution)
x 250 (volume of supernatant in inoculum) = $2·29 \times 10^8$

Cell lysate portion: 100,000 (number of infected cells per coverslip)
x 50 (average bacteria per cell)
x 175/1.33 (ratio of flask/coverslip area)
x 18 (number of flasks) = $1·18 \times 10^{10}$

Total = $1·2 \times 10^{10}$ in 350 ml = $3·4 \times 10^8$ in 10 ml inoculum
+++ Numerous heavily infected cells present

Further sections of the intestinal tissues were stained with Young's silver stain for intracellular bacteria.

Results

Inoculum

Stained cytospin smears of the supernatant portion of the inoculum showed numerous bacteria resembling *L intracellularis* within all the detached cells, and many free bacteria. Culture of the inoculum on agar and broths showed no evidence of bacterial growth after two, five or seven days. The counts of cells heavily infected with *L intracellularis* in co-cultures derived from a 1/80 dilution of the supernatant portion of the inoculum are shown in Table 2. The counts of heavily infected cells in coverslip co-cultures incubated in parallel with the 18 final inoculum flasks are also shown in Table 2. The combination of the two values gave an estimated number of *L intracellularis* in the final inoculum of 3·4 $\times 10^8$ per pig.

Clinical findings

Three pigs in group 1 developed mild to moderate diarrhoea two weeks after challenge. All the other pigs remained clinically healthy. Several pigs in group 1 failed to gain weight as quickly as those in group 4. The mean weight of the group 1 pigs at necropsy (11·1 kg, average daily gain 248 g per day) was 19·6, 9·0 and 14·0 per cent, respectively, below that of the pigs in group 2 (13·8 kg, average daily gain 362 g per day), group 3 (12·2 kg, average daily gain 295 g per day) and group 4 (12·9 kg, average daily gain 314 g per day).

Necropsy findings

The results of the examinations are presented in Table 3. The challenged animals clearly received *L intracellularis* in sufficient quantities to cause disease because all the untreated animals in group 1 developed disease. Six of the pigs in this group had grossly visible lesions (Table 3) and three had relatively severe changes (Fig 1), even though they had been dosed only three weeks earlier. The histological lesions of proliferative enteropathy observed in the pigs of group 1 have been described and illustrated fully by McOrist and others (1993). No lesions were evident in any of the pigs in groups 2, 3 or 4, and all intestinal sections appeared normal.

TABLE 3: Results of necropsy examinations

	Gross findings*		% Histological lesions of proliferative enteropathy†	
	Ileum/caecum	Other sites	Ileum	Caecum
1	Mucosa Hpl	–	1	0
2	Mucosa Hpl	–	100	100
3	Mucosa Hpl	–	10	50
4	Mucosa Hpl	–	50	40
5	–	–	2	0
6	Mucosa Hpl	–	100	100
7	Mucosa Hpl	–	30	30
8-13	–	–	0	0
14	Ileal lesions?	–	0	0
15	Ileal lesions?	–	0	0
16	–	–	0	0
17	–	Serositis	0	0
18-20	–	–	0	0
21-27	–	–	0	0

*Gross findings
Mucosa Hpl, Thickening and/or hyperplasia of the intestinal mucosa of the ileum and/or caecum; – No apparent lesions detected; Serositis, Multifocal deposition of creamy serofibrinous plaques over serosa of liver, spleen and intestines
†Histological findings
Results expressed as the approximate percentage of the total number of intestinal crypts in sections of each area that were enlarged and positive for intracellular *L intracellularis* bacteria

The serositis observed in one pig was probably due to a previous septicaemic event and was considered to be incidental and unrelated to the treatment or challenge.

Discussion

To test the effect of oral treatment and prevention strategies in porcine proliferative enteropathy, an accurate, semi-quantitative challenge model was used. It was found that continuous oral medication with 50 ppm tiamulin in the feed could prevent pigs challenged with pathogenic *L intracellularis* from developing the disease. A therapeutic effect was also evident when 150 ppm tiamulin was given orally to pigs seven days after the challenge, indicating that tiamulin could treat the disease. The results of the trial also show that proliferative enteropathy can be readily reproduced in pigs challenged with *L intracellularis*. This is the first time that a challenge model with pure cultures has been used to test any compounds for their ability to prevent or treat the disease.

The results of this trial can be compared with the results of in vitro determinations of the minimum inhibitory and bacteriocidal concentration (4 µg/ml) for tiamulin hydrogen fumarate against *L intracellularis* (McOrist and Gebhart 1995, McOrist and others 1995b). That value was considered low to moderate, indicating that tiamulin was capable of inhibiting the organism's growth within cultured enterocytes. To be effective in vivo, however, a compound must attain inhibitory concentrations of the active constituent within enterocytes in the lower bowel of the pig after oral dosing. Other factors in this process include the passage of the compound through the gastrointestinal tract, the appropriate localisation of the compound in the lower bowel, the internalisation of the compound into the cytosol of enterocytes at the site, and the activity of the compound in the cells in the conditions of the lower bowel of the pig, a site of bacterial fermentation and low oxygenation. The results of this trial indicate that tiamulin is capable of overcoming these factors and retaining a high activity against *L intracellularis* in the intestinal cells of the pig. The in vivo test may be considered a more valid indicator of antimicrobial activity than the in vitro tests, which are not always an accurate guide.

No specific field or experimental infection trials have been reported for the use of tiamulin in proliferative enteropathy, but some previous reports of the clinical use of various compounds for the treatment of naturally occurring disease have suggested that tiamulin can be of benefit (Loula 1992, Straw and Henry 1994). There was, however, no scientific basis for the clinical selection of drugs against this disease before this trial was conducted. It may be of benefit to conduct further animal trials, using this challenge model and natural outbreaks of the disease to assess the efficacy of different inclusion levels of tiamulin in the feed and alternative delivery systems such as injections or medication of the drinking water.

Until recently, the only challenge model available for this disease relied on the use of inocula consisting of homogenised intestinal mucosa derived from naturally occurring cases of proliferative enteropathy (Mapother and others 1987). That type of

FIG 1: Proximal colon and caecum of pig 6 from group 1. There was marked thickening of the mucosa of both areas of large intestine

inoculum has several possible disadvantages, including the likelihood of considerable variations in the infective dose of *L intracellularis* derived from the donor pigs and administered to the challenged pigs, the presence of other infective agents in the inocula and the potential for gut material in the inocula to delay or interfere with the passage of the infective portion of the inoculum to the lower bowel. Of greatest concern for treatment trials, in which strict comparisons between groups need to be made, would be any variation in the infective dose administered to the challenged pigs. With homogenised mucosa as the inoculum, no clear estimation of the infective dose or viability of the causative agent received by the animals can be made. Possibly for these reasons no reports have been published of the use of such materials for treatment trials. The development of the challenge model using an oral inoculum of pure bacteria therefore provides a breakthrough in the investigation of this disease, because the inoculum for each pig contained the same number of the same strain of viable organisms. As a result the model can yield specific and valid conclusions for drug usage.

The reduction in the growth rate of the piglets observed in this trial agree with the reductions which have been observed in field and experimental studies of proliferative enteropathy (Roberts and others 1977, Gogolewski and others 1991). Moreover, it is the first trial to compare the growth rate of affected pigs with the growth rate of challenged pigs also receiving oral antibiotic in a premix formulation. The markedly higher growth rate of the pigs receiving 50 ppm tiamulin from two days before the challenge could indicate a useful economic benefit from the use of the

antibiotic. Although tiamulin has been widely used in the British pig industry since 1981 for the control of swine dysentery, there has been no firm evidence, either in this trial or elsewhere, of resistance to pleuromulins (Taylor 1995).

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Comparison of the absorbed ELISA and agar gel immunodiffusion test with clinicopathological findings in ovine clinical paratuberculosis

C. J. Clarke, I. A. P. Patterson, K. E. Armstrong, J. C. Low

Thirty-two sheep with clinical paratuberculosis and 43 normal, healthy control animals were tested for serum antibodies to *Mycobacterium avium* subspecies *paratuberculosis* with the absorbed enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) test. All the sheep were necropsied and the diseased cases were categorised as having either multibacillary ('lepromatous') or paucibacillary ('tuberculoid') intestinal lesions. The ELISA and AGID test were highly sensitive when testing the multibacillary group (86·4 per cent and 100 per cent, respectively) but the sensitivity of the tests in the paucibacillary group was significantly lower (10 to 50 per cent and 30 per cent, respectively). These findings were related to the ELISA optical density readings, with the multibacillary samples having values significantly greater than those of the paucibacillary and control groups, and the

optical density values appearing to correlate with the numbers of mycobacteria present in the intestinal lesions. These results indicate both the usefulness of serological testing in the diagnosis of the multibacillary form of paratuberculosis and the difficulty in identifying animals with the paucibacillary form of the disease.

OVINE paratuberculosis is characterised by a chronic granulomatous enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M paratuberculosis*). The disease has a long incubation period and clinically affected sheep lose weight progressively, but only a minority of animals have diarrhoea (Chiodini and others 1984). Diagnosis can be difficult on the basis of clinical signs alone and there is no single ante mortem test with absolute sensitivity and specificity (Hietala 1992). Procedures for detecting mycobacteria in faeces, by microscopic examination, polymerase chain reaction assay or culture, are of limited use either because of poor sensitivity or because of technical demands (Hurley and others 1989, Collins and others 1993). The culture of organisms from sheep can be especially difficult (Collins and others 1993). The evaluation of cell-mediated immunity either by skin testing or by the responses of peripheral blood lymphocytes is helpful in identifying the early stages of disease but anergy may occur in the later clinical stages. Furthermore, such assays may simply indicate exposure and the presence of stimulated memory cells (Hietala 1992). Serological tests for circulating antibody, such as the complement fixation test (CFT), the agar gel immunodiffusion (AGID) test and the absorbed enzyme-linked immunosorbent assay (ELISA) are currently used for cattle. Assessments of these tests have

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(Nolan and others 1989, Beugnet and Chardonnet 1995). Similar values were found with susceptible populations of *D gallinae*. The resistance factors for pyrethroids are variable among strains of *Boophilus*, from 3·2 for the Lamington strain and 6·0 for the Marmor strain to 114 for the Parkhurst strain. A similar variability was observed with the populations of *D gallinae*.

It is now necessary to manage resistance in order to control mite infestations of poultry. There is cross-resistance between pyrethroids, which are a relatively homogeneous group (Beugnet and Chardonnet 1995). For example, permethrin-resistant mites would rapidly become resistant to flumethrin if it were used (Doti and Muruzeta 1989). The development of resistance must be avoided because of the lack of new acaricides. The strategy of resistance management should be based on a change to another chemical group as soon as resistance is detected. This should permit a reduction in the frequency of resistance genes within the mite population. Three groups of acaricides could be used in rotation: organophosphates/carbamates, pyrethroids and endectocides (ivermectin, moxidectin or doramectin) (Zeman 1987b). A reduction in the frequency of acaricide applications might help to keep the prevalence of genes for resistance low, and thus maintain the genes for susceptibility among mite populations. Other possible treatments should be examined, for example, the activity of 'insect growth regulator' on some mites such as *D gallinae* (Oliver and others 1985), or the use of mineral oil (Guimaraes and Tucci 1992).

The management of poultry flocks should also be adapted to reduce the use of acaricides. For example, the interval between depopulating and repopulating the houses should not be less than four weeks, in order to allow the mite population to decline. Vacuum cleaning the houses between flocks would eliminate dust, which is necessary for the survival of the mites. Strategies for controlling *D gallinae* should be investigated further, otherwise the development of resistance could have serious effects on the economic viability of poultry farms.

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Short Communications

Estimate of direct financial losses due to porcine proliferative enteropathy

S. McOrist, S. H. Smith, L. E. Green

IN the past 20 years, major reductions have occurred in the incidence in North America and Europe of epidemic pig diseases associated with high mortality. In the past 10 years, further changes in management systems have resulted in marked reductions in several endemic diseases, despite the increasing intensification of farms. For example, there is now a widespread availability of pigs free of enzootic pneumonia and swine dysentery through specific programmes conducted by major commercial pig breeders. These programmes have included the use of caesarean-derived piglets for subsequent breeding and the early weaning of piglets into clean, separately sited premises. Use of these relatively disease-free pigs under intensive farm management has allowed further marked improvements in productivity. This new productivity means that it is important to assess the possible economic impact of 'emerging' diseases, such as proliferative enteropathy, which inhibit production rather than the numbers of pigs.

The causative agent in proliferative enteropathy has now been established as the obligately intracellular bacterium *Lawsonia intracellularis* (McOrist and others 1993, 1995). Previous reports of production losses due to proliferative enteropathy have suggested that the growth of weaned pigs may be reduced by up to 50 per cent of normal (Gogolewski and others 1991, Mackinnon 1993). To provide specific estimates of output losses that occur in individual pigs during the course of proliferative enteropathy, this study uses measurements taken from disease challenge studies rather than from field data, thereby eliminating the compounding effect of concurrent diseases and increasing the sensitivity of comparison with controls.

Five studies (1 to 5) measuring production parameters in groups of pigs, either challenged with *L intracellularis* or dosed with a control buffer solution, were conducted. To assist comparability between the studies, all the pigs were derived from one genetic origin (Large White x Landrace) and weaned from the sow, without the use of creep feed, at 24 days old. After weaning, the pigs were housed in identical indoor pens (one pen per group) and allocated to groups of four to eight pigs (Table 1) on a stratified randomised weight basis, to eliminate initial bias. The pigs had access to a proprietary feed (wheat/barley base with soybean meal) and water ad libitum. Pigs were either challenged orally with 3·4 to 3·7 x 10^8 *L intracellularis*, or dosed orally with a sucrose-potassium-glutamate buffer solution, six days after weaning, using methods described previously (McOrist and others 1993). All pigs challenged with *L intracellularis* were expected to develop lesions of proliferative enteropathy, with or without clinical signs (McOrist and others 1993).

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TABLE 1: Mean weight gain and feed conversion ratio, with likely feed and facility costs, in studies of groups of pigs challenged with *Lawsonia intracellularis* and unchallenged control pigs

Study number and challenge status	n	Mean start, finish weight (kg)	Average daily gain (kg/day) during study*	Feed conversion ratio during study[†]	Likely feed cost to market[‡]	Annual number of batches and likely cost per pig space[§]
1						
Yes	7	5·9,11·1	0·248 (21%)	2·0 (20%)	£52	2·07 - £9·7
No	7	6·3,12·9	0·314	1·6	£47	2·28 - £8·8
2						
Yes	7	5·3,12·3	0·250 (9%)	1·6 (6%)	£48	2·19 - £9·2
No	7	5·5,13·2	0·275	1·5	£47	2·28 - £8·8
3						
Yes	5	6·9,12·9	0·168 (20%)	2·6 (15%)	£50	2·09 - £9·6
No	4	6·7,14·5	0·210	2·2	£47	2·28 - £8·8
4						
Yes	8	8·3,14·8	0·310 (31%)	1·5 (13%)	£50	1·99 - £10
No	8	8·2,17·6	0·450	1·3	£47	2·28 - £8·8
5						
Yes	7	7·3,11·4	0·205 (28%)	·0 (25%)	£53	2·02 - £9·9
No	7	7·4,13·1	0·285	1·5	£47	2·28 - £8·8

n Number of pigs per group
* Value in parentheses is the percentage reduction in daily gain of challenged pigs compared to controls
[†] Results are the number of kg of feed eaten per kg of weight gain in each group. The value in parentheses is the percentage worsening of challenged pigs compared to controls
[‡] Feed cost per pig calculated as the current cost of feed (£0·175 per kg) x likely feed conversion ratio (3·0 x 0·5 percentage worsening in each group) x 90 kg weight gain required from weaning to market
[§] Annual number of batches is determined by dividing 365 by number of days required to reach market weight at the likely weight gain in each group, plus 10 days for cleaning. The cost of replacing a standard breeding pig space in the UK (£400 to £650) is then divided by the number of weaned pigs and pig batches per year to give a likely cost of pig facilities per pig in each group

The production parameters measured were the average daily weight gain of individual pigs; the group feed intake, which was measured by weighing feed offered and refused each day; and the feed conversion ratio for each group, which was calculated as kg feed consumed/kg of weight gain.

No mortality was observed and the studies (1 to 5) were terminated four, five, eight, four and four weeks after weaning, respectively, and each pig was examined at necropsy.

The presence of lesions indicative of proliferative enteropathy and the presence of *L intracellularis* were investigated by standard diagnostic methods (McOrist and others 1993).

The target weight gain between weaning and marketing is generally 90 kg (Anon 1995). This was multiplied by the projected feed conversion ratio value and an assumed current cost of 1 kg of British pig feed of £0·175 (Anon 1995, 1996) to give an estimate of the likely feed cost in each group. The likely feed conversion ratios were estimated by assuming that the recorded change would persist for one half of the growing period only.

The likely costs arising from the changes in average daily weight gain, in terms of days to slaughter, were also estimated by assuming that the recorded change in weight gain would persist for one half of the growing period. Both estimates were used to make proportional adjustments to commercial targets (Anon 1995) of 3·0 for feed conversion ratio and 150 for days to slaughter. The replacement cost of one unit of breeding pig space in the UK, or the actual cost of one sow unit, including breeding, labour, management and financial costs, is considered to be £400 to £650 (Anon 1995). If one sow, on average, produces 20 pigs/year, then the cost per growing pig is £20 to £33. This cost is then divided by the number of pigs available per unit of housing space per year; that is, the number of 'batches' ('turns', 'cycles') of pigs per year, plus 10 days per batch for cleaning on an 'all-in, all-out' basis, and assuming each batch is handled similarly with respect to restocking.

The rate of mortality of pigs near to slaughter weight in the UK and elsewhere is considered to be around 2 per cent, or 290,000 pigs/year (Anon 1995). Several surveys of the causes of mortality in the UK and elsewhere have identified acute proliferative enteropathy as causing 2 to 5 per cent of this mortality, or 5800 to 14,500 pigs per year (Roberts and others 1979, Duran 1994, Christensen and others 1995). These data were used to calculate the annual costs due to fatal episodes of acute proliferative enteropathy in the UK.

To extrapolate from the estimation of loss components derived for each pig affected with chronic proliferative enteropathy to the possible direct costs for the British pig industry, a survey of the prevalence of chronic proliferative enteropathy (porcine intestinal adenomatosis) was conducted. A specific questionnaire was prepared and sent to 572 convenience-selected farms, approximately 2·8 per cent of the total of about 20,000 British pig farms. Farmers were asked whether 'porcine intestinal adenomatosis' had been diagnosed by their veterinary surgeon in the past three years. This was a semi-open question, the possible replies were 'yes', 'no' or 'don't know'.

The production results of the five studies (Table 1) indicate clear reductions in the mean weight gain of groups of pigs affected with proliferative enteropathy, ranging from 9 per cent (study 2) to 31 per cent (study 4) less than the control animals. The ratio of feed conversion (weight gain to feed) was also markedly worsened in pigs affected with proliferative enteropathy, ranging from 6 per cent (study 2) to 25 per cent (study 5) compared with the normal pigs. The likely costs of the extra feed required to finish these pigs (£1 to £6) and the costs of the reduced facility space available (£0·5 to £1) because of the increased days to slaughter, are outlined in Table 1. Addition of these costs gives an estimate of the direct financial loss due to proliferative enteropathy of £2 to £7 per pig.

Of 343 farmers who replied to the survey (60 per cent response rate), 103 (30 per cent) indicated that a veterinary diagnosis of chronic proliferative enteropathy had been made on their farm in the past three years. It is estimated, therefore, that of the 14·5 million pigs slaughtered in the UK each year approximately 1·5 million are at risk of proliferative enteropathy. Diagnostic investigations on affected farms have indicated that approximately 35 per cent of growing pigs are affected with proliferative enteropathy (McOrist and Lawson 1989, Pointon 1989) and thus approximately 0·5 million pigs are estimated to be affected each year with chronic proliferative enteropathy in the UK. Multiplying this number by the estimated cost per pig gives a total direct cost of £1 million to £3·5 million, depending on the degree of effect on production.

The market value of a pig at slaughter in February 1996 (Anon 1996) was £130 per pig, so the total loss for 5800 to 14,500 pigs affected with acute proliferative enteropathy per annum could be between £0·75 and £1·9 million. A proportion of this loss is discounted due to the necessary costs incurred with or without the pres-

ence of disease. The total direct loss due to proliferative enteropathy in the UK may therefore be from £2 million to £4 million.

This study has attempted to estimate the direct costs of an important emerging disease which limits production in growing pigs. While there are numerous assumptions within the calculations, derived partly from current knowledge of the disease process and partly from current industry costs, both of which may alter in the future, the authors consider that the figures of an annual cost to the British pig industry of £2 million to £4 million are realistic. This estimate is in line with two previous estimates, one in the USA of $20 million per annum (Mapother and others 1987) and another in Australia of $25 AUS per sow (Cutler and Gardner 1988). However, the inherent weaknesses in this type of exercise preclude close comparison to previous studies. While the present estimate is based on specific challenge trials and is likely to provide somewhat more accurate figures for the direct cost of this disease, there are likely to be some differences between the trial results and those seen in the field. Previous reports of the weight gains of pigs naturally affected with proliferative enteropathy measured reductions of up to 50 per cent compared to normal pigs (Gogolewski and others 1991, Mackinnon 1993). As was clear from the present study, there can be variation in the impact of disease challenge between groups of apparently similar pigs. The impact appeared to be greater when pigs were infected at a higher initial weight (Table 1). It is likely that the impact of the disease will be variable between farms. It was not feasible in this study to use large numbers of pigs to obtain an estimate of the statistical variation of the effect of *Lawsonia* species infection, whereas field studies can incorporate large numbers of animals in calculating the costs of this disease.

The estimate of direct losses presented here is considered to be an underestimate of the full impact of this disease. Possible additional costs include those of an increased variation in carcase composition or dead weight and costs to breeding programmes. Currently in the UK there is no nationally recognised benefit for producing finished carcases with a specific lean meat composition, or dead weight, although there are local contracts between suppliers and abattoirs where carcase grading is important. The costs of reduced carcase lean meat composition and variations in dead weight due to changes in growth rates at the critical period of lean meat deposition in the growing pig, at 12 to 20 weeks old (Stahly 1994), could therefore not be estimated. If there is reduced growth during this period, such as occurred in this trial and as occurs in the field disease (Roberts and others 1979, Gogolewski and others 1991), then there may be a detrimental effect on carcase quality, with an increased back-fat thickness evident. Other direct costs not measurable in this study were those associated with an increased variability in the size and weight of pigs within an affected group. This variability could have a measurable impact on the weight range of pigs offered for slaughter, and on the quality of pigs selected for subsequent breeding programmes (Christian and others 1973). Further costs not measurable in this study were the public costs of animal disease, such as the cost of the State Veterinary Service and other support groups. These indirect costs are borne by farmers and others to support regulatory and advisory roles in services such as nutrition and disease surveillance and can be quite large compared to the direct costs in some cases (McInerney and others 1992).

In studies of the economic impact of animal disease it is vital to recognise that the figure given for these direct costs does not represent the figure saved if the disease were to be eradicated or controlled. This is because there is a certain limit to the likely spending on measures to control the disease, such as antibiotic provision. Actual levels of expenditure on normal farms and on those suffering proliferative enteropathy may not reflect the apparent differences given between the groups of pigs in the present study, so the figures may not quantify the true avoidable cost of proliferative enteropathy. The new emerging production diseases may therefore have a serious impact on the profitability of affected farms and are worthy of attempted control. The likely impact of serious mortality and even endemic diseases may now be of lesser importance to advanced sectors of the pig industry than production diseases.

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Evidence of *Theileria buffeli* infection in cattle in southern Italy

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CASES of tropical theileriosis have been recorded in cattle in the Apulia area of southern Italy since 1954 (Battelli and others 1954). In June 1995 veterinarians in this area reported theileriosis-like symptoms in cattle. The clinical symptoms included fever, anorexia, prostration, failure of rumination and very pale mucous membranes. Haematological examination revealed marked anaemia, anisocytosis, poikilocytosis and leucopenia. Animals with a subclinical form showed mild anaemia and hypogalaxia (Ceci and others 1995).

To identify which *Theileria* species were involved in this outbreak, two techniques were used: (i) evaluation of the erythrocytic form of the parasite (the piroplasm) from cattle exhibiting clinical signs of an infection; and (ii) serological definition of the parasite using *Theileria annulata*, *T buffeli* and *Babesia bigemina* antigens

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Development of persistent intestinal infection and excretion of *Lawsonia intracellularis* by piglets

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SUMMARY

Challenge experiments using *Lawsonia intracellularis* as oral inocula have established its aetiological role in porcine proliferative enteropathy. Thirty piglets, in four groups, were weaned at 21 days of age and inoculated orally at 24 days. Six piglets were challenged with 1.0×10^8 *L intracellularis* strain 916/91 (NCTC 12657) passaged 12 times in vitro, six with 5.0×10^8 of the same strain, seven with 3.0×10^8 *L intracellularis* strain LR189/5/83, passaged nine times, and 11 controls were dosed with sucrose-potassium glutamate buffer. An immunofluorescence assay for *L intracellularis* was applied to faecal smears and a polymerase chain reaction (PCR) incorporating *L intracellularis*-specific primers was applied to extracts of bacterial DNA derived from the faeces samples. Up to five pigs in each challenge group excreted detectable *L intracellularis* in faeces, in samples taken between two and 10 weeks after challenge. Some of the pigs had up to 7×10^8 *L intracellularis* g^{-1} faeces. The average weight gains of the higher dose challenge groups were moderately below those of the control pigs between three and nine weeks after challenge; diarrhoea was also observed in six pigs, two to four weeks after challenge. Numerous *L intracellularis* were detected in the intestines of all the pigs challenged with strain LR189/5/83 and two of the pigs challenged with 916/91, but not in other tissues.

PROLIFERATIVE enteropathy is a common disease of weaned pigs worldwide which is characterised by marked proliferation of ileal and colonic enterocytes into a hyperplastic to adenomatous state. A clinical or subclinical reduction in weight gain is its primary effect in live, affected pigs, usually at six to 12 weeks of age (Connor 1991, Rowland and Lawson 1992) A consistent feature is the occurence of intracytoplasmic, non-membrane-bound curved bacilli within enterocytes in affected portions of intestine (Rowland and Lawson 1974). Antigenic and DNA analyses and growth in cell cultures have indicated that these organisms are a novel genus of obligate intracellular bacteria (McOrist et al 1987, 1989, Gebhart et al 1993, Lawson et al 1993). The successful culture of the intracellular organism in enterocyte cell cultures (Lawson et al 1993) led to the availability of experimental inocula which reproduced the disease in conventional, specific pathogen-free piglets and in gnotobiotic pigs pre-dosed with a minimal gut flora, but not in gnotobiotic pigs (McOrist et al 1993, 1994a). Taxonomic studies of cell culture-derived intracellular organisms (McOrist et al 1995a) and organisms purified from pig intestines (Gebhart et al 1993) established that they are a novel obligately intracellular bacterium, with the definitive name of *Lawsonia intracellularis* (McOrist et al 1995a), previously known as Ileal symbiont intracellularis (Gebhart et al 1993).

Although these previous experiments were able to fulfil Koch's postulates concerning the aetiology of the disease, they did not consider important issues in its clinical manifestation. Similarly, other experiments used oral inocula consisting of crude intestinal homogenates to reproduce the disease (Mapother et al 1987, Jones et al 1993c), and initial polymerase chain reaction (PCR) and DNA probe methods were used to follow the course of the infection in its early stages (Jones et al 1993a,b,c). The further development of a PCR-based assay for *L intracellularis* (Jones et al 1993b, McOrist et al 1994b), capable of detecting the organism in tissues and faeces, made it possible to design extended experiments to monitor the course of the infection after challenge with pure cultures of *L intracellularis*. Blood based assays have yet to prove fully effective for monitoring the course of infection with *L intracellularis* (Lawson et al 1988). In particular, there was little previous information on the duration of infection in pigs, the level of *L intracellularis* in faeces, the possibility of carrier animals or the exact correlations between strain, dose and clinical effect. All this information is of great relevance to pig clinicians, who have lacked the knowledge to enable them to deal scientifically with this important disease.

MATERIALS AND METHODS

Conventional pigs

Thirty piglets were delivered from specific pathogen-free sows (commercial synthetic breed) and maintained in hygienic conditions separate from other pigs. The piglets were weaned from the sows at 21 days, without the use of any supplementary creep feed, into separate sanitary pens for the three challenge groups and the control group. The piglets were dosed once orally, at 24 days of age, and fed commercial feed free of any antibiotics ad libitum. The pigs were not medicated with immunosuppressive or other drugs.

Tissue culture-derived inocula for piglet infections

IEC-18 cells, rat enterocytes (American Type Culture Collection CRL 1589) were grown to a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, amphotericin B (Fungizone), and 10 per cent (v/v) fetal calf serum. The infection of these cells by the microaerophilic, intracellular bacterium *L intracellularis* derived from pig intestines has been described in detail by Lawson et al (1993). In that study isolates from 10

pigs from four farms had been cultured. Two strains isolated from separate pigs with acute haemorrhagic proliferative enteropathy were selected for this study – 916/91 (NCTC 12657) for groups one and two and LR189/5/83 for group 3 – on the basis of their suspected enteropathogenicity. The infected cells were passaged by treatment with potassium chloride. as described by Lawson et al (1993), and were then removed from each flask with a cell scraper. The scraped cells were ruptured by needle passage and the cell lysate, containing bacteria, was used to infect fresh IEC-18 cells in new flasks.

The infected cells were grown for five to seven days before they were prepared for either infecting piglets or repassaging to expand the culture. For each flask. the supernatant fluid was removed and centrifuged at 4000 *g* for 20 minutes. The bacterial pellet was resuspended in sucrose-potassium glutamate buffer with 5 per cent (v/v) fetal calf serum. The cells left in the flasks were removed. and the cell lysate bacteria and the supernatant bacteria from each passage were combined for use as a challenge inoculum. The piglets were each dosed orally with 10 ml, from a syringe and tube.

Control inoculum

Control piglets (group 4) were dosed once orally with 10 ml of sucrose-potassium glutamate buffer (Lawson et al 1993). In previous studies, control pigs dosed with uninfected IEC-18 cells had remained normal (McOrist et al 1994a)

Monitoring of inocula

First. 1 ml of a 1/80 dilution (in DMEM) of 1 ml of the supernatant portion of each inoculum was added to three separate IEC-18 cell cultures grown on 13 mm diameter glass coverslips in small bottles. The inoculated cells were incubated by the standard methods for a further five days. Secondly, as the flask cell cultures were being inoculated with cell lysate containing *L intracellularis* for incubation for one week before the final harvesting, two separate cultures of IEC-18 cells grown on 13 mm coverslips in bottles were inoculated and incubated in parallel for five days.

After harvesting and washing, the coverslip cultures were immunostained for *L intracellularis* with a specific monoclonal antibody (mAb Ig4) as the primary antibody (McOrist et al 1987) and an anti-mouse peroxidase conjugate as the secondary antibody in an indirect immunoperoxidase test with haematoxylin counterstaining. The number of heavily infected cells (>30 bacteria per cell, estimated average 50 per cell) was counted for each coverslip and multiplied by the initial dilutions and the relative area of the flask cultures, to give an estimate of the number of organisms present in each experimental inoculum. Previous studies had indicated the accuracy of this enumeration procedure (Lawson et al 1993).

Portions of each inoculum were incubated at 37°C on blood agar and nutrient broths in anaerobic, microaerobic and aerobic atmospheres. Infected and uninfected IEC-18 cells stained for the presence of *Chlamydia* species and *Mycoplasma* species by means of antibody kits obtained commercially, were uniformly negative. Samples of each inoculum were incorporated into the specific PCR testing, as described below for faeces.

Clinical and faecal monitoring

The piglets were weighed and a faecal sample was collected at dosing and at one week intervals thereafter. The piglets were examined daily for evidence of diarrhoea or other clinical signs.

Triplicate smears of each faecal sample, including those from control piglets, were prepared on glass slides, air-dried and acetone-fixed. Ten-fold dilutions of 0·1 g of each sample were prepared and smeared similarly. Each smear was immunostained for *L intracellularis*, using antibody Ig4 incorporated into an indirect immunofluorescence assay (McOrist et al 1987), and the number of brightly fluorescing curved bacteria in each smear was counted under a fluorescence microscope (brightly fluorescing *L intracellularis* in faecal smears are illustrated in McOrist et al [1987]). Multiplication of the number of organisms detected by the dilution gave an approximate estimate of the number of *L intracellularis* in the faeces.

Bacterial DNA was extracted from 0·2 g of each faecal sample by the use of a commercial kit (Bioline UK, Finchley) which incorporated guanidine lysis, DNA immobilisation on a mineral carrier and washing and elution steps. The extracted DNA was incorporated into a PCR, using the primers and conditions developed for reaction with the *L intracellularis* genome (Jones et al 1993b, McOrist et al 1994b). Separate PCR reactions and subsequent detection of PCR products were set up for each sample. The positive PCR product in this reaction is 319 bp derived from a *L intracellularis*-specific genomic target (Jones et al 1993b), and was detected by electrophoresis of products in a 1 per cent agarose gel. The identity of the positive PCR products was confirmed by DNA sequencing of selected 319 bp amplified products. The PCR products were purified from an agarose gel by using a commercial DNA binding kit (Geneclean II; Invitrogen: CA, USA). The PCR products were sequenced by the dideoxynucleotide extension/termination method, using the fmol DNA Sequencing System (Promega: Madison, WI, USA). The sequence was determined unequivocally on both strands. The product sequences obtained were compared to the known *L intracellularis* sequence in this region of its genome (Genbank accession L08049).

Necropsy

A full necropsy was performed on all the pigs at either three, eight, 11 or 14 weeks after oral dosing. Samples of tonsil, stomach, small and large intestine, rectum and ileocecal lymph node were fixed in formalin, sectioned by routine methods and stained with haematoxylin and eosin. Young's silver stain and indirect immunofluorescence assay for *L intracellularis* (McOrist and Lawson 1989b). Further sections of these fixed tissues were added to separate tubes and incorporated into the PCR reaction as described above. In selected pigs in group 2, the intestinal mucosa was incorporated into culture procedures for *L intracellularis* as described by Lawson et al (1993).

RESULTS

Inocula

Bacterial culture of the inocula on cell-free media revealed no detectable isolates. Immunoperoxidase staining of infected IEC-18 cells set up in parallel to the flasks used to challenge the piglets and cultures derived from the

supernatant fluid of inocula showed numerous curved bacteria reactive with the primary mAb. Accepting the assumptions concerning bacterial counts within heavily infected cells and across flasks compared with coverslips, the piglets within the different groups received different doses of *L intracellularis*. The six pigs in group 1 received 1.0×10^8 of 916/91, the six in group 2 received 5.0×10^8 of 916/91, passaged 12 times in vitro, and the seven pigs in group 3 received 3.0×10^8 of LR189/5/83 passaged nine times in vitro. The infected monolayers showed no discernible cytopathic effects during infection and passage of the bacteria. PCR testing of samples from each inoculum revealed a 319 bp PCR product consistent with the *L intracellularis* genome (see below).

Clinical findings

The average weight changes of the piglets in each group are shown in Fig 1. The weights of the piglets in groups 1 and 2 at each weighing, between three and eight weeks for group 2 and at three weeks for group 1 after the initial challenge, were significantly below those of the controls (P<0.01). No other significant changes were recorded. All the control pigs remained clinically healthy throughout. Three piglets in group 2 and three in group 3 had moderate diarrhoea, with the faeces being pale green to dark red with a loose, watery consistency. In all cases, this diarrhoea began two weeks after challenge but persisted only for one to two weeks.

Faecal monitoring

The examination of immunostained faecal smears for *L intracellularis* indicated that numerous brightly staining bacterial organisms were present in one piglet in group 1 and in three piglets in both groups 2 and 3 at samplings taken between two and 10 weeks after challenge. The samples from individual pigs were consistently positive or neg- ative within the triplicate samples in the test and control samples in this test, and the results were consistent with the PCR results (see below). Dilutions of faecal samples taken from these pigs at the same times revealed detectable *L intracellularis* within the range of 1/10 to 1/10,000 dilutions, indicating that *L intracellularis* was present at concentrations in the range of 5×10^4 to 7×10^8 organisms g^{-1} faeces in each positive sample. A comparison of the faecal counts with the original challenge dose indicated a trend towards higher counts in group 2 pigs which received the higher dose.

The detection of the 319 bp PCR product consistent with the presence of the *L intracellularis* genome in piglet faeces taken during the course of the experiment is illustrated in Fig 2. In summary, three weeks after challenge there was one piglet in group 1 positive for the *L intracellularis* PCR product, and there were three each in groups 2 and 3. Six to seven weeks after challenge, one of the five pigs remaining in group 1 and four of the five in group 2 (two pigs during week six and two others during week seven) were positive for *L intracellularis* PCR product in faeces. Ten weeks after challenge none of the three pigs in group 1 was positive and one of the five pigs in group 2 was positive. Only four pigs were maintained for more than 10 weeks after challenge (for up to 14 weeks) but none of these was PCR positive. Direct sequencing of the 319 bp PCR products derived from the DNA prepared from bacteria in the inocula and in the faeces revealed readable DNA sequences 250 to 300 bp. A comparison of the sequences of these DNA fragments with the published sequence of *L intracellularis* at this site showed over 99 per cent similarity.

Necropsy

Gross changes were detected in one of the six pigs in group 1, two of the six in group 2 and all seven pigs in group 3 in necropsies performed either three weeks (groups 1 and 3) or 14 weeks (group 2) after challenge. *L intracellularis* was re-isolated in small numbers from the intestines of the two affected pigs in group 2; the pigs in the other groups were not cultured.

The ileal and colonic mucosa of all the pigs in group 3 had numerous enlarged crypts lined by proliferating, immature enterocytes. There were numerous mitotic figures but few or no goblet cells in the affected crypts. In grossly affected areas these changes were diffuse and severe, with the mucosa having an adenomatous appearance. The
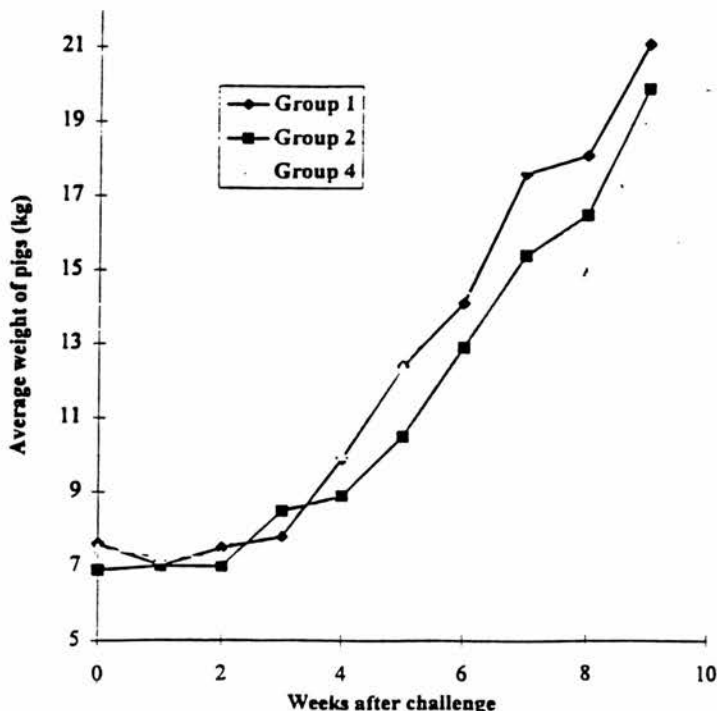

FIG 1: Average weights of piglets after an oral challenge with *Lawsonia intracellulans* (groups 1 and 2). Control piglets (group 4) were dosed orally with sucrose-potassium glutamate buffer. Group 3 piglets were euthanased three weeks after challenge
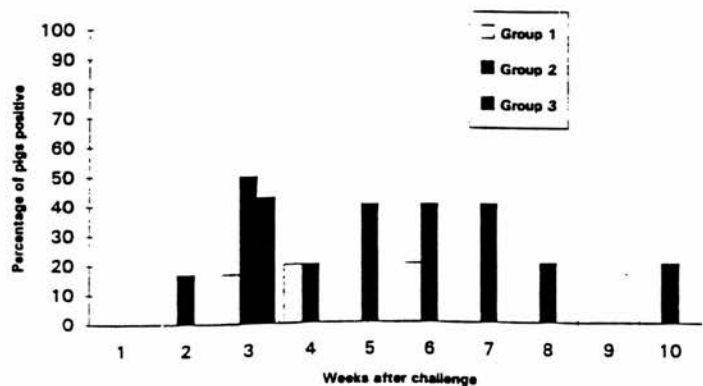

FIG 2: Percentage of piglets in each group positive for the specific 319 bp PCR product of *Lawsonia intracellularis* in faecal samples collected weekly after challenge. Numbers per group given in text. All group 4 piglets were negative at each sampling. Group 3 piglets were euthanased three weeks after challenge

intestines of the pigs with gross lesions in groups 1 and 2 showed healing lesions of proliferative enteropathy, with the crypts in affected areas being lengthened by immature enterocyte and goblet cells extending from the base of the crypt. The intestine of all the other pigs appeared normal. There was a mild to moderate infiltration of lymphocytes. macrophages and neutrophils in the mucosa and lamina propria of the ileum and large intestine of all the pigs. An examination of sections of mesenteric lymph nodes and other major organs showed no significant lesions in any of the pigs.

Silver impregnation stains showed that there were many curved bacilli in the apical cytoplasm of affected portions of the intestine of all the group 3 pigs. When sections of affected portions of the intestine of these pigs were treated with antisera to *L intracellularis* they revealed brightly reacting curved bacilli within enterocytes (this type of lesion is illustrated in McOrist et al [1993]). Intracellular bacteria were not demonstrated by silver staining or immunostaining either in other tissues or in other pigs.

DISCUSSION

This study provides new information on the nature and course of infection of pigs by the enteropathogen. *L intracellularis*, the causative agent of porcine proliferative enteropathy. Because the incubation period of the disease is two to three weeks (Mapother et al 1987. McOrist and Lawson 1989a), the disease is transmitted faecal-orally (McOrist and Lawson 1989b. Rowland and Lawson 1992), and the main clinical signs of the disease generally occur in growing weaner pigs six to 12 weeks old (Rowland and Lawson 1992), it was considered likely before this study that most pigs became infected shortly after weaning. developed the disease and passed it on to other weaned pigs (Rowland and Lawson 1992). The modern pig industry has developed excellent controls over the inappropriate mixing of pigs. however, which have led to the relative isolation of different age groups. The occurrence of many outbreaks of proliferative enteropathy has therefore not fitted into the suggested pattern of infection. In addition to the features mentioned above, the results of this study suggest that the infection of young piglets with *L intracellularis* can persist for at least 10 weeks. Although the infection can produce relatively mild clinical signs. faeces containing ample infective organisms to be passed on to other pigs can be excreted persistently.

These features suggest that once an infected animal is introduced naturally into a group of weaned pigs, the infection is likely to spread quickly and may persist in that group for a considerable portion of the growing period. In previous studies, piglets of seven or 15 days of age were successfully infected with *L intracellularis* and developed active proliferative enteropathy (McOrist and Lawson 1989a. McOrist et al 1994a), further suggesting that young piglets are fully susceptible to the disease. This study also indicates that the disease can then persist until pigs are mixed into groups after weaning, with the weaned pigs commonly excreting the organism. Previous faecal monitoring in field and experimental challenge studies have indicated similar findings (McOrist and Lawson 1989b, Jones et al 1993c). The further persistence of the organism in the weaned pigs. which could possibly be enhanced by its excretion by one or more pigs. leading to further newly infected pigs. makes it possible that some female pigs may

remain infected until their first pregnancy. If so, then their offspring could possibly be infected in the late suckling period and the infection could be transferred to the next generation. It is possible that the organism has developed a successful method for its survival and transmission, adapted from its requirement for intracellular growth in enterocytes. Somewhat similar methods for persistence in the host have been developed by other intracellular organisms living in the enterocytes of pigs, such as *Isospora suis. I suis* is also thought to infect young piglets and has developed encystment methods to allow it to persist in the environment (Lindsay et al 1992).

Further aspects of the nature of infection with *L intracellularis* were examined. The organism was first apparently excreted after two weeks. supporting the previous estimates of the incubation period of two to three weeks for the disease (McOrist and Lawson 1989a). The estimated numbers of *L intracellularis* in the faeces of some pigs were clearly above the infective dose of 10^7 organisms previously observed to transmit the disease (McOrist et al 1993. 1994a). Because some pigs could apparently excrete over 10^8 organism g^{-1} of faeces. it would therefore appear that one infected pig might provide ample organisms to infect other pigs in the same group. This result agrees with the findings of previous field and experimental challenge studies of infected pigs' faeces. in which numerous *L intracellularis* organisms were detected during outbreaks of the disease (McOrist and Lawson 1989b. Jones et al 1993b,c), either by PCR or immunofluorescence assays. The result also supports the reliability of these assays in suggesting that a positive result indicates the presence of viable, infectious agents as further suggested by the correlations observed between the results of PCR, immunofluorescence assays and post mortem findings.

There was some evidence of a difference in the ability of the two strains of the organism to cause disease, with strain LR189/5/83 producing moderate to severe disease only three weeks after dosing. It is possible that the pathogenicity of some strains are influenced by being passaged in vitro (McOrist et al 1993). In both the higher dose challenge groups. there was evidence of a failure to gain weight as quickly as the controls. an effect which would represent considerable economic loss to pig farmers. Nevertheless. in many infected pigs the reduction in weight gain was relatively modest. making the clinical detection of the disease difficult. Furthermore. the diarrhoea, which coincided with the incubation period of the disease. did not develop in every infected pig. The development of diarrhoea in some pigs was a valuable marker for the disease. however, a feature not emphasised in some clinical texts (Rowland and Lawson 1992). Proliferative enteropathy is of economic importance. yet difficulty in diagnosis is a prominent feature of the disease (Connor 1991, Rowland and Lawson 1992). Unfortunately, the PCR test used in this study would be difficult to apply as a routine diagnostic test, owing to its high cost. Further field studies are needed to correlate the results of this study with disease patterns.

This study also examined some of the tissues infected by *L intracellularis* with the intention of detecting possible healthy carrier animals. The small and large intestines were infected, providing the basis for the excretion of the organism. but no other site was clearly demonstrated by the PCR test to harbour *L intracellularis*. Although it is possible that the tonsils or other sites may be infected in severe field cases of the disease. and another test may prove to be more sensitive. the results of this study suggest that the clearance

of the organism from the intestines should lead to a cure, that is, carrier animals are not evident. This result further emphasises the remarkable predilection of *L intracellularis* for the cytoplasm of enterocytes, as suggested by previous in vitro studies (Lawson et al 1993, 1995). The organism has developed mechanisms for its rapid entry into and multiplication within these cells (McOrist et al 1995b). These mechanisms have apparently helped it to survive readily in the types of pigs now favoured by the modern pig industry.

ACKNOWLEDGEMENTS

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