DEVELOPMENT OF A REVERSE PASSIVE LATEX AGGLUTINATION TEST FOR SWINE DYSENTERY

by

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SUMMARY

Two diagnostic reverse passive latex agglutination tests for swine dysentery were produced and evaluated. Such tests are faster and potentially more specific than current diagnostic tests.

In the first test the antibody used was raised in a rabbit to whole *Serpulina hyodysenteriae*, S80/5 cells. The antiserum was absorbed with non-*S. hyodysenteriae* spirochaetes PWS/A and 4/71 and used to coat 0.46μm latex beads. These beads were suspended in glycine buffered saline pH 8.2. This reagent was found to detect *S. hyodysenteriae* at 10^7 organisms/ml and the type strain B78 at 10^8 organisms/ml. The test also cross reacted with PWS/A and 4/71 at levels of 10^9 organisms/ml and 10^{11} organisms/ml respectively. When cultured *S. hyodysenteriae* was suspended in normal pig faeces, levels of 10^8 organisms/ml could be detected. When faeces from experimentally infected pigs was examined, RPLA positive faeces was found to contain 6 x 10^8 organisms/ml.

In the first instance, the test specificity was examined for its ability to distinguish between *S. hyodysenteriae* and other intestinal porcine spirochaetes and the sensitivity was looked by determining the lowest concentration of organisms detectable by the test.

However when dealing with diagnostic tests, the sensitivity of the test should also be looked at as the number of positive results from animals with the disease. The specificity as the proportion of animals without the disease which have a negative result. This was looked at using field samples from pigs with suspected swine dysentery.

Twenty eight field samples of dysentery or diarrhoea were examined by the RPLA and culture. Twenty three were positive by RPLA but only one yielded *S. hyodysenteriae*. Eleven others yielded spirochaetes shown by the haemolysis pattern and API ZYM not to be *S. hyodysenteriae*. The remaining eleven samples contained spirochaetes visible in smears but which could not be grown.

In an attempt to improve specificity antiserum was prepared in a rabbit to the 16 kDa antigen of *S. hyodysenteriae* purified by SDS PAGE and immunoblotting. The resulting polyclonal antiserum was used to prepare a second test. This was more sensitive detecting *S. hyodysenteriae* at levels of 10^6 organisms/ml and non-*S. hyodysenteriae* (PWS/A and 4/71) at 10^9-10^{10} organisms/ml. It was used on the samples previously tested with the addition of 17 further fresh samples. The test appeared to be more sensitive and more specific than before, but false positives still occurred.

Examination by SDS PAGE of 14 of the non-*S. hyodysenteriae* isolates revealed that they contained a 16 kDa antigen. Negative staining of 1 of these spirochaetes confirmed that their ultrastructure differed from that of *S. hyodysenteriae*. It appeared that the false positives might result from the possession of a 16 kDa antigen by non-*S. hyodysenteriae* spirochaetes.

INTRODUCTION

The study described here is concerned with the development of a reverse passive latex agglutination (RPLA) test for swine dysentery. RPLA tests are made by coating a suspension of tiny latex particles with specific antibody or antigen. When a drop of the latex suspension is added to a drop of the prepared sample and mixed on a slide, the latex particles agglutinate forming visible granules if the sample contains the relevant antigen or antibody. If however there was no antigen or antibody present in the sample then the particles remain as a smooth suspension.

RPLA tests are extremely rapid, very sensitive and potentially very specific. They are particularly relevant in swine dysentery where secondary infections may be present altering the clinical signs and pathology. Cultural confirmation of this disease may be required and take days rather than the minutes required by RPLA tests which would shorten the time taken by current diagnostic tests.

Two RPLA tests were constructed and evaluated in this study. The first RPLA test described in the study involves the coating of the latex particles with polyclonal antiserum raised against the aetiological agent of swine dysentery, *Serpulina hyodysenteriae*. This antiserum had also been absorbed with strains of the non-pathogenic spirochaete, *Serpulina innocens*.

The second RPLA test used polyclonal antiserum against the 16kDa antigen of *S. hyodysenteriae* to coat the latex particles.

The RPLA tests were then evaluated for specificity and sensitivity using cultured organisms and dysentery samples from experimentally inoculated pigs. Field samples were also tested and the results were compared with results from smear, culture, API ZYM and SDS PAGE gels of the spirochaetes to determine whether or not the organisms possessed the 16 kDa antigen considered to be specific for *S. hyodysenteriae*.

CHAPTER ONE

LITERATURE REVIEW

HISTORY OF THE DISEASE.

Swine Dysentery was first described by Whiting *et al* in 1921. Successful transmission of the disease was achieved by the administration of infected faeces or colonic material from diseased pigs to healthy ones (Whiting, 1924).

For fifty years after the description of swine dysentery, its aetiology remained a mystery despite the fact that the disease was becoming a serious problem in North America. Outbreaks of the disease in the United Kingdom were first reported in Dorset in 1957 (Birrell, 1957) and in South-East Scotland in 1960 (Deas,1960). In 1944 Doyle suggested that the causative organism was a vibrio, *Vibrio coli*. However he also observed that the dysentery that followed the feeding of pure cultures of the vibrio to pigs was less severe though not unlike that following administration of dysenteric colon (Doyle, 1944).

Several other groups of workers agreed with this suggestion (James and Doyle, 1947; Roberts, 1956; Lussier, 1962) although many also disagreed having failed to produce even a transient diarrhoea in pigs after administration of pure cultures of the organism (Andress *et al*, 1968; Andress and Barnum, 1968; Deas, 1960)

Terpstra *et al* (1968) achieved transmission of the disease using faecal and colonic material containing large spirochaetes. In 1969 Vallejo reported the finding of large numbers of spirochaetes in faecal samples taken from pigs with swine dysentery and produced the first electron micrograph of one. He did not, however, think that these spirochaetes were the primary pathogens. Roberts and Simmons (1970) also reported finding large numbers of spirochaetes in dysenteric faeces which resembled those described by Vallejo (1969).

It was not until 1971 that Taylor and Alexander reported the isolation and culture of a pathogenic anaerobic spirochaete (Taylor and Alexander,1971). This finding was later confirmed by workers at Iowa State University and the organism was named *Treponema hyodysenteriae* (Glock and Harris, 1972; Harris *et al* 1972)

It has since been renamed *Serpula hyodysenteriae* (Stanton *et al*, 1991). *Treponema hyodysenteriae* and the non-pathogenic *Treponema innocens* were reclassified in a new genus, *Serpula* due to their genetic, physiological and morphological characteristics which differentiated them from the other members of the genus *Treponema*. However since this work was published, it was discovered that a fungal genus had already been given the same name.

This meant that the name of the bacterial genus had to be changed from *Serpula* to *Serpulina* meaning "belonging to the class of little snakes" (Stanton, 1992).

PATHOGENICITY

Early work on the pathogenicity of *S. hyodysenteriae* seemed to indicate that the organism was only able to cause the disease in the presence of other anaerobic bacteria such as *Fusobacterium necrophorum* and *Bacteroides vulgatus* (Harris *et al*, 1978; Whipp *et al* 1979). However, Whipp *et al* (1982) demonstrated that *S. hyodysenteriae* can colonise and express pathogenicity in the colon of gnotobiotic pigs in the absence of any other microbial contamination. This leads to the conclusion that in conventional pigs the synergism between *S. hyodysenteriae* and certain other anaerobes facilitates colonisation and not pathogenicity. Therefore only *S. hyodysenteriae* is able to initiate the disease.

S. hyodysenteriae is the only pathogen involved in swine dysentery and infection is by the faecal oral route. The organisms pass through the stomach undamaged because they are coated with the thick mucus present in dysenteric faeces (Taylor, 1989). In the large intestine, the organisms have been shown to colonise the mucus layer covering the colonic epithelium (Kennedy *et al*, 1988). The organisms

multiply in the mucosal crypts, causing crypt elongation, outpouring of mucus, pseudomembrane formation and surface necrosis. Although Wilcock and Olander (1979) demonstrated the attachment of *S. hyodysenteriae* to the cell surface *in vitro*, Kennedy *et al* (1988) failed to find any significant evidence of *S. hyodysenteriae* adhering to the intestinal epithelium *in vivo*.

Following colonisation, lesions develop on the epithelial surface of the colon, and typically remain as active superficial necrosis without deeper penetration, fibrosis or any of the other changes normally accompanying a prolonged colitis. *S. hyodysenteriae* invades the goblet cells and epithelial cells, disrupting them, but does not spread beyond the lamina propria, hence the confinement of the lesions to the intestine (Glock *et al*, 1974; Taylor and Blakemore, 1971). The spirochaetes can be seen both within and between the cells of the colonic mucosa and may continue to multiply within the cells which as a result become contracted and vacuolated (Glock *et al*, 1974; Taylor and Blakemore, 1971).

It is possible that two toxins of *S. hyodysenteriae* may be involved in the development of the lesions. Saheb *et al*, (1980) described the haemolysin of *S. hyodysenteriae* which was later purified and its molecular weight was found to be 19kDa (Kent and

Lemcke,1984; Kent *et al*, 1988). The haemolysin resembles Streptolysin S in many ways (Loridan and Alouf, 1986; Lysons *et al*, 1991) e.g. they are of similar molecular weights and they both require a carrier molecule for showing *in vitro* haemolytic activity from metabolically active organisms. Avirulent strains produce a smaller quantity of haemolysin which has less cytotoxic activity and this may account for the difference in pathogenicity (Kent and Lemcke,1984). Lysons *et al* (1991) demonstrated the cytotoxic effect of the haemolysin in ligated loops of the colon and ileum. The effect on the epithelial cells was thought to be direct. They observed that the internal organelles of the cells were disrupted first, followed by the swelling and then shedding of the epithelial cells.

The second toxic factor produced by *S. hyodysenteriae* is the lipopolysaccharide (LPS) which was described by Baum and Joens (1979(a)). Nuessen *et al* (1982(a)) showed that the LPS extracted from *S. hyodysenteriae* was biologically active *in vitro*. They demonstrated that when used in low concentrations, it is weakly mitogenic for murine splenocytes, and it was found to be as active as *E. coli* endotoxin in producing chemotactic factors for leucocytes. The LPS was also shown to be toxic for mouse peritoneal macrophages at doses similar to that for enterobacterial LPS. The intraperitoneal inoculation of *S. hyodysenteriae* LPS

also gave rise to the appearance of macrophages which were capable of enhanced IgG-Fc and C3 receptor mediated phagocytosis. Nuessen *et al* (1983) showed that the LPS may have a direct effect on the epithelial cells of the large intestine. Evidence produced by Albassam *et al* (1985), also supported that of Nuessen *et al* (1982(a)) by demonstrating the cytotoxic effect of *S. hyodysenteriae* on mouse peritoneal phagocytes. They also demonstrated that in mice the intraperitoneal injection of the non-pathogen *S. innocens* caused macrophage aggregation and a transient neutrophilia, but failed to produce cytotoxicity of peritoneal phagocytes. This would indicate that the endotoxin of *S. innocens* is much less potent than that of *S. hyodysenteriae*.

The extensive fluid losses observed in swine dysentery are due to the reduction in the transport of Na^+ and Cl^- from the lumen of the colon to the blood (Argenzio, 1980; Argenzio *et al*, 1980; Schmall *et al*, 1983). This fluid and electrolyte loss can be fatal because around 50% of the extracellular fluid volume is presented daily to the colon of the pig for reabsorption (Low *et al*, 1978). In weanling pigs it has been shown that there is little net absorption of water in the small intestine (Hamilton and Roe, 1977) and in the absence of any substrate, the jejunum of the pig actively secretes Na^+ and anions (McClung *et al*, 1976). This explains why the fluid and electrolyte

absorption in the colon is so important and consequently why the disease can lead to death by dehydration or ion imbalance.

Serum antibody to *S. hyodysenteriae* can be demonstrated within seven to fourteen days after the start of clinical disease (Burrows *et al*, 1984). Repeated infection with *S. hyodysenteriae* results in high levels of serum immunoglobulin G (IgG). The level of serum IgG is linked to the duration of the disease and secretory IgA in the colon is indicative of recent exposure (Rees *et al*, 1989). In faecal samples, only IgA can be detected as it is far more resistant to proteolysis than the other immunoglobulins present in the intestinal secretions (Rees *et al*, 1989). Joens and Marquez (1986) compared a pathogenic strain of *S. hyodysenteriae* with a strain of the non-pathogen *S. innocens*. They carried out immunoblots using antiserum from pigs recovering from swine dysentery. Using this method, a 16kDa antigen was identified which was present on *S. hyodysenteriae* but not on *S. innocens*. This same antigen was also identified when immunoblots were carried out using colonic secretions from convalescent pigs but not when sera from normal pigs were used. Sera from pigs which have recovered naturally from the disease also respond strongly against this particular antigen when used for immunoblotting.

Wannemuehler *et al* (1988), found that pigs vaccinated with *S. hyodysenteriae* outer membrane extract developed antibody responses qualitatively similar to those of convalescent pigs, and in particular against low molecular mass antigens in the range of 14-19kDa.

These views are supported by Sellwood *et al* (1989). They examined eleven pathogenic strains of *S. hyodysenteriae* and two non-pathogens and found the 16kDa antigen in all eleven pathogens but in neither of the non-pathogens. Using immunogold labelling, Sellwood *et al* (1989) located the antigen in the outer membrane of the organism. These results were further confirmed by Wannemuehler *et al* (1990) when they found that immunity against swine dysentery is related to the presence of antibody against the 16-19kDa antigen. In their investigations they used a proteinase K digest of a lysate of *S. hyodysenteriae* as a vaccine preparation. It therefore appears that this antigen may prove a valuable tool for the diagnosis of swine dysentery and it is possible that antibody against this 16kDa antigen may be protective against swine dysentery.

However other workers consider that different antigens are responsible. Chatfield *et al* (1988) carried out immunoblots using hyperimmune antiserum to *S. hyodysenteriae* which had been absorbed with whole *S. innocens* cells. This showed 3 distinct bands with

molecular weights of 68, 36 and 31 kDa. They found these antigens to be conserved amongst different *S. hyodysenteriae* isolates. Chatfield *et al* (1988) thought that these other polypeptides might also be involved in protection. It is possible that multiple antigens may be involved as there have been findings of 16 kDa (Sellwood *et al*, 1989; Joens and Marquez, 1986), and 31 kDa (Chatfield *et al*, 1988) proteins on *S. hyodysenteriae* although it has not been established that they are related or found on every serotype. Chatfield *et al* (1988) found a 31kDa protein but no 16kDa protein. LPS antigens were also found in the molecular weight range of 14-24 kDa although they were serotype specific. Smith *et al* (1990) also found the LPS antigens to be serotype specific. They also found major polypeptide antigens with molecular weights of 34 and 30 kDa which were conserved between the various isolates. They also noted that the Australian isolates used possessed an extra common antigen with a molecular weight of 39 kDa which was not found on any of the British isolates.

CLINICAL SIGNS

The first signs of swine dysentery in a pig are the twitching of the tail, abdominal discomfort and a slight loss of appetite. Some animals may have an increased rectal temperature of 40-40.5 ^{o}C but this is not seen in every animal and usually disappears with the onset of the diarrhoea which remains through the

course of the disease. The diarrhoea is yellowish in colour and sometimes contains streaks of fresh blood, undigested food and mucus. As the disease progresses the amount of blood in the diarrhoea increases, deepening the colour to a dark reddish brown and shreds of necrotic material may be present. After seven to fourteen days the pigs start to recover and produce faeces containing large amounts of mucus (Taylor, 1989; Harris and Glock, 1986).

Affected pigs are gaunt in appearance and suffer a severe loss of bodily condition. The coat becomes rougher and the hind quarters become stained with faeces. However, in general they remain alert and only mildly depressed. Advanced cases may develop a generalised congestion of the skin and become very depressed. Affected pigs all show some degree of loss of appetite but they all continue to drink, (Taylor, 1989; Harris and Glock, 1986).

POST MORTEM FINDINGS

At post mortem examination, pigs which have died from swine dysentery are found to be in poor bodily condition. Their coats are rough and stained with faeces. Some degree of dehydration is usually evident (Taylor, 1989).

Lesions are consistently found in the large intestine but not in the small intestine and there may be a

distinct demarcation line at the ileocaecocolic junction. There may also be some lesions present in the fundic area of the stomach but this is not always the case (Harris and Glock, 1986).

Further effects of the disease include oedema of the walls and mesentery of the large intestine and the mesenteric lymph nodes are often pale and swollen but not congested. There is also hyperplasia of the goblet cells and the large intestinal epithelium is covered with thick mucus which may contain some blood. As the disease develops, the oedema in the large intestinal wall decreases and the severity of the lesions on the mucosa increases. Older lesions on the mucosa later become covered in a thin layer of necrotic material which may be dislodged to reveal the bleeding mucosa beneath. The distribution of the lesions becomes more diffuse as the duration of the disease increases. Other areas which may be affected include the liver, which can become swollen and congested and the fundic region of the stomach which may haemorrhage (Harris and Glock, 1986).

The microscopic lesions are limited to the swollen colonic mucosa. The spirochaetes may be found in the goblet cells as well as in and between the epithelial cells and adjacent microvilli may become degenerate (Glock *et al*, 1974). The organisms appear to be viable and free within the cytoplasm of the cells with no

evidence of loss of the spirochaetal envelope membranes (Glock, *et al* 1974). Once the organisms had entered the cells they continued to multiply although they did not penetrate beyond the lamina propria, explaining why these lesions are limited to the large intestine.

EPIDEMIOLOGY

Swine dysentery occurs world wide (Roncalli and Leaning, 1976). In Britain approximately 25% of sows are in infected herds and almost every fattening enterprise is affected by the disease (Taylor, 1984; 1989).

All ages of pigs can be infected with the disease but it is found most commonly in six to twelve week old weaned pigs (Harris and Glock, 1986). The disease is usually transmitted by the ingestion of infected faeces.

A carrier state has been found to exist in the disease so that clinically normal pigs may be shedding *S. hyodysenteriae* in their faeces. Songer and Harris (1978) demonstrated that asymptomatic convalescent pigs could transmit the disease to susceptible pigs by pen contact. Pigs which had acute swine dysentery were the positive controls and they transmitted the disease to eight contact pigs in ten to sixteen days. This incubation time agrees with that observed by others

(Taylor and Alexander, 1971). Negative control pigs were maintained in the herd of origin and were inoculated with *S. hyodysenteriae* at the end of the experiment. All of these pigs developed typical swine dysentery. Carrier pigs which had been allowed to recover for 10-25 days and had remained asymptomatic were able to transmit the disease to contacts over a period of 14-51 days. Pigs that had a recovery period of 70 days were still able to transmit swine dysentery to contact pigs in 28 days. Songer and Harris (1978) also showed that the levels of *S. hyodysenteriae* shed in the faeces of carrier pigs were at a culturally detectable level before the contact pigs became ill. These findings were supported by Fisher and Olander (1981). They also observed that the frequency of shedding of the organism decreases with time and only a small proportion of the convalescent pigs may be carriers of the disease. They found that on average faecal shedding of *S. hyodysenteriae* occurred at the rate of 0.108 culturally positive faecal samples/carrier pig/day (Fisher and Olander, 1981).

Windsor and Simmons (1981) carried out an investigation into the spread of swine dysentery. In 23 of the 25 outbreaks investigated, the evidence pointed to the source of the disease as being bought in pigs. In none of these outbreaks were pigs seen to be ill on entry to the farm, although if the pigs had been receiving medication in their feed then this

could suppress the disease (Goodwin and Whittlestone, 1984). The past history of the herd is a more accurate indicator of the presence of the disease than the appearance of the pigs.

The disease has also been shown to be transmitted by other means. Joens (1980) demonstrated the experimental transmission of *S. hyodysenteriae* to pigs from mice which had been infected intragastrically. On exposure to the infected mouse droppings the pigs developed swine dysentery 11-13 days later. Joens and Kinyon (1982) investigated the presence of *S. hyodysenteriae* in wild rodents found on pig farms. It was found that mice could shed *S. hyodysenteriae* for 180 days and rats for only 2 days, indicating that rodents may be involved in the spread and transmission of swine dysentery. Dogs have also been implicated in the spread of swine dysentery. Songer *et al* (1978) and Glock *et al* (1978) have reported the isolation of pathogenic *S. hyodysenteriae* from dog faeces. Stockmen have also been shown to be responsible for carrying the organism into the farm on their boots (Windsor and Simmons, 1981).

The ability of *S. hyodysenteriae* to survive in the environment is also another factor which contributes to the spread and transmission of the disease. Chia and Taylor (1978) examined the effects of storage at different temperatures on the survival of *S.*

hyodysenteriae in dysenteric faeces. It was found to survive best at 0-10 ^{o}C lasting for around thirty days. At 20-25 ^{o}C and above the survival was markedly reduced. A one in ten dilution of the samples with tap water proved to enhance the survival of the organism especially if stored at 5 ^{o}C. Any further dilution served to reduce the survival of the organism. *S. hyodysenteriae* was not recovered from dried faecal samples. However these times may be extended using the newly available highly selective media (Kunkle and Kinyon, 1988).

S. hyodysenteriae has also been isolated from the waste handling system of a pig farm (Songer *et al*, 1978). Glock *et al* (1975) showed that lagoon water contaminated with effluent from a herd infected with swine dysentery would cause the disease in susceptible pigs. *S. hyodysenteriae* has also been reported to survive for 18 days at 4 ^{o}C in the soil, although attempts at transmission of the disease by putting susceptible pigs on contaminated areas of ground were unsuccessful (Harris and Glock, 1986). These findings suggest that although *S. hyodysenteriae* is an anaerobic organism it has the potential to survive in diverse environments.

CLINICAL DIAGNOSIS OF SWINE DYSENTERY

The onset of an outbreak of swine dysentery in a herd is characteristically insidious, distinguishing it

from T.G.E. and epidemic diarrhoea (Taylor, 1989), and so it may be easy for the first few cases of the disease to escape the notice of the farmer especially if there has not been a history of the disease on his farm. The diagnosis is usually made on the history of the outbreak such as the introduction of new pigs, presumably carriers, the clinical signs presented by affected pigs and any post mortem findings.

Swine dysentery may be distinguished from many other enteric diseases by the presence of blood and mucus in the faeces. Clinical findings can however only offer a presumptive diagnosis. Further confirmation results from the post-mortem findings of a mucohaemorrhagic typhlocolitis. Swine dysentery can, however, occur in conjunction with other diseases and as a result the clinical signs may be atypical. This may also happen if incomplete drug treatment has been administered (Taylor, 1989; Harris and Glock, 1986)

These problems can be avoided by detecting the presence of *S. hyodysenteriae* or antibody against it. However the difficulty here lies in ensuring that the method used is specific enough to distinguish *S. hyodysenteriae* or the relevant antibody from the closely related non-pathogens or cross-reacting antibodies concerning them.

THE LABORATORY DIAGNOSIS OF *S. hyodysenteriae*

a) Animal Transmission.

A variety of characteristics of *S. hyodysenteriae* have been exploited to distinguish it from other non-pathogenic spirochaetes such as the ability of *S. hyodysenteriae* to produce swine dysentery on oral administration of pigs (Kinyon *et al*, 1977). Some non-*S. hyodysenteriae* spirochaetes have also been shown to produce diarrhoea (Taylor *et al*, 1980) which could lead to confusion. The inoculation of colonic loops and subsequent examination of the lesions produced has also been used for the testing of *S. hyodysenteriae* isolates, (Whipp *et al*, 1978). Joens *et al* (1980) suggested the use of the CF1 mouse as a model for differentiation between the *S. hyodysenteriae* and *S. innocens*. However these methods are both costly and cumbersome for routine diagnosis.

b)Staining of faecal smears

Using staining methods such as dilute carbol fuchsin or Victoria Blue 4R (Olson, 1978) it is possible to see both *S. hyodysenteriae* and *S. innocens* on smears but impossible to distinguish between them. This may only be useful when the clinical signs are typical and suggestive of swine dysentery.

Both indirect and direct fluorescent antibody tests have been evaluated for detecting *S. hyodysenteriae* (Terpstra *et al*, 1968; Saunders and Hunter, 1974; Hunter and Clark, 1975). However after further

investigation these tests were found not to be specific for the organism (Hudson *et al*, 1976). Hunter and Saunders (1977) attempted to improve the specificity by absorbing the fluorescent antiserum with non-pathogenic spirochaetes. However Smit and Jongerius (1982) reported that they frequently failed to isolate *S. hyodysenteriae* from samples that had been positive when tested with the absorbed fluorescent antiserum. Lysons and Lemcke (1983) investigated the specificity of the direct fluorescent antibody test. They found that absorption with two non-pathogenic spirochaetes was necessary to eliminate any false positives. In doing this however the test would then not react with all the isolates of *S. hyodysenteriae*. This means that any specimens producing positive fluorescent antibody test results require isolation and identification of *S. hyodysenteriae* by another method.

c)Culture

Songer *et al* (1976) devised a selective isolation medium which uses 400ug/ml of spectinomycin hydrochloride incorporated into trypticase soy agar and 5% bovine or horse blood. Plates should be incubated anaerobically in an atmosphere of 5% Carbon dioxide and 95% Hydrogen for 48 hours at 37^{o}C. The organisms show little surface growth and powerful haemolysis. With the primary culture of a faecal sample on spectinomycin agar, it may prove difficult

to distinguish between *S. hyodysenteriae* and *S. innocens* although *S. innocens* is only weakly beta haemolytic. Serial passage on selective agar can help to distinguish between them although it can take some time to carry out.

A further modification of this medium involves the addition of NaRNA (Lemcke and Williams, 1984). Ribonucleic acid is a very efficient carrier molecule for the haemolysin of *S. hyodysenteriae*. When added to the selective medium it enhances the growth of *S. hyodysenteriae* and the zones of haemolysis surrounding the colonies increase. There was also a slight increase in the viable count suggesting that the supplement may increase the sensitivity of selective culture. The addition of NaRNA had little or no effect on the growth of *S. innocens*.

Kunkle and Kinyon (1988) developed a selective medium for the isolation of *S. hyodysenteriae* involving trypticase soy agar containing five anti-microbial agents and 5% pig faeces extract. In their experiments they were able to detect the presence of the organisms in the faeces of asymptomatic pigs using this agar but not using the conventional agar containing 400ug/ml spectinomycin.

If the presence of *S. hyodysenteriae* is to be detected by culture methods then the faecal samples must arrive

at the laboratory quickly so that any organisms present will still be viable. Taylor *et al* (1985) investigated the survival of *S. hyodysenteriae* in faecal samples sent by post and stored at room temperature. This led to the finding that unless samples were diluted 1/10 in phosphate buffered saline (PBS) or swabs placed in Amies medium then the viable count would diminish. Given that those animals that are either carriers, have been treated or are recovering have a smaller number of organisms to start with it is possible that culture methods will fail to detect them on arrival at the laboratory.

Olson and Fales (1983) compared stained smears and culturing for the identification of *S. hyodysenteriae*. They used sheep blood agar containing 400 ug/ml of spectinomycin for the culturing experiments. Victoria Blue 4R was used to stain the smears. In their experiments they took samples from pigs with no clinical evidence of the disease and found that 15.1% of the samples tested had large spirochaetes present in the stained smears and 11.2% that produced either strong or weak beta haemolysis. However 40% of samples that were taken from pigs with haemorrhagic or non-haemorrhagic dysentery had no large spirochaetes in stained smears and a similar percentage was negative for *S. hyodysenteriae* by culture methods. Some samples taken from pigs infected with swine dysentery were also shown to produce weak beta haemolysis on blood

agar. Some samples taken from pigs infected with *S. innocens* produced strong beta haemolysis. Olson and Fales (1983) concluded that neither staining rectal smears nor culturing rectal swabs is sufficient either together or alone for the diagnosis of swine dysentery, although they give supportive evidence to the clinical diagnosis.

d)The detection of serum antibody

Hunter and Saunders (1973) devised a serum agglutination test for swine dysentery. Their results indicated that this test may be useful on a herd basis. Joens *et al* (1978(a)) also developed a serum agglutination test that could detect antibody against *S. hyodysenteriae* in pigs 5-7 weeks after recovery. However the ranges of serum titres were such that the agglutination test was again found only to be useful on a herd basis. A cost analysis showed that this test was less expensive per sample than culture methods although it cannot be used for individual pigs.

Jenkins *et al* (1976) devised a passive agglutination test for the detection of antibody to *S. hyodysenteriae*. This involved two fold serial dilutions of test sera to each of which was added *S. hyodysenteriae* sensitised sheep red blood cells and guinea pig complement. The titres of the sera were then expressed as the last dilution of serum resulting in 50% haemolysis of the indicator cells. This test

will however only indicate the presence or absence of immunity, not the presence or absence of the organism. There may also be problems encountered with different serotypes not reacting.

Joens *et al* (1982) developed an enzyme linked immunosorbent assay (ELISA) for the detection of serum antibody to *S. hyodysenteriae*. They concluded that this test was sensitive enough to be used for diagnosis of *S. hyodysenteriae* in individual pigs. Egan *et al* (1983) found that the ELISA was only useful determining the overall infection status of a herd. This supports the view of Joens *et al* (1978(b)) and Rees *et al* (1989) who found that there was a wide range of antibody titres produced in response to the organism. Such an ELISA will not detect those infected animals which have not yet developed an immune response.

IDENTIFICATION OF *S. hyodysenteriae* BY BIOCHEMICAL AND ENZYMATIC DIFFERENCES.

Biochemical differences between *S. hyodysenteriae* and *S. innocens* have also been investigated (Lemcke and Burrows, 1981). It was concluded that biochemical tests do not provide a suitable means for identifying *S. hyodysenteriae*. The only point of distinction proved to be that the three strains examined in these experiments tended to produce more n-butyric acid than the non-pathogens. The production of indole is

suggestive but not an infallible indication that an organism is *S. hyodysenteriae*. Both *S. hyodysenteriae* and *S. innocens* were able to ferment carbohydrates especially fructose.

Hunter and Wood (1979) examined the possibility of using the API ZYM system as a means of identifying spirochaetes associated with swine dysentery. This test involves the isolation and culture of the organism before testing with the spectrum of enzymatic reactions involved in the API ZYM system. *S. hyodysenteriae* strains were found to be consistently lacking in alpha galactosidase whereas they always produced alpha and beta glucosidase.

IDENTIFICATION OF *S. hyodysenteriae* BY SEROLOGICAL AND ANTIGENICITY TESTS IN CULTURE

Lemcke and Burrows (1979) developed a disc growth inhibition test for the identification of *S. hyodysenteriae*. This involves the impregnation of antibiotic assay discs with anti- *S. hyodysenteriae* antiserum. The suspected *S. hyodysenteriae* is then spread over a trypticase soy agar plate containing unheated rabbit serum, 200 ug/ml spectinomycin, and cysteine hydrochloride. One of the antibiotic assay discs is then placed on the surface of the plate which is then incubated anaerobically for 3-5 days. The test proved to be more specific than the fluorescent antibody test although the anti-serum was not absorbed

with non-pathogens. It is however a time consuming method as at least two subcultures have to be done before the actual test. Positive results proved easy to pick out ,although how easy it was depended on such things as the thickness of the agar and how heavy the inoculum was. In some cases false negatives could occur because the anti-*S. hyodysenteriae* anti-serum did not inhibit the particular strain of *S. hyodysenteriae* being tested. This test also requires a medium not generally found in veterinary diagnostic laboratories.

The rapid slide agglutination test (Burrows and Lemcke, 1981) requires only one subculture and therefore is much quicker than the growth inhibition test. In this test the antiserum used was absorbed with non-pathogenic spirochaetes. A drop of absorbed anti-serum is placed on a glass slide and a drop of spirochaetal growth added. A positive reaction is indicated by agglutination. However the use of absorbed anti-serum will not necessarily eliminate all the cross reactions with non-pathogens or be able to react with every pathogenic strain. A lot of work remains to be done in defining the range of serologically distinct strains both in *S. hyodysenteriae* and in *S. innocens* (Baum and Joens, 1979(b); Hampson, 1990).

A microscopic agglutination test for the rapid identification of *S. hyodysenteriae* has been developed (Lysons, 1991). This test involves the addition of absorbed polyclonal antisera to a suspension of spirochaetes. The preparations were then examined under phase contrast or dark field microscopy at a magnification of 400X for any agglutination. Of the 21 strains examined, the test correctly identified all twelve of the known *S. hyodysenteriae* strains and the 9 strains of *S. innocens*. However this test also suffers from the fact that the organisms must first be isolated and then grown in pure culture - a procedure which can take more than a week.

The presence of *S. hyodysenteriae* antigen can be detected in faeces and slurry by a faecal ELISA test (Taylor and Stevenson, 1986). This test has the added advantages of being non-invasive and being able to detect those animals that have been recently infected and have not had time to develop an immune response. It is specific for *S. hyodysenteriae* and can detect organisms at levels of 10^2 organisms per gram. This ELISA test does not require viable organisms, only the presence of the antigen, although the scope of such a test may be limited by the quality of the antiserum. However this test is not as fast as the RPLA test and still requires that the sample be sent to a laboratory for testing.

Jensen *et al* (1990) developed a diagnostic test using oligodeoxynucleotide probes complementary to *S. hyodysenteriae* 16S rRNA. The 28 base probe was shown to detect the organism in faeces, although the strains it was detecting were those for which the probe had been developed. Further studies involving field samples have yet to be carried out.

Multilocus enzyme electrophoresis has been investigated as a means for the identification and typing of *S. hyodysenteriae* (Lymberry *et al*, 1990). This system is an improvement on the serotyping method suggested by Baum and Joens (1979(b)) which uses extracted LPS and could lead to a greater understanding of the epidemiology of the disease. Hampson *et al* (1989) found that some isolates contain multiple major LPS antigens while others lose them. This restricts the use of the scheme and limits its ability to identify natural clones. Using only 5 enzyme systems, Lymberry *et al* (1990) were able to distinguish between several isolates which had been previously found to be the same serotype. More enzyme loci would improve the sensitivity of the system.

THE ECONOMIC IMPORTANCE OF SWINE DYSENTERY

Swine dysentery is an economically important disease world-wide (Roncalli and Leaning, 1976). The lack of a vaccine and the presence of asymptomatic carriers are

problems which can be overcome by enforcing strict control schemes and maintaining high standards of hygiene (Goodwin and Whittlestone, 1984). The costs of eradicating the disease from a farm without such drastic measures as depopulation are high, although they have been shown to be recouped within the next twelve months due to the increased feed conversion ratio and there being no requirement for medication in the feed (Wood and Lysons, 1988; Briggs, 1989; Windsor and Simmons, 1981). However it would be easier to maintain these high standards if pigs could be tested more quickly for the presence of *S. hyodysenteriae* as they were bought and sold thereby helping to prevent the spread of the disease. This could be done by using a reverse passive latex agglutination test (RPLA).

THE ADVANTAGES OF AN RPLA TEST FOR SWINE DYSENTERY.

The problems of identifying *S. hyodysenteriae* at present are compounded by its slow growth rate and the requirement for a pure culture with which to work. A reverse passive latex agglutination test for swine dysentery would have a much faster turn-around time than the current diagnostic methods. It could be carried out on the farm as no laboratory equipment is required and the results would be known within minutes. This would avoid the delays necessary while waiting for culture results and would enable appropriate action to be taken sooner rather than later.

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An RPLA test for swine dysentery would also be able to detect asymptomatic carriers which are a major cause of the spread of the disease (Windsor and Simmons, 1981). This could enable the testing of pigs as they were bought and sold.

From the point of view of diagnostic laboratories, RPLA tests do not require the presence of the live organism, only the antigen, so that whether the sample arrives containing viable organisms or not becomes irrelevant. In short an RPLA test for swine dysentery would be a simple, quick and effective diagnostic test for use both in the laboratory and in the field.

REVERSE PASSIVE LATEX AGGLUTINATION TESTS

Since the advent of reverse passive latex agglutination (RPLA) tests in 1957 (Singer and Plotz, 1957) these tests have been in widespread use. The ease with which the test can be carried out in the field and the quick production of results have made this type of test favourable for routine testing and screening procedures. RPLA tests can avoid or limit delays incurred by a conventional approach involving the isolation and identification of an organism, a criticism of which would be that the results would not be available until relatively late in the clinical illness which may limit the overall value of the test.

The test can be used for the detection of antibody or antigen. Test kits have been devised to detect antibody to such organisms as cytomegalovirus (Feng *et al*, 1986; McDonald *et al*, 1990), infectious mononucleosis virus (Levey *et al*, 1980) and *Toxoplasma gondii* (Mazumder *et al*, 1988; Seamon *et al*, 1977). Various antigens such as streptococci (Isada and Grossman, 1987), *Escherichia coli* (March and Ratnam, 1989), salmonella and shigella (McGowan and Rubenstein, 1989) and rotavirus (Brandt *et al*, 1987; Dennehy *et al*, 1988) can also be detected by RPLA tests.

Several different RPLA tests can be incorporated in the same suspension using different coloured particles for each test, each colour being coated with antibody or antigen for a different test (Thorns *et al*, 1989; Hadfield *et al*, 1987).

RPLA tests are versatile and can be used on a variety of samples such as blood, faeces, urine or food with a minimum of processing. Specimens generally require dilution in a buffer of a relevant pH, serum samples may need a pH around 7 whereas urine or faecal samples may require a pH nearer 8.0. The addition of a large excess of antigen or antibody to the test beads may prevent the beads agglutinating so that several dilutions may need to be done. Samples containing large amounts of particulate matter which may

interfere with the test can be suspended in buffer and then centrifuged or filtered before testing. The use of positive pressure filters which can be fitted onto the end of a syringe enables the test to be carried out anywhere.

The sensitivity and specificity of RPLA tests can vary from test to test. Some tests such as that for group B streptococci can detect as little as 30 colony forming units per ml of the organism with a specificity of 93% (Isada and Grossman, 1987).

The latex agglutination test for *E. coli* serotype 0157 is 100% sensitive and specific (March and Ratnam, 1989). They do however recommend it for presumptive identification and stipulate that further serotyping with H7 antiserum is necessary for definitive identification. This test proved to be more sensitive in detecting very low numbers of the organism in food than the current culture methods.

The RPLA test designed for cholera toxin is able to detect 1-2 ng of toxin per ml. When compared with ELISA results, it had a sensitivity of 97% and a specificity of 100% (Almeida *et al*, 1990).

Rotavirus can be detected in faecal specimens by using RPLA tests. Dennehy *et al* (1988) surveyed a total of nine methods of detecting rotavirus in clinical

samples including four RPLA tests and compared them to a reference immunoassay. This revealed that the commercial latex tests had sensitivities ranging from 70% to 90% and specificities of 80%-100%. Brandt *et al* (1987) also investigated rotavirus detection by RPLA. Their conclusion was that when the number of virions present in the sample was 2 or less per minute as seen by EM viewing then the test was no longer able to detect this level. However with 3 or more virions per minute the sensitivity is 96.3% with 100% specificity.

Haemophilus influenzae type b can also be detected by a latex test (Riera, 1985). This test can detect as little as 0.2ng/ml (Marcon *et al*, 1984) compared with the coagglutination test limit of 25ug/ml.

When compared with its ELISA counterpart, the RPLA test for *Toxoplasma gondii* antibodies has a sensitivity of 86% and a specificity of 100% (Mazumder *et al*, 1988).

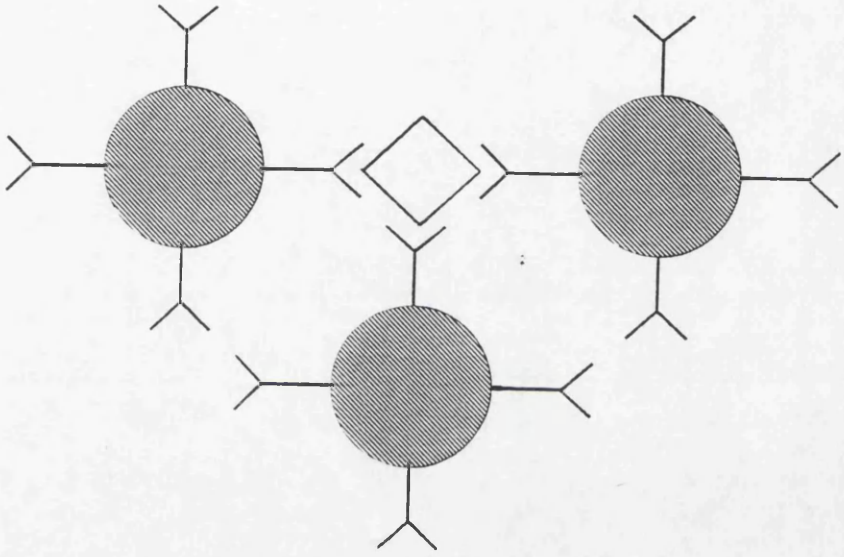
RPLA tests may also render false negative results. In some cases this is due to an excess of the antigen or antibody, and further dilution is necessary to produce a positive result. However this is not always the case and in the study involving the cholera toxin RPLA an overall 3.4% false negative rate was noted (Almeida *et al*, 1990). The coating of the antigen by antibody

present in a sample e.g. in a faecal specimen does not appear to present a problem.

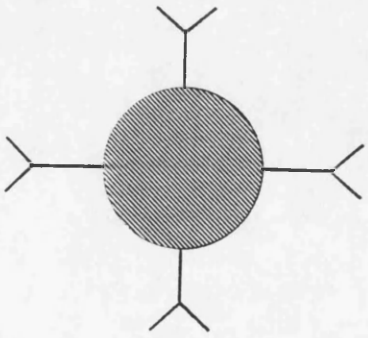
Results which cannot be interpreted by this method may occur. This is when non-specific agglutination occurs in both the test latex and the negative control latex. Miles *et al* (1988) found non-specific agglutination in about 1% of samples tested for *C. difficile* toxin and so it is not thought to be a significant problem with this test. They showed this type of result to be due to *S.aureus* reacting with both the control and test latex. When serum samples are being tested false positives may occur due to the presence of rheumatoid factor (Hechemy and Michaelson, 1984).

Uncoated latex particles by virtue of their charged surfaces are able to form stable lyophobic suspensions. However these can be non-specifically destabilised by the addition of trichloroacetic acid. If the particles are coated with a specific ligand they adopt the properties of that ligand, forming stable lyophilic suspensions. When particles are coated with antigen or antibody they remain in suspension until they are mixed with the relevant antigen or antibody causing them to agglutinate, (Figure 1) (Hechemy and Michaelson, 1984).
Only a small amount of ligand is adsorbed to the surface of the particles relative to the amount added to the suspension. This means that the process of

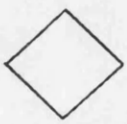
FIGURE 1: MECHANISM OF RPLA AGGLUTINATION



AGGLUTINATION OF LATEX PARTICLES



ANTIBODY COATED LATEX



ANTIGEN

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There are three methods of coating the latex particles: passive adsorption, forced adsorption and coupling. Passive adsorption involves the random adsorption of a solute molecule onto the surface of the latex particles. This creates a double molecular layer, the inner layer of which is strongly bound and the more loosely bound outer layer which can be easily washed off (Singer *et al*, 1963). The adsorption process is thought to occur in two stages. Firstly on the addition of a small amount of ligand the surface charge of the particles is neutralised causing the particles to agglutinate spontaneously. This is

followed by the addition of more ligand until the particles cease to agglutinate. Addition of excess ligand past this point causes further agglutination (Hechemy *et al*, 1976).

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The addition of a limited excess of the ligand can help to stabilise the solution. In some cases the free ligand can be replaced, and albumin used to stabilise the solution. If this is to be done then the ligand

should be added first and then the stabilising agent, otherwise the ligand will not be able to bind to the latex as the stabilising agent will be occupying all the sites (Hechemy and Michaelson, 1984)

The length of time that the passive adsorption of the latex particles takes appears not to be critical (Singer *et al*, 1961; Oreskes and Singer, 1961). In the first five minutes about 98% of the total ligand will have been adsorbed and the remaining 2% will be adsorbed within 1-2 hours.

Should the passive adsorption method be unsuitable, other methods for coating the particles should be investigated. Forced adsorption involves the use of a precipitating agent to force precipitate the ligand onto the surface of the latex particle. This eliminates the need for a highly purified ligand (Hechemy and Michaelson, 1984).

Carboxyl, hydroxyl and amide groups present on the surface of the latex particles can be used to couple the ligand covalently to the particles. This can be done by using agents such as carbodiimide, cyanogen bromide and glutaraldehyde (Singer and Plotz, 1956; Hechemy and Michaelson, 1984).

A number of buffers may be used with RPLA tests both for suspending the coated particles and diluting the

test sample. Sample preparation may also involve centrifugation or filtration. The most commonly used buffers are glycine buffered saline (GBS) (Riera, 1985; Hechemy *et al*, 1981; Levey *et al*, 1980) and phosphate buffered saline (PBS) (Hadfield *et al*, 1987; Mazumder *et al*, 1988). The buffers can also be of varying pH, usually from 7 to 9.2, latex particles being unstable below pH 7 (Hechemy and Michaelson, 1984). The pH of the buffer will also depend on the nature of the sample, e.g. serum samples will have a decreased activity above a pH of 8 (Hechemy and Michaelson, 1984).

RPLA kits typically comprise a test latex suspension which has been sensitized to the relevant antigen or antibody, a control latex suspension, a buffer and a positive control. The positive control is a solution of the relevant antigen or antibody against which the test latex has been sensitized. The control latex consists of latex particles which have been sensitized with non-immune anti-serum. Either a slide or a disposable card can be used to carry out the test. In carrying out the test, two separate drops of the prepared sample are placed on the slide and a drop of the test latex is added to one and to the other a drop of control latex. A drop of the positive control is also placed on the slide and a drop of the test latex added. A positive test should involve agglutination in the test latex and in the positive control, with no

agglutination in the control latex. A negative result is indicated by agglutination only in the positive control. The entire test takes about 10 minutes to perform.

Because RPLA tests are this quick and easy to carry out, they could be completed on the farm, greatly improving the turn-around time of current diagnostic methods for swine dysentery. It therefore remains to isolate an antigen specific to *S. hyodysenteriae* against which either polyclonal antiserum can be raised or monoclonal antibodies can be generated.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

INTRODUCTION

The materials and methods described in this Chapter were used throughout the study. Where they were modified the variations are described in the relevant chapter. Methods which were developed for a particular study are also described in the appropriate Chapter.

MATERIALS AND METHODS

ANTIGEN PREPARATION AND ANALYSIS

ORGANISMS

The organisms used in this study were *S. hyodysenteriae*, strains, S80/5 and B78 and *S. innocens* strains, PWS/A and 4/71 and the dog spirochaete, Reddicap. S80/5 and B78 were stored freeze dried and kept at 4°C. PWS/A and 4/71 were obtained from Dr. R. Lysons, Compton and were stored both freeze dried and at -70°C.

CULTURE MEDIA AND CONDITIONS

The organisms were grown on blood agar (7% horse blood, blood agar base No.2 (Oxoid)). S80/5 was also grown in trypticase soy broth (Oxoid) containing 1% VPI salt solution, 0.5% yeast extract (Oxoid), 0.05% cysteine hydrochloride (BDH), and 5% horse serum, (Wannemuehler *et al*, 1988). VPI A salt solution contains 0.04% $CaCl_2$ and 0.04% $MgSO_4$. VPI B salt solution contains 0.2% K_2HPO_4, 0.2% KH_2PO_4, 2% $NaHCO_3$ and 0.4% NaCl. The completed medium was prereduced overnight in an anaerobic cabinet (Mark three

anaerobic work station, Don Whitley Scientific Limited) before use. The cultures were all incubated in an anaerobic cabinet for 48-72 hours at 37°C in an atmosphere of 10% hydrogen, 10% carbon dioxide, 80% nitrogen.

SUBCULTURE AND MAINTENANCE OF ORGANISMS

The organisms were subcultured onto horseblood agar plates every three to four days and were maintained in an anaerobic environment as described above.

SDS POLYACRYLAMIDE GELS (SDS PAGE)

The SDS polyacrylamide gels were made up using the method of Sambrook *et al*, 1989 and the materials described below. The running gel was a 12.5% SDS PAGE gel which contained 18.7 ml of a stock solution containing 30% acrylamide (Sigma) and 2.67% N-N bismethylene acrylamide (BDH); 14.9 ml of 1M tris/HCl (BDH) pH 8.7; 0.2 ml of 20% (w/v) SDS (BDH) and 6.2 ml of distilled water. This solution was degassed and 30 µl of tetramethyl ethylenedimine (TEMED) (Sigma) and 135 µl of freshly made up 10% ammonium persulphate (BDH) were added to complete the running gel. Once the running gel had been poured the surface was levelled by pipetting a 50:50 mixture of butanol and water on top of the gel. The gel was then left to set for half an hour. After pouring off the butanol/water mixture, the stacking gel was made up and pipetted on top. The stacking gel contained 2.4 ml of the solution

containing 30% acrylamide and 2.67% N-N bismethylene acrylamide; 1.25 ml of 1M tris/HCl pH 6.9; 50 µl of 20% (w/v) SDS and 6.35 ml of deionised water. As soon as the stacking gel had been poured, the comb was set in place. If the gel was not to be run that day, it was stored overnight in a plastic bag in the fridge.

PREPARATION OF ORGANISMS FOR SDS PAGE

48-72 hour cultures of organisms were harvested from blood agar plates by washing with approximately 1 ml of sterile saline. The resulting suspensions of organisms from each plate were then centrifuged (approximately the same amount of each organism was used each time) and the supernatant replaced with sample buffer (Sambrook *et al*, 1989). The sample buffer contained 0.0625M Tris/HCl (BDH) pH 6.8; 2% SDS (BDH); 10% glycerol (M&B); 5% 2-mercaptoethanol (BDH) and 0.001% bromophenol blue (BDH). The samples and standard molecular weight markers were heated at 100°C for 4 minutes before use. The molecular weight markers used were ß lactoglobulin, 18,400 Da (Sigma) and lysozyme, 14,300 Da (Sigma).

RUNNING SDS PAGE GELS

The gel apparatus (Bethesda Research Laboratory Vertical Gel Electrophoresis System) was set up and the two tanks were filled with electrode buffer pH 8.3. The electrode buffer contained 25 mM tris, 192 mM glycine and 5 ml of 20% (w/v) SDS in 1 litre of

distilled water. 30 μl of sample were put into each well. 10 μl of molecular weight markers were put in one well at either end of the gel which was then run for approximately 3.5 hours at 25 mA or overnight at 4mA.

COOMASSIE BLUE STAINING OF GELS

One corner of the gel was marked and then it was stained with the following solution: 50% methanol; 10% acetic acid; 40% deionised water and 0.05% Coomassie blue. It was left in the stain for 30 minutes before destaining in the following solution: 50% methanol; 10% acetic acid and 40% distilled water. Gels were examined visually and stored in the destain solution. The molecular weight of the relevant band was determined by measuring and comparing with the distance migrated by the molecular weight markers.

SILVER STAINING OF GELS

The gel to be silver stained was rinsed for 8 hours in a solution of 50% v/v methanol. The rinsing solution was changed 4 or 5 times. Two separate solutions were made up for the silver stain. Solution A contained 1.6g silver nitrate (JMC) in 8 ml distilled water. Solution B contained 42 ml of 36% (w/v) NaOH (Formachem) mixed with 2.5 ml of "0.88", (17.1M) ammonia solution (BDH). Solution A was then added slowly to solution B with continual mixing to prevent the formation of precipitate and the volume was then

made up to 200 ml with distilled water. The methanol/water mixture was poured off and enough of the staining solution was added to cover the gel and it was left for 15 minutes. Before developing, the gel was washed twice for five minutes in distilled water. The developing solution was made up with 2.5 ml 1% w/v citric acid (BDH) and 0.6 ml formaldehyde. This solution was then made up to 500 ml with distilled water. After the gel had been left to soak in the developer for 10-15 minutes, 45% methanol 10% glacial acetic acid (BDH) was added to stop the developing process. The molecular weight of the relevant band was determined by measuring the distance migrated and comparing with the distance migrated by known molecular weight markers. Gels were then stored in the "stop" solution.

WESTERN BLOTTING

Gels which were to be blotted were rinsed first in transfer buffer (25mM tris/HCl; 192 mM glycine; pH 8.3) to remove any remaining SDS. Two pieces of filter paper and a piece of nitrocellulose (BDH) were cut to the size of the gel and were then soaked in buffer along with the supports (Scotchbrite pads). The various layers were then put together in the following order: scotchbrite pad, filter paper, gel, nitrocellulose, filter paper, and scotchbrite pad. The bottom left hand corner of the nitrocellulose was marked and placed over the bottom left hand corner of

the gel. The blot was carried out in such a way as to transfer proteins from the gel onto the nitrocellulose, i.e. from cathode to anode. The blot was carried out in a BIO-RAD Trans blot cell overnight at 20 volts at and 4°C to prevent overheating and the formation of bubbles.

The nitrocellulose was then transferred to a dish containing blocking solution. This consisted of TNT buffer (10mM Tris/HCl; 0.15mM NaCl (FSA); 0.05% Tween 20 (BDH); pH 8.0) and 10% skimmed milk powder. This was then left on a platform shaker for one hour. The nitrocellulose was then given three five minute rinses with TNT buffer after which a 1/500 dilution of the rabbit anti-*S. hyodysenteriae* antiserum described below in antibody buffer (5% skimmed milk in TNT buffer) was added. It was then left on a platform shaker overnight at 4°C. The nitrocellulose was again rinsed three times for five minutes in TNT buffer. A 1/3000 dilution of goat anti-rabbit horseradish peroxidase conjugate (Biorad) in antibody buffer was then added to the nitrocellulose. The nitrocellulose was then left in the cold room for one hour. Before it was developed the nitrocellulose was washed once in TNT buffer and twice in NT buffer (10mM tris/HCl; 0.15mM NaCl; pH 8.0).

The horseradish peroxidase colour development solution was made up as follows: 10 ml of methanol was measured

into a universal which was wrapped in tin foil and left on ice. When it was cold 30 mg of 4 chloro-1-naphthol was added. 50 ml of NT buffer was measured into a small flask to which 30 μl of cold H_2O_2 (100 volumes) was added. These two solutions were then poured over the nitrocellulose and left to develop for 15-30 minutes. The blots were stored in distilled water at 4^{o}C in the dark.

PRODUCTION OF ANTISERUM

Anti S80/5 antiserum was produced by the method of Burrows and Lemcke (1981). Some of the antiserum was absorbed using PWS/A and 4/71. This was done by harvesting the growth from two plates of pure cultures of each strain with PBS (0.5ml of each strain) and adding to 1 ml of the antiserum and leaving it on ice for 20 minutes. This was repeated three times. Reactivity of the antiserum was assessed by western blotting. (Absorption of the antiserum with this quantity of organisms for four hours at 37^{o}C completely absorbed the antiserum removing all antibody).

PURIFICATION OF ANTIBODY

Antibody was extracted from the serum by ammonium sulphate precipitation. Firstly the volume of the antiserum was determined and then it was centrifuged at 3000g for 30 minutes. A pre-precipitation step was carried out in order to remove any large proteins and

proteins that would precipitate at a lower concentration of ammonium sulphate. The supernatant was transferred to a small container and while stirring, half the volume of antiserum of ammonium sulphate was added. Once it was all added, the serum was left at 4°C for 6 hours. After centrifugation for a further 30 minutes at 3000g, the supernatant was transferred to a clean container. Half the starting volume of ammonium sulphate was added with stirring to bring the solution to a final concentration of 50% saturation. This was then left overnight at 4°C.

The supernatant was then removed and the pellet resuspended in PBS in 0.5 volume of the starting volume. Then a further precipitation step was carried out as before and the final pellet was then redissolved in 0.3 volume PBS. The antibody was then transferred to dialysis tubing and left overnight. The protein concentration of the final solution was estimated using a Lowry protein assay kit (Sigma) in the recommended manner. The purified antibody was then stored at -20°C.

COATING OF LATEX BEADS

White polystyrene latex beads (0.45μm diameter; aqueous suspension; 10% solids content) were obtained from Sigma. The quantity of antibody that was required to coat the surface of the beads was calculated by checkerboard titration. Glycine buffered saline (GBS)

was used as a dilulent and for suspending the coated latex particles. GBS consisted of 0.05M glycine; 0.05M NaCl and 0.05g sodium azide per litre in distilled water (Kimura, 1980). The pH was then adjusted to 8.2 by addition of 1N sodium hydroxide. Antibody was then added to a concentration of 3μg/ml. Then a 1/10 dilution of the latex beads in antibody containing solution was made. The completed suspension was then left to incubate at room temperature for two hours with occasional shaking before being refrigerated overnight to ensure that the adsorption process was complete. The suspension was then stored at 4°C. A control latex suspension was also made by the same method using antibody derived from non-immune rabbit antiserum.

THE EXAMINATION OF FAECAL SAMPLES

FORM OF THE SAMPLES

Samples for testing arrived as pots of faeces or colon contents in normal saline, or as swabs. Each sample or set of samples came with its own history.

EXAMINATION OF FAECAL SAMPLES

Faecal and colon samples were tested by RPLA for both *S. hyodysenteriae* and for rotavirus. This was carried out in order to determine whether or not the disease present was caused solely by any spirochaetes present. Samples which came as swabs were cultured before RPLA testing. Smears were also made of the samples which

were then Gram stained. The samples were then examined for *S. hyodysenteriae* by culture on spectinomycin blood agar. The plates were then incubated anaerobically for 48-72 hours before examination. Other routine examinations were carried out for ß haemolytic *E. coli* and salmonellae and other possible pathogens. Any spirochaetes that did grow were isolated and sub-cultured. They were subsequently examined by API ZYM and SDS PAGE techniques.

TESTING OF SAMPLES BY RPLA

Both faecal samples and colonic contents from pigs from various sources were tested. This involved making a one in ten dilution of the sample with GBS pH 8.2 and the filtering it with a positive filter (Sartorius ministart, 0.45 μm pore size) attached to the end of a syringe. Two separate drops of the filtrate were placed on a black tile which had been marked into four into two of the separate areas. One drop of the test suspension was added to one of the drops of filtrate and to the other one drop of the control suspension was added. The tile was then tilted by hand for approximately two minutes. This was then repeated and if the results did not agree then the test was repeated a third time.

INTERPRETATION OF TEST RESULTS

If the test suspension was seen to agglutinate in three or two out of the three times and the control

did not agglutinate then the sample was regarded as positive. If both of the suspensions agglutinated every time the test was carried out then the sample was regarded as uninterpretable by RPLA. When neither of the suspensions agglutinated the result was regarded as negative. However because negative results may be due to an antigen excess then the sample was further diluted to 1/100 before a conclusive result was drawn. A positive result (+) was regarded as being a good strong agglutination of the beads. A stronger positive result involving the formation of large floccules of beads is indicated by (++). This may indicate the presence of a nearer optimum number of organisms (approximately 10^8 organisms per ml) or perhaps those of the same serotype. A weak positive result was indicated by the formation of a few small floccules of beads which were best seen when the slide was tilted gently and the initial weak positive result was not due to an excess of antigen present in the sample. The RPLA results were also interpreted in the light of the culture results and the presence or absence of spirochaetes in smears. The morphology and the numbers of any spirochaetes were also taken into account when interpreting the smears. It was also neccessary to take note of how long it had taken for the samples to arrive at the laboratory and whether or not it was reasonable to expect *S. hyodysenteriae* to grow in culture.

SPECIFICITY AND SENSITIVITY ASSAYS

The known pathogens, S80/5 and B78 and the two non-S. hyodysenteriae spirochaetes, PWS/A and 4/71 were grown on horse blood agar as previously described. The organisms were harvested from each plate with sterile PBS and counted by the method described below. Once the suspensions of organisms had been brought to a standard concentration of organisms/ml the RPLA tests were carried out. Known numbers of organisms were added to spirochaete-free faecal samples in order to determine whether or not the RPLA tests could detect the same levels of spirochaetes in faeces as in culture.

ESTIMATION OF NUMBERS OF ORGANISMS

This was done by the method of Miles *et al* (1938). Cultures of *S. hyodysenteriae* S80/5 and B78 and *S. innocens* PWS/A and 4/71 were grown on horse blood agar as previously described. The organisms were harvested from the surface of the agar with 1 ml sterile PBS. A series of 1/10 dilutions of each strain was carried out and a 5μl drop of each dilution was placed onto a quartered plate. Each dilution was plated out in triplicate. After anaerobic incubation at 37^{o}C as previously described, the number of organisms was then counted enabling the total number per millilitre to be calculated.

The number of organisms in a faecal sample was estimated using the following method. A gram of the faecal sample was weighed out and diluted one in ten with sterile PBS and shaken. A series of one in ten dilutions was then prepared and 5μl of each dilution was plated out onto spectinomycin blood agar and left to incubate anaerobically as before. This experiment was also done in triplicate and then the numbers of spirochaetes in each sample could be calculated.

CHAPTER THREE

DEVELOPMENT AND TESTING OF THE ABSORBED POLYCLONAL RPLA TEST

INTRODUCTION

The development of the RPLA was carried out in a previous study (Thom, 1990). In order to assess the potential use of the RPLA test for *S. hyodysenteriae* several criteria had to be investigated. Firstly the test had to be able to detect *S. hyodysenteriae* not only in culture but also in faecal filtrates, after which the specificity of the test for *S. hyodysenteriae* had to be determined. The sensitivity of the test was investigated using both cultured organisms and those present in both faecal samples and colonic washings from experimentally infected pigs. This was done in order to assess the effect, if any, of coproantibody on the test.

MATERIALS AND METHODS

PREPARATION OF LATEX REAGENTS

The RPLA reagents for *S. hyodysenteriae* detection and the negative control reagents were produced as described in Chapter Two. They consisted of latex coated with absorbed rabbit anti *S. hyodysenteriae* antibody, latex coated with a similar preparation from non-immune rabbit serum and GBS buffer pH 8.2.

EVALUATION OF SENSITIVITY AND SPECIFICITY

Spirochaetes, S80/5, B78, 4/71 and PWS/A were cultured and counted as described in Chapter Two. The organisms were then brought to a standard concentration of 10^{10} organisms/ml. A series of tenfold dilutions in GBS was

prepared with each organism and each dilution was tested with the RPLA test as before. Known numbers of organisms were added to spirochaete-free faeces and dilutions of these samples were then tested.

The numbers of organisms found in faecal samples was estimated by the method described in Chapter Two. Each dilution of the faecal sample was then tested by RPLA.

FAECAL AND COLONIC SAMPLES

Faecal and colonic samples were obtained from pigs from a swine dysentery free herd which had been experimentally inoculated with *S. hyodysenteriae*, S80/5. The pigs concerned were part of a control group involved in a study to determine the effective level of monensin required to control swine dysentery (Taylor, 1991) and as such did not receive any medication. The pigs were inoculated in the following manner: For one week prior to infection, all pigs were maintained on an antimicrobial free diet. After weighing all pigs were inoculated with *S. hyodysenteriae*, S80/5. Each pig received a saline suspension of 3 thickly inoculated 48 hour plate cultures of *S. hyodysenteriae* on day 8 of the experiment. Two hours later, enough feed was given to each pen to allow each pig 0.5 kg feed per head. This infection was repeated again the following day. The resulting disease first appeared in the unmedicated

control animals from day 22 and typical swine dysentery eventually developed.

S. hyodysenteriae was sought in the faeces of pigs in pens where swine dysentery occurred. At post mortem, the pigs were examined at 10 sites for the presence of *S. hyodysenteriae*. The RPLA results were then compared with the bacteriological and post mortem findings. Spirochaete free faeces was also obtained for use as a negative control.

DETERMINATION OF SHELF LIFE OF THE RPLA TEST.

In order to determine the shelf life of the RPLA test, two preparations were set up, one to remain at room temperature and one to be stored at +4°C. The two preparations were examined on a weekly basis. The preparation was considered to be effective if latex beads which had agglutinated spontaneously were easily separated to form a smooth suspension once again and remain that way. If they were not easily separated when added to an equal volume of GBS pH8.2 then the preparation was considered unusable. Once this had been done and the latex suspensions proved to be either smooth or have lumps that could be removed with very little effort, then the RPLA test was carried out using cultured organisms.

RESULTS

RESULTS OF SPECIFICITY AND SENSITIVITY TESTS

A Western blot of the four strains of organisms using the absorbed anti *S. hyodysenteriae* antiserum is shown in Figure 2 from which it can be seen that there is some cross-reaction between *S. hyodysenteriae* and *S. innocens*.

The results of specificity and sensitivity testing when cultured material was used are shown in Table 1. It was noticeable that the RPLA tests carried out on *S. hyodysenteriae* reacted rapidly - they became positive within seconds provided that there was an optimum number of organisms present (approximately 10^8 organisms). The non-*S. hyodysenteriae* spirochaetes did cause agglutination but did not react until there were at least 10^9 organisms present for PWS/A or 10^{10} for 4/71. The RPLA results of normal faeces to which organisms had been added were also carried out, the results of which are shown in Table 2. The RPLA results of dilutions of faecal samples from infected pigs of known *S. hyodysenteriae* content is shown in Table 3.

RESULTS OF SHELF-LIFE EXPERIMENT

The RPLA test that was stored at $4^{\circ}C$ was found to last for approximately one year and that which was stored at room temperature remained useable for approximately

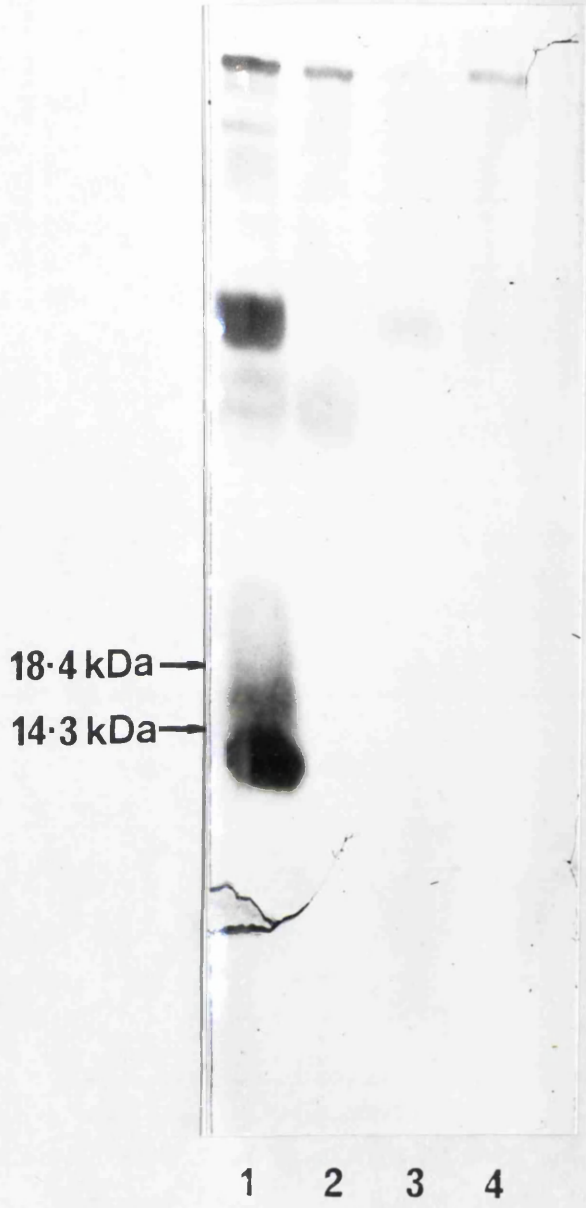

Figure 2: Western blot of four strains of spirochaetes against absorbed polyclonal anti-S80/5 antiserum. Lane 1, S80/5; Lane 2, B78; Lane 3, 4/71; Lane 4, PWS/A. Molecular weight markers are indicated on the left hand side.

TABLE 1 SENSITIVITY AND SPECIFICITY OF THE ANTI-*S. hyodysenteriae* RPLA TEST

No. OF ORGS/ML	STRAIN OF ORGANISM S80/5	B78	PWS/A	4/71
0	-	-	-	-
1	-	-	-	-
10	-	-	-	-
10^2	-	-	-	-
10^3	-	-	-	-
10^4	-	-	-	-
10^5	-	-	-	-
10^6	-	-	-	-
10^7	+	W	-	-
10^8	+	+	-	-
10^9	+	+	W	-
10^{10}	ND	ND	ND	ND
10^{11}	ND	ND	ND	+

+=POSITIVE; -=NEGATIVE; W=WEAK POSITIVE

TABLE 2 RPLA TEST RESULTS OF FAECAL SAMPLES WITH ADDED ORGANISMS

No. OF ORGS/ML	STRAIN OF ORGANISM S80/5	PWS/A	4/71
0	-	-	-
1	-	-	-
10	-	-	-
10^2	-	-	-
10^3	-	-	-
10^4	-	-	-
10^5	-	-	-
10^6	-	-	-
10^7	-	-	-
10^8	W	-	-
10^9	+	W	-
10^{10}	+	+	W

+= POSITIVE; -=NEGATIVE; W=WEAK

TABLE 3 RPLA RESULTS OF DILUTIONS OF SAMPLES OF KNOWN *S. hyo* CONTENT

SAMPLE	No. ORGS/ML	RPLA RESULT
No.1	6×10^8	WEAK
	6×10^7	++
	6×10^6	++
	6×10^5	WEAK
	6×10^4	-
No.2	7.5×10^5	WEAK
	7.5×10^4	++
	7.5×10^3	+
	7.5×10^2	-
	7.5×10	ND

ND= not done; *S. hyo*= *S. hyodysenteriae*

one month. The results of the experiment are shown in Table 4.

RESULTS OF RPLA TESTS ON SAMPLES FROM EXPERIMENTALLY INFECTED PIGS.

The bacteriological and post mortem findings from the experimentally infected pigs are shown in Table 5a. They also revealed that one of the pigs, 1224 was normal. No *S. hyodysenteriae* was isolated from it and no further evidence of swine dysentery was evident at post mortem. Pig number 868 was shown to be a normal carrier of the organism as only small numbers of organisms could be isolated from 2 sites. The results in Table 5 show that the RPLA test using the absorbed polyclonal antiserum was able to detect S80/5 in both faecal samples and colonic washings from experimentally infected pigs. RPLA results on dilutions of up to 1/1000 give weak or positive results in both faecal and colonic samples. All the controls which involved spirochaete free pig faeces and the latex beads which had been coated with non-immune rabbit antibody were negative unless otherwise stated.

DISCUSSION

These experiments clearly demonstrate the ability and the potential of the RPLA test to detect the presence of *S. hyodysenteriae* in faecal samples. However it must also be said that there are some limitations to

TABLE 4 SHELF LIFE OF RPLA TEST

TEMPERATURE	RPLA	MONTH												
		1	2	3	4	5	6	7	8	9	10	11	12	13
4 °C	S. hyo	+	+	+	+	+	+	+	+	+	+	+	+	X
	-VE C	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-	-
ROOM (16-25 °C)	S. hyo	+	+	+	+	X	ND	ND	ND	ND	ND	ND	ND	ND
	-VE C	+	+	+	+	X	ND	ND	ND	ND	ND	ND	ND	ND

ND=not done; X=non-usable test; -VE C=neg. control

TABLE 5a DETAILS OF INFECTED PIGS

PIG No.	DYSENTERY	(DAYS)	P.M. FINDINGS GROSS	HIST	*S. hyo* IN PIGS	SITES
1	+	NR	NR	NR	NR	NR
2	+	NR	NR	NR	NR	NR
868	+	(9)	-	-	+	2
1229	+	(2)	+	+	+	10
844	-		+	+	+	9
862	+	(10)	+	+	+	10
1211	+	(3)	+	+	+	10
1224	-		-	-	-	0
846	+	(5)	+	+	+	10
845	+	(2)	+	+	+	10

SITES: Ten sites throughout the colonic mucosa were sampled at post mortem for *S. hyodysenteiae*. This is the number of sites in which *S. hyodysenteriae* was found.

DAYS: Number of days out of the 40 day trial that the pigs had diarrhoea/dysentery.

NR=not recorded

TABLE 5b RESULTS OF RPLA TESTS ON SAMPLES FROM EXPERIMENTALLY INOCULATED PIGS

PIG NUMBER	SAMPLE TYPE	RPLA RESULTS DILUTION OF SAMPLE						No. OF ORGS/ML
		10	10	10	10	10	10	
1	F	W	++	++	W	-	ND	6x10
2	F	W	++	+	-	ND	ND	7.5x10
868	F	W	++	W	W	-	-	
1229	F	W	++	W	-	-	-	
868	C	++	++	+	W	-	ND	
1229	C	W	++	W	-	-	-	
844	C	+++	++	W	-	-	ND	
862	C	++++	++	W	-	-	ND	
1211	C	+++	++	+	-	-	ND	
1224	C	++	++	W	-	-	ND	
846	C	++	+	W	-	-	ND	
845	C	++	+	W	-	-	ND	

F=faecal; C=colonic contents; W=weak; ND=not done

the test. It has been shown that the RPLA test using polyclonal antiserum to *S. hyodysenteriae* which had been absorbed with *S. innocens* encountered the same problems of non-specificity as did the fluorescent antibody test. While the reaction of the RPLA test to some of the non-*S. hyodysenteriae* spirochaetes was slightly delayed when compared with that of *S. hyodysenteriae*, it cannot be said that it would be the same for all the non-*S. hyodysenteriae* spirochaetes that would be encountered. However this is not perhaps altogether surprising considering that there is some cross-reaction between the species as seen from immunoblots carried out with the absorbed polyclonal antiserum. In theory the absorption of the antiserum with a sufficient amount of *S.innocens* should remove all the antibodies directed against both species leaving only those specific to *S. hyodysenteriae*. This also indicates that there was perhaps insufficient absorption and so this may need further investigation in order to determine the right length of time and the correct amount of organisms to use for absorption of this particular antiserum.

The RPLA test proved to more sensitive in faecal samples than it did with cultured organisms. This could be due to the fact that there may be more soluble antigen present in a faecal sample from an *in vivo* situation than there would be in a sample of cultured *S. hyodysenteriae*. How large a part the

soluble antigen plays in the agglutination of the test could be worked out using cell-free extracts from *S. hyodysenteriae* positive faecal samples.

Weak results in the 1/10 dilution of the faecal samples are likely to be due to antigen excess and the fact that the antibody used in the test is anti-S80/5 corresponding directly to the organism in the samples. Much stronger positive reactions were seen in the 1/100 dilution of samples but reaction was weak or unconstant at dilutions of 1/1000. The other factor which may complicate the situation is the presence of free antibody in the faecal sample. The RPLA results of faecal samples to which spirochaetes had been added revealed that the sensitivity had fallen to 10^8 organisms per ml compared to the sensitivity with cultured organisms and faecal samples from experimentally infected pigs. This again supports the view that either the free antigen or antibody produced in the colon is also playing some part in the agglutination of the RPLA. The loss of sensitivity may perhaps be explained by some organisms being trapped in the faeces after mixing and so fewer organisms than were added are actually detected. Positive RPLA results were obtained from pig No. 1224 although no *S. hyodysenteriae* was isolated from any of the ten sites sampled at post mortem. This may be explained by the presence of other cross-reacting spirochaetes or that there were too few organisms present to be detected by

culture. However as the animal was known to have been exposed to *S. hyodysenteriae*, a combination of the two explanations may also be likely.

The fact that the RPLA seems at this stage to be dependant to a certain extent on time and concentration effects can be put down to the antigen(s) on the non-*S. hyodysenteriae* spirochaetes being only slightly similar to that of the *S. hyodysenteriae* and consequently there are fewer antibodies against such epitopes. This means that they are distributed throughout the latex suspension and so take longer to react and the effect may be seen to be quicker with a larger concentration.

If, however sensitivity is regarded in the light of proportion of animals with the disorder that test positive then the test has been shown to be very sensitive.

It may also be possible that the cross-reactions could be due to non-specific reactions in the suspension. This could occur if there were any unreacted sites on the beads that allowed the binding of other proteins causing autoagglutination. These sites can be blocked by using non-specific agents such as BSA or Tween 20 although these have been tried without success (Thom, unpublished observations), it does not mean to say that other such agents do not exist.

CHAPTER FOUR

FIELD TESTING OF THE ABSORBED POLYCLONAL RPLA TEST

INTRODUCTION

In these experiments, samples of faeces from suspected swine dysentery cases were tested for *S. hyodysenteriae* by various methods including selective culture and smear, and isolated organisms were examined by API ZYM and SDS PAGE gel electrophoresis in order to compare them with the RPLA results.

MATERIALS AND METHODS

SAMPLES

Samples of pig faeces and colonic material were obtained from the sources shown in Table 6.

RPLA TESTING

RPLA testing was carried out as described in Chapter Three. In order to test the swab samples by RPLA, the swab was cultured on spectinomycin blood agar. If there were any spirochaetes present then after removing a small amount and inoculating a fresh plate, the original plate was washed with GBS (approximately 1.5 ml) and tested by RPLA.

CULTURE

Each sample was examined by selective culture on spectinomycin blood agar as described in Chapter Two. Colonies of *S. hyodysenteriae* are about 1mm in diameter and are surrounded by a ring of ß haemolysis after 48 hours incubation in an anaerobic atmosphere.

TABLE 6 FIELD SAMPLES

SAMPLE NUMBER	DESCRIPTION OF SAMPLES
16029	Faecal sample from either a finishing unit or a breeding unit. *S. hyodysenteriae* had already been isolated and identified from another finishing unit belonging to the same owner.
16469	Blood/mucus from floor of pen in PBS. 12 week old pig. No tiamulin for 4 weeks
16470	Diarrhoeic faeces from floor of pen in PBS. 12 week old pig. No tiamulin for 4 weeks.
16471	Fresh colon contents in PBS from a sacrificed 12 week old pig. No tiamulin for 4 weeks.
16472	Section of colon in PBS from a sacrificed pig. No tiamulin for 4 weeks.
16473	Fresh colon contents in PBS from a sacrificed 9 week old pig originally from the same farm. No tiamulin for 2 weeks
16474	Colon from a sacrificed piglet.
16665-16667	Faecal swabs in transport medium.
16668-16671	Diarrhoeic faecal samples.
16706-16711	Faecal samples from gilts.
16759-16763	Faecal samples in normal saline.
17045-17047	Colon samples.

If spirochaetes grew, the degree of haemolysis present was noted as *S. innocens* is only weakly ß haemolytic.

A series of tenfold dilutions in PBS was made from those samples which contained a large amount of mucus in order to dilute out the effect of any antibody and allow the organisms to grow in culture.

SMEARS

Smears were made from each sample and these were stained by Gram's stain and examined for the presence or absence of spirochaetes.

API ZYM TESTING

This was carried out on spirochaete isolates in the manner recommended by Hunter and Wood (1979) except that the organisms were cultured on horse blood agar instead of sheep blood agar. Those spirochaetes lacking the enzyme, alpha galactosidase (enzyme No. 13) are 95% likely to be associated with swine dysentery and those possessing the enzymes alpha and ß glucosidase (enzymes Nos. 16 and 17) are likely to be associated with swine dysentery (Hunter and Wood, 1979).

SDS PAGE GEL ELECTROPHORESIS

SDS PAGE gels were run according to the method described in Chapter Two in order to determine whether

or not the isolates possessed a 16 kDa antigen and to correlate these results with the API ZYM results.

RESULTS

Field samples of suspected swine dysentery were tested with the RPLA test and the results were then compared with those of the smears and cultures (Table 7). In all cases where spirochaetes were isolated or seen in smears the RPLA was positive with the exception of case 16669. However culture methods were unable to detect organisms present in sample numbers 16469-16474. These samples contained a large amount of mucus and had also been delayed in the post. The result was that even after diluting the samples with PBS before culture, the spirochaetes evident in the smears could not be grown.

The API ZYM findings are shown in Table 8. The correlation of API ZYM results and SDS PAGE gel electrophoresis results are shown in Table 9. A 16kDa antigen was present in the majority of the isolates even though only one isolate, 16780 appeared to be *S. hyodysenteriae*. The SDS PAGE gel of the isolates is shown in Figure 3.

DISCUSSION

When absorbed polyclonal antiserum was used to coat the latex particles, it can be seen that when spirochaetes were present in the smears then the RPLA

TABLE 7 COMPARISON OF RPLA TEST RESULTS WITH SMEAR AND CULTURE RESULTS

SAMPLE	SAMPLE TYPE	RPLA	SMEAR	CULTURE	DEGREE OF HAEMOLYSIS
16029	FAECAL	+	+	+	WEAK
16469	FAECAL	+	+	-	NA
16470	FAECAL	+	+	-	NA
16471	COLON	++	++	-	NA
16472	COLON	+	+	-	NA
16473	COLON	+	+	-	NA
16474	COLON	+	+	-	NA
16665	SWAB	+	+	+	WEAK
16666	SWAB	+	+	+	WEAK
16667	SWAB	+	+	+	WEAK
16668	SWAB	+	+	+	WEAK
16669	FAECAL	-	-	+	WEAK
16670	FAECAL	+	+	+	WEAK
16671	FAECAL	++	++	+	WEAK
16759	FAECAL	+	+	-	NA
16760	FAECAL	+	+	+	WEAK
16761	FAECAL	+	+	+	WEAK
16762	FAECAL	+	+	-	NA
16763	FAECAL	+	+	+	WEAK
16780	FAECAL	+	+	+	STRONG
16781	FAECAL	-	-	-	NA
16782	FAECAL	-	-	-	NA
17045	COLON	+	+	-	NA
17046	COLON	+	+	-	NA
17047	COLON	+	+	-	NA

NA= not attempted

TABLE 8 RESULTS OF API ZYM TESTING

SAMPLE	ENZYME NUMBER																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
16029	-	+	+	+	-	-	-	-	-	-	+	+		+	-	+	-	+	-	-
16665	-	+	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16667	-	+	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16669	-	+	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16670	-	+	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16671	-	+	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16760	-	-	+	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16761	-	-	-	+	-	-	-	-	-	-	+	+		+	-	+	+	-	-	-
16763	-	-	-	+	-	-	-	-	-	-	+	+		+	-	-	-	-	-	-
16780	-	+	-	+	-	-	-	-	-	-	+	+		+	-	+	+	-	-	-
B78	-	+	+	+	-	-	-	-	-	-	+	+		+	-	+	+	-	-	-
S80/5	-	+	+	+	-	-	-	-	-	-	+	-		+	-	+	+	-	-	-
PWS/A	-	+	+	+	-	-	-	-	-	+	+	+		+	+	+	+	-	-	-

+= POSITIVE -=NEGATIVE

TABLE 8b

ENZYMES AND SUBSTRATES USED IN THE API ZYM SYSTEM

No.	Enzyme assayed for	Substrate	pH
1	Control	-	-
2	Alkaline phosphatase	2-naphtyl phosphate	8.5
3	Esterase (C4)	2-naphtyl butyrate	6.5
4	Esterase lipase (C8)	2-naphtyl caprylate	7.5
5	Lipase (C14)	2-naphtyl myristate	7.5
6	Leucine arylamidase	L-leucyl-2-naphtylamide	7.5
7	Valine arylamidase	L-valyl-2-naphtylamide	7.5
8	Cysteine arylamidase	L-cystyl-2-naphtylamide	7.5
9	Trypsin	N-benzoyl-DL-arginine -2-naphtylamide	8.5
10	Chymotrypsin	N-glutaryl-phenyl-alanine-2-naphtylamide	7.5
11	Phosphatase acid	2-naphtyl phosphatate	5.4
12	Phosphoamidase	Naphtol AS-B1 phospho-diamide	5.4
13	Alpha galactosidase	6-Br-2-naphtyl-alpha D galactopyranoside	5.4
14	Beta galactosidase	2-naphtyl-BD-galactopyranoside	5.4
15	Beta glucuronidase	Naphtol-AS-B1-BD glucoronate	5.4
16	Alpha glucosidase	2-naphtyl-Alpha D glucopyranoside	5.4
17	Beta glucosidase	6-Br-2-naphtyl BD glucopyranoside	5.4
18	N-acetyl-B glucosaminidase	1-naphtyl-N-acetyl-BD glucosaminide	5.4
19	Alpha mannosidase	6-Br-2-naphtyl-alpha B mannopyranoside	5.4
20	Alpha fucosidase	2-naphtyl-alpha L-fucopyranoside	5.4

TABLE 9 API ZYM RESULTS AND POSSESSION OF 16kDa ANTIGEN IN 8 SPIROCHAETAL ISOLATES

SAMPLE	16kDaAg	API ZYM	HAEMOLYSIS
16029	+	?	WEAK
16667	+	NS	WEAK
16668	+	NS	WEAK
16669	+	NS	WEAK
16671	+	NS	WEAK
16761	+	NS	WEAK
16763	+	NS	WEAK
16780	+	S	STRONG

S=95% chance *S. hyo*; NS=non *S. hyo*

Figure 3: Coomassie blue stained SDS PAGE gel of field isolates. Lane 1, 16669; Lane 2, 16668; Lane 3, 16667; Lane 4, 16780; Lane 5, 16029; Lane 6, 16763; Lane 7, 16761; Lane 8, PWS/A; Lane 9, B78; Lane 10, S80/5; Lane 11, molecular weight markers. The 16kDa antigen of S80/5 and B78 is difficult to see on gels stained with Coomassie blue (see Figure 4).

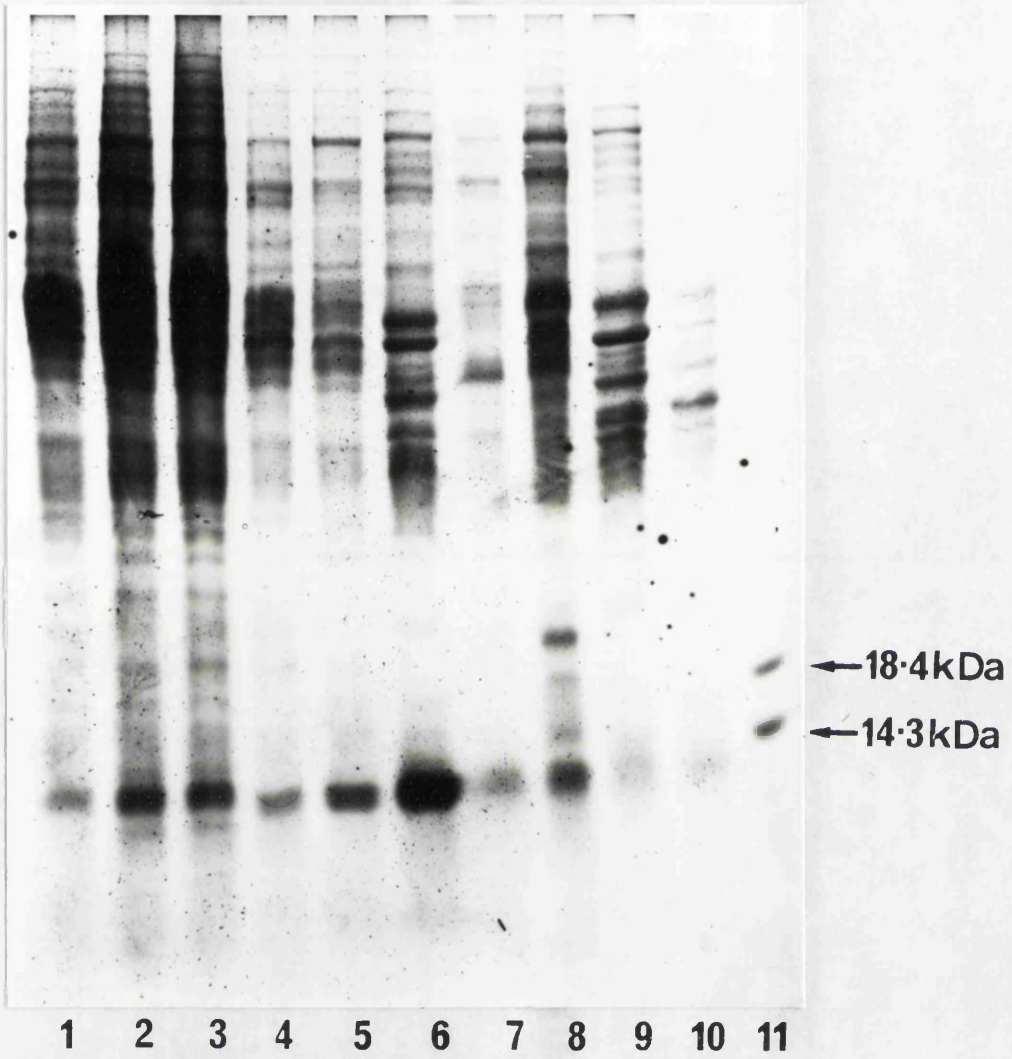

Figure 3: Coomassie blue stained SDS PAGE gel of field isolates. Lane 1, 16669; Lane 2, 16668; Lane 3, 16667; Lane 4, 16780; Lane 5, 16029; Lane 6, 16763; Lane 7, 16761; Lane 8, PWS/A; Lane 9, B78; Lane 10, S80/5; Lane 11, molecular weight markers. The 16kDa antigen of S80/5 and B78 is difficult to see on gels stained with Coomassie blue (see Figure 4).

test would also be positive. Spirochaetes were seen in Gram stained smears of 16759 and 16762 and antigen was demonstrated by the RPLA test. The organisms could not, however, be grown on spectinomycin blood agar. Spirochaetes were also visible in Gram stained smears of samples numbered 16760, 16761 and 16763. These were isolated on spectinomycin blood agar. These organisms all produced different API ZYM results. Isolate 16761 was definitely not *S. hyodysenteriae*. Isolates 16760 and 16763 did not possess alpha galactosidase like *S. hyodysenteriae* but neither did they possess alpha and ß glucosidase which are considered to be characteristic of that species. All of these organisms (16760, 16761 and 16763) were weakly ß haemolytic and only one, isolate 16760 did not possess a 16 kDa antigen.

Sample number 16029 was seen to contain spirochaetes when a Gram stained smear was examined. Spirochaetes were isolated on spectinomycin blood agar. The API ZYM results showed that the organism was alpha galactosidase negative and produced alpha glucosidase like *S. hyodysenteriae* but unlike *S. hyodysenteriae* it did not produce ß glucosidase and was weakly ß haemolytic. It did however possess a 16kDa antigen when examined by SDS PAGE.

16469-16474 were faecal and colon samples which were delayed in the post. These samples were also very

mucoid. When Gram stained smears of these samples were examined, large numbers of spirochaetes could be seen, especially in sample number 16471. However, the organisms failed to grow even after tenfold dilutions had been made of the samples and it is possible that the presence of mucus affected the isolation in some way. The faecal samples were reported by the vet as being bloody and mucoid when seen on the floor of the pen. The clinical signs together with the presence of large numbers of spirochaetes present in the samples led to the supposition that the spirochaetes were *S. hyodysenteriae*.

Samples numbered 16665-16671 (not including 16669) were shown by Gram staining of positive smears to contain spirochaetes and these samples all produced positive results with the RPLA test. API ZYM tests were carried out on isolated spirochaetes (16665, 16666, 16667, 16670, and 16671) and showed that these organisms are not related to *S. hyodysenteriae*. However despite the fact that the organisms are weakly haemolytic and are not related to *S. hyodysenteriae* by API ZYM testing, they apparently possess a 16kDa antigen. This antigen does however stain better with Coomassie blue than does the 16kDa antigen of *S. hyodysenteriae*. This may be due to variation in the amount of antigen produced.

Isolate 16780 was clearly an isolate of *S. hyodysenteriae*. It was typical in producing strong ß haemolysis on blood agar. When tested by API ZYM, it did not produce alpha galactosidase but did produce alpha and ß glucosidase. It was also seen to possess a 16kDa antigen when subjected to SDS PAGE. It was detected using the RPLA, but the reaction was only + whilst 16671, a non-*S. hyodysenteriae* isolate was ++.

The selective culture of the organisms on spectinomycin blood agar proved to be more accurate than the RPLA test using whole serum for indicating the presence of *S. hyodysenteriae* based on the higher degree of haemolysin production. *S. innocens* was also identified by this method.

API ZYM results also confirmed that the test was non-specific and was detecting almost any spirochaete. It therefore seems that the absorbed polyclonal RPLA test is only useful for screening for spirochaetes or for confirming a suspected case of swine dysentery quickly.

It was then decided to raise an antiserum to an antigen of *S. hyodysenteriae* which was said to be specific for the organism, the 16kDa envelope protein and to repeat the study.

CHAPTER FIVE

THE DEVELOPMENT AND TESTING OF THE ANTI-16kDa RPLA TEST

INTRODUCTION

S. hyodysenteriae has been shown to cross-react with *S. innocens* in serological tests such as the fluorescent antibody test which cross-reacts with strains of non-*S. hyodysenteriae* spirochaetes (Hudson *et al*, 1976). Even when the antiserum had been absorbed with non-*S. hyodysenteriae* spirochaetes cross-reaction was reported (Lysons and Lemcke, 1983). However all strains of *S. hyodysenteriae* so far examined for this purpose have been shown to possess a unique 16 kDa antigen (Sellwood *et al*, 1989; Joens and Marquez, 1986). This antigen is found in the outer membrane of the organism (Sellwood *et al*, 1989). Antiserum against this antigen should then provide the means to produce a highly specific diagnostic test for swine dysentery, by-passing the problems of absorption with non-pathogenic spirochaetes and those attributed to the quality of the antiserum. The finding of a 16kDa antigen in so many of the non *S. hyodysenteriae* spirochaetes made this study particularly interesting, allowing investigation into whether these were cross-reactive proteins or there was a family of 16kDa proteins which may be involved in pathogenicity but which were distinct.

MATERIALS AND METHODS

PRODUCTION OF ANTIGEN

The antigen was produced in two ways. Firstly an entire SDS PAGE gel of S80/5 was run and then blotted

onto nitrocellulose (see Chapter Two). Molecular weight markers were located at either end. The antigen was located by Western blotting and staining the portions of the nitrocellulose containing the molecular weight markers with either amido black or Coomassie blue.

The second method involved the purification of the antigen from the outer membrane. This involved the removal of the outer membrane from the organism (Penn *et al*, 1985). *S. hyodysenteriae* was grown in trypticase soy broth as described in Chapter Two. Batches of the azide killed organisms were centrifuged at 10,000g for 20 minutes and washed once in 10 ml PBS. The pellet was then resuspended in PBS containing 10 mM $MgCl_2$ and 0.2% (v/v) Triton X-100 (BDH) to a density of 2 x 10^{10} ml^{-1} and incubated at 37°C for 30 minutes. The suspensions of treated organisms were then centrifuged as before. Both the supernate and the pellet were retained for examination by SDS PAGE although the 16 kDa antigen is in the triton soluble fraction. The antigen was then purified by SDS PAGE which enabled the production of a larger concentration of the antigen on the same amount of nitrocellulose (Fig 4).

PREPARATION OF ANTIGEN FOR INJECTION

Eight nitrocellulose strips were finely chopped and mixed with 200 μl of sterile PBS. The mixture was then

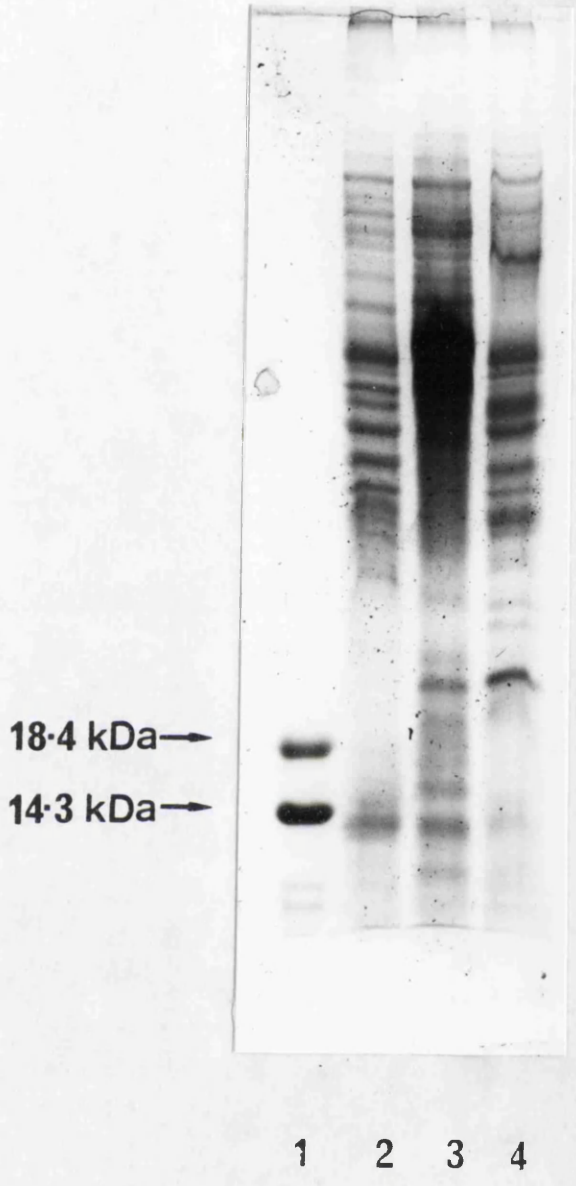

Figure 4: Coomassie blue stained SDS PAGE gel showing the 16kDa antigen in the outer membrane preparation of S80/5. Lane 1, molecular weight markers; Lane 2, S80/5; Lane 3, outer membrane preparation; Lane 4, residual pellet from the preparation of the outer membrane.

sonicated repeatedly for 10 seconds with 30 seconds cooling time until the mixture was fine enough to pass through a needle suitable for injecting a rabbit. The antigen was then mixed with an equal volume of Freund's incomplete adjuvant (Sigma) and sonicated for 1 second. The mixture was then injected into the rabbit (Harlow and Lane, 1988). Three weeks later the rabbit was reinjected with a similar mixture and three weeks later the rabbit was bled.

Antibody was separated from the rabbit serum by ammonium sulphate precipitation as described in Chapter Two. Its specificity was then checked by Western blotting and it was then stored at -20°C.

COATING OF LATEX PARTICLE SUSPENSION

The coating of the latex particles was carried out in the same manner as before (Chapter Two). The amount of antibody that had to be added to the suspension was ascertained by checkerboard titration (Chapter Two) and was again found to be 3μg/ml.

EVALUATION OF SENSITIVITY AND SPECIFICITY OF RPLA TEST

These were determined as described in Chapter Two. A Western blot was carried out to determine the specificity of the anti-serum (Figure 5). In order to determine whether or not any cross-reactions that occurred with the RPLA tests were due to the presence of anti-horse antibodies in the serum, a series of

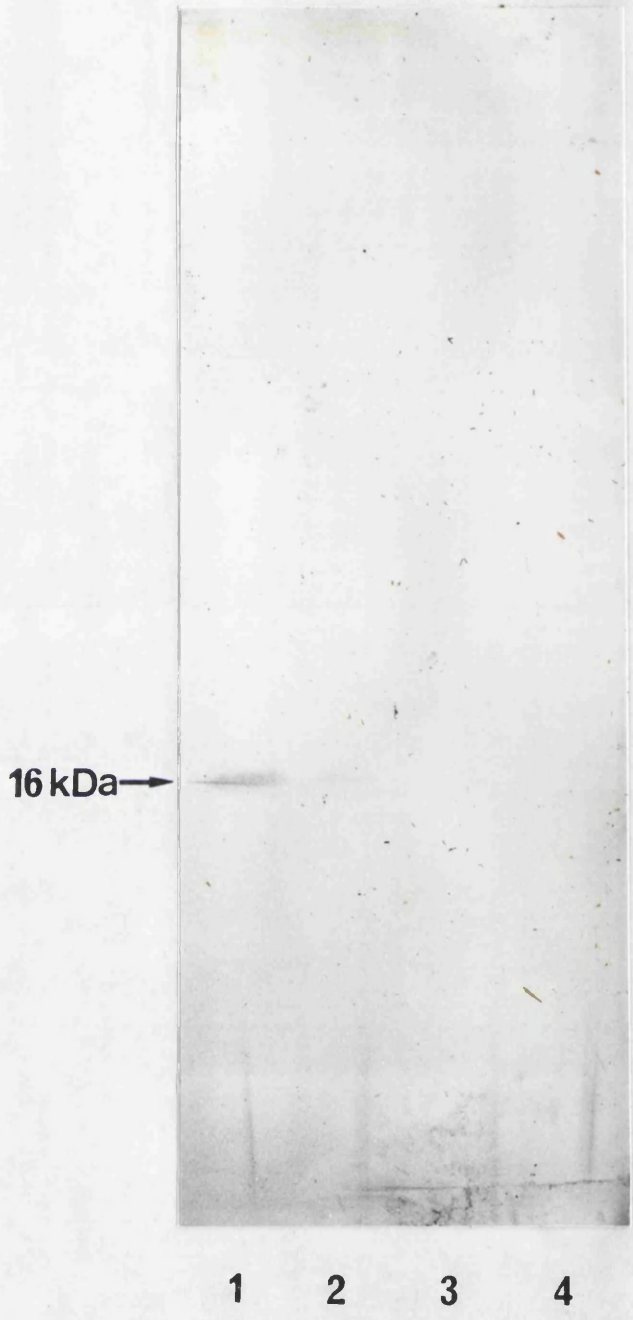

Figure 5: Western blot showing the specificity of the anti-16 kDa antiserum against whole cell antigens. Lane 1, S80/5; Lane 2, B78; Lane 3, 4/71; Lane 4, PWS/A. Molecular weight markers are indicated on the left hand side.

1/10 dilutions of horse serum in PBS was made and then subjected to SDS PAGE and Western blotting.

FAECAL AND COLONIC SAMPLES

Faecal and colonic samples were obtained from the sources described in Table 10.

CONVALESCENT PIG SERA

These were obtained from pig no 868 which had been involved as part of a control group in the monesin drug trial previously mentioned in Chapter Three. This pigs recovered from the disease.

FIELD TESTING

The results of RPLA tests were compared with smear, culture, API ZYM and SDS PAGE results as described in Chapter Four. Electron Microscopy was also carried out on some samples in order to confirm their identity. Spirochaetes were harvested from blood agar plates in physiological saline. After fixing to a grid they were negatively stained with 2% Tungstate (Biorad) before examination. *S. hyodsenteriae* may be distinguished from some other spirochaetes by the following criteria: *S. hyodysenteriae* seen under E.M. is 6-10 μm in length and 350nm in diameter. It has pointed ends and has 8-16 fibrils all surrounded by an envelope.

TABLE 10 SOURCES AND FORM OF FIELD SAMPLES

SAMPLE	DESCRIPTION OF SAMPLES
16029	Faecal sample from a finishing unit or a breeding unit. *S. hyo* had already been isolated and identified from another finishing unit belonging to the same owner.
16665-16667	Faecal swabs in transport medium.
16668-16671	Diarrhoeic faecal samples.
16706-16711	Faecal samples from gilts.
16759-16763	Faecal samples in normal saline.
17045-17047	Colon samples
17445-17458	Faecal samples in normal saline. Sample numbers 17449, 17450, 17453 and 17454 were all from sows with soft faeces.
17594	Faeces in normal saline from a grower pig.
17607-17608	Faecal samples in PBS from pigs showing signs of colitis.

RESULTS

SDS PAGE AND WESTERN BLOTTING

SDS PAGE gels of S80/5, B78, PWS/A and 4/71 were carried out in order to determine the precise location of the 16 kDa antigen. One gel was stained with Coomassie blue and the other with silver nitrate (Figures 6 and 7). This showed the presence of the 16 kDa antigen in *S. hyodysenteriae* and its absence from the non-*S. hyodysenteriae* spirochaetes.

Immunoblotting with hyperimmune rabbit serum raised against whole cell S80/5 revealed that there was extensive cross reaction between the two species (Figure 8). Absorption of the hyperimmune serum with PWS/A and 4/71 before carrying out the immunoblot failed to remove all the cross reactions (Figure 2; Chapter 4). Although there was a lot of cross reaction, immunoblotting revealed that there were more differences in the lower molecular weight range. The 16kDa antigen was shown to be present only in the pathogens, B78 and S80/5 and not on the non-*S. hyodysenteriae* spirochaetes, 4/71 and PWS/A. These antigens were also evident when serum from convalescent pigs was used for immunoblotting (Figure 9). This information enabled the antigen to be excised on the nitrocellulose for injection into the rabbit as described in Chapter Two.

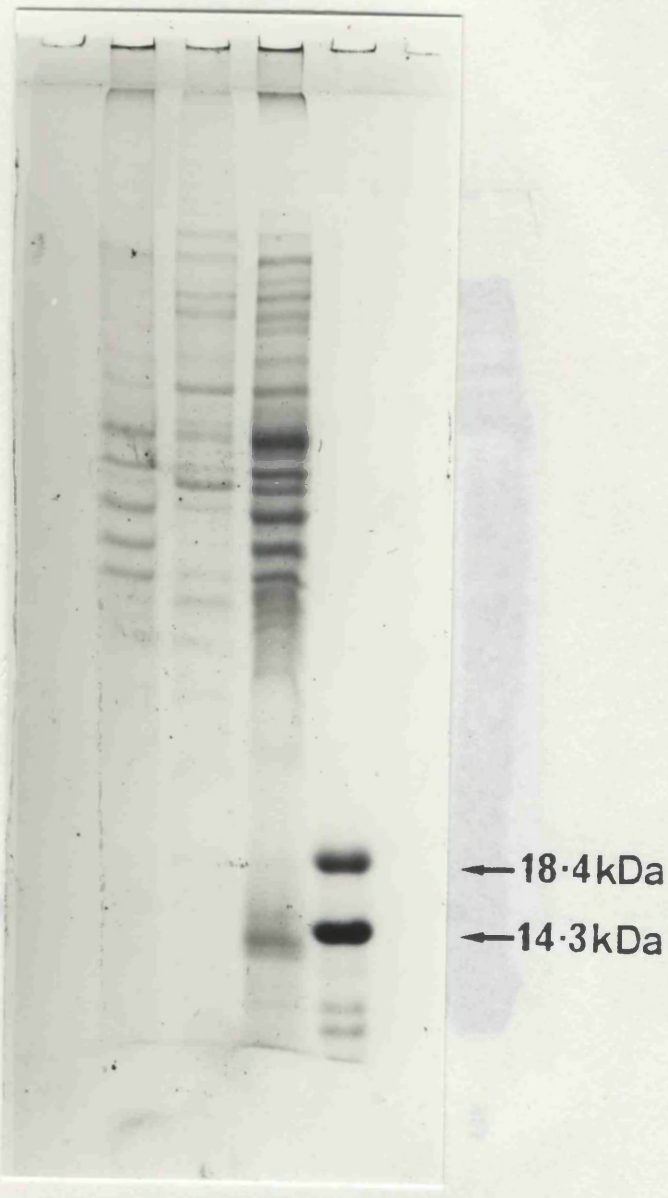

Figure 6: Coomassie blue stained SDS PAGE gel of three strains of spirochaetes. Lane 1, PWS/A; Lane 2, 4/71; Lane 3, S80/5; Lane 4, molecular weight markers.

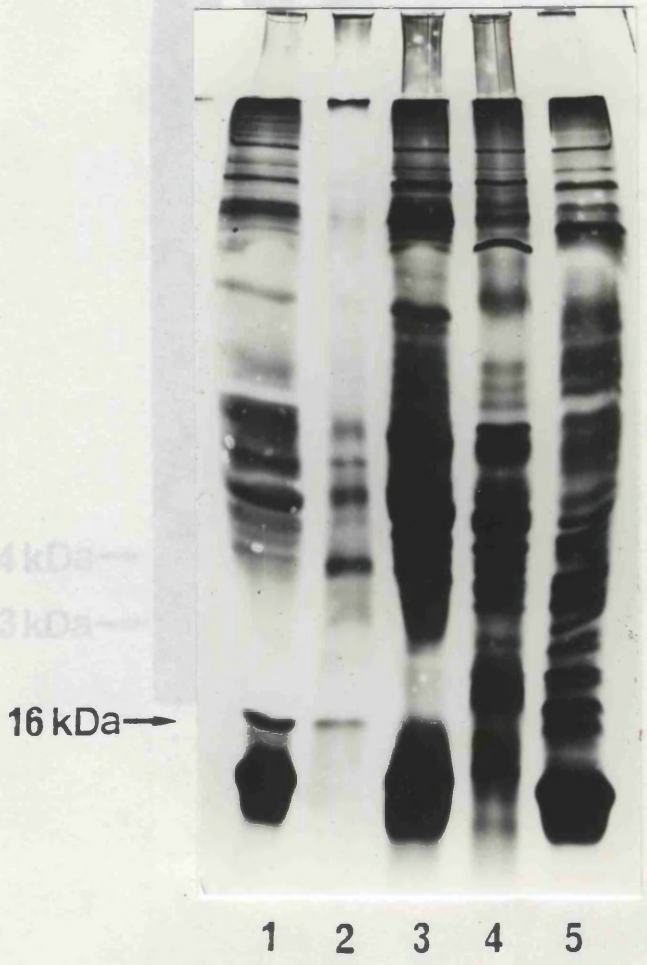

Figure 7: Silver stained SDS PAGE gel of the four strains of spirochaete. Molecular weight markers are indicated on the left hand side. Lane 1, S80/5; Lane 2, outer membrane preparation of S80/5; Lane 3, B78; Lane 4, PWS/A; Lane 5, 4/71.

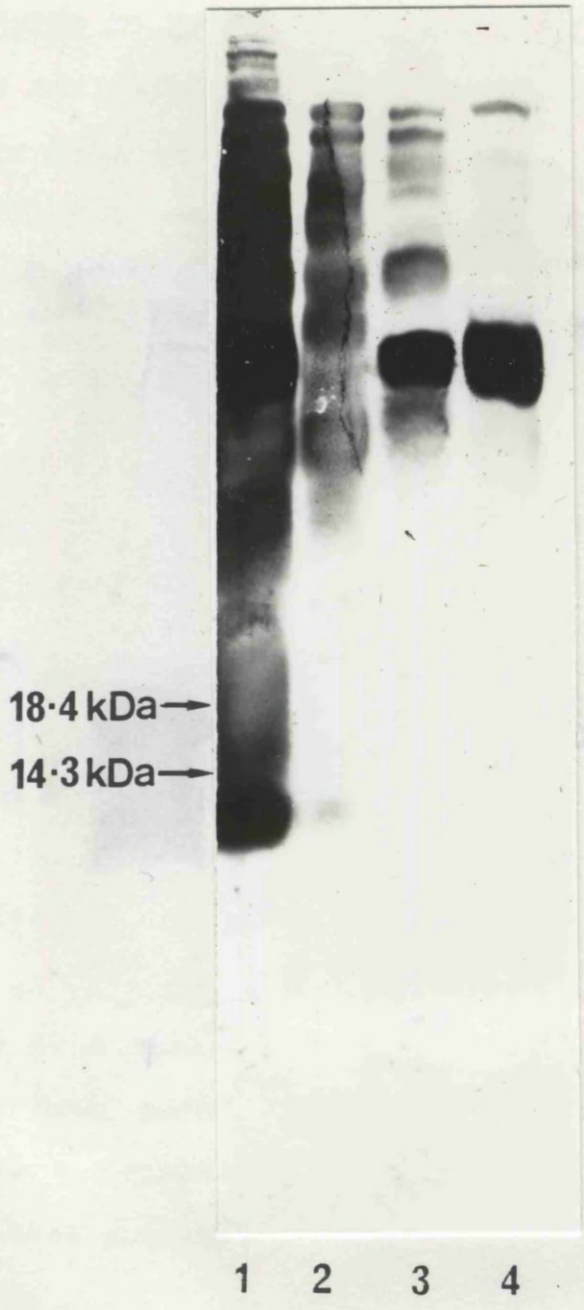

Figure 8: Whole cell preparations of the four strains of spirochaetes blotted against antiserum to S80/5. Lane 1, S80/5; Lane 2, B78; Lane 3, 4/71; Lane 4, PWS/A.

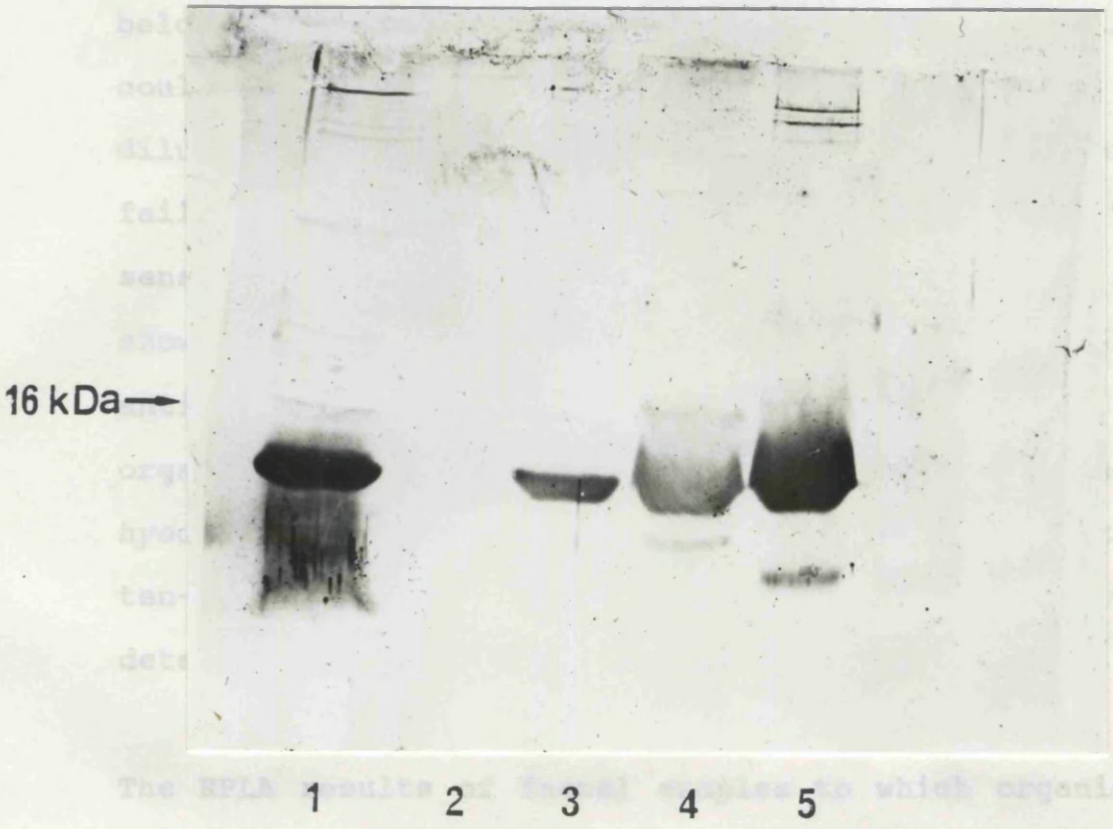

Figure 9: Western blots of strains of spirochaetes against convalescent pig serum. Lane 1, S80/5; Lane 2, Reddicap; Lane 3, 4/71; Lane 4, B78; Lane 5, PWS/A. Molecular weight markers are indicated on the left hand side.

RESULTS OF SENSITIVITY AND SPECIFICITY

The anti-16kDa antiserum was blotted against the four organisms in order to determine its specificity. The antiserum was able to detect the 16kDa antigen belonging to S80/5 but only a very faint reaction could be produced against B78. Western blotting of dilutions of horse serum against 16kDa antiserum failed to demonstrate any cross reactions. The sensitivity and specificity of the RPLA results are shown in Table 11. They show that the anti-16kDa antiserum coated latex beads could detect at least 10^6 organisms per ml for S80/5 and 10^8 for B78. The non-*S. hyodysenteriae* spirochaete PWS/A was detected at a ten-fold greater concentration than 4/71 which was detectable at a level of 10^{10} organisms per ml.

The RPLA results of faecal samples to which organisms had been added are shown in Table 12. These results show a decrease in sensitivity when compared to the culture results.

THE RESULTS OF FIELD TESTING

The results of the work carried out on field samples is shown in Table 13. As can be seen from the Table in general, the presence of spirochaetes in the smear suggests that a positive result may be expected from the RPLA test. This is true of all samples except 17448-17450 and 17453. No spirochaetes were observed

TABLE 11 SENSITIVITY AND SPECIFICITY OF ANTI-16kDa RPLA

No. OF ORGS/ML	STRAIN OF ORGANISM S80/5	B78	PWS/A	4/71
0	-	-	-	-
1	-	-	-	-
10	-	-	-	-
10^2	-	-	-	-
10^3	-	-	-	-
10^4	-	-	-	-
10^5	-	-	-	-
10^6	+	-	-	-
10^7	+	-	-	-
10^8	+	+	-	-
10^9	+	+	W	-
10^{10}	ND	ND	ND	W
10^{11}	ND	ND	ND	+

+=POSITIVE; -=NEGATIVE; W=WEAK

TABLE 12 RPLA RESULTS OF FAECAL SAMPLES WITH ADDED ORGANISMS

No. OF ORGS/ML	STRAIN OF ORGANISM S80/5	PWS/A	4/71
0	-	-	-
1	-	-	-
10	-	-	-
10^2	-	-	-
10^3	-	-	-
10^4	-	-	-
10^5	-	-	-
10^6	-	-	-
10^7	-	-	-
10^8	+	-	-
10^9	+	W	-
10^{10}	+	+	W

+=POSITIVE; -=NEGATIVE; W=WEAK AGGLUTINATION

TABLE 13 COMPARISON OF ANTI-16kDa RPLA RESULTS WITH SMEAR AND CULTURE RESULTS

SAMPLE NUMBER	SAMPLE TYPE	RPLA	SMEAR	CULTURE	DEGREE OF HAEMOLYSIS
16029	FAECAL	+	+	+	WEAK
16665	SWAB	+	+	+	WEAK
16666	SWAB	+	+	+	WEAK
16667	SWAB	+	+	+	WEAK
16668	SWAB	+	+	+	WEAK
16670	FAECAL	+	+	+	WEAK
16671	FAECAL	+	+	+	WEAK
16759	FAECAL	+	+	-	NA
16761	FAECAL	+	+	+	WEAK
16762	FAECAL	+	+	-	NA
16763	FAECAL	+	+	+	WEAK
16780	FAECAL	+	+	+	STRONG
16781	FAECAL	-	-	-	NA
16782	FAECAL	-	-	-	NA
17045	COLON	+	+	-	NA
17046	COLON	+	+	-	NA
17047	COLON	+	+	-	NA

NA=not applicable; W=weak agglutination

TABLE 13 cont. COMPARISON OF ANTI-16 kDa RPLA RESULTS WITH SMEAR AND CULTURE RESULTS

SAMPLE NUMBER	SAMPLE TYPE	RPLA	SMEAR	CULTURE	DEGREE OF HAEMOLYSIS
17445	FAECAL	-	-	+	WEAK
17446	FAECAL	-	-	-	NA
17447	FAECAL	-	-	-	NA
17448	FAECAL	+	-	?	?
17449	L/FAECAL	+	-	?	?
17450	L/FAECAL	W	-	-	NA
17451	FAECAL	-	-	-	NA
17452	FAECAL	-	-	+	STRONG
17453	L/FAECAL	W	-	+	STRONG
17454	L/FAECAL	-	-	-	NA
17455	FAECAL	-	-	-	NA
17456	FAECAL	-	-	+	WEAK
17457	FAECAL	-	-	-	NA
17458	FAECAL	-	-	-	NA
17594	FAECAL	+	+	+	STRONG
17607	FAECAL	+	+	+	STRONG
17608	FAECAL	+	+	+	STRONG

NA=not attempted; W=weak agglutination; L=loose
?=unsure if there were spirochaetes present

in the smears of these samples but the RPLA tests were positive.

The results of the API ZYM tests are shown in Table 14, showing the results obtained with isolates 17452, 17594, 17607 and 17608. The correlation of the API ZYM results, degree of haemolysis and possession of a 16kDa antigen is shown in Table 15. Gels of field isolates showing the 16kDa antigen are shown in Figures 10 and 11.

ELECTRON MICROSCOPY RESULTS

The diameter of these organisms varied from 123nm to 361nm. All the spirochaetes measured had 4-6 fibrils (Figure 12). Only one spirochaete with a diameter of 295nm was seen to have 8 fibrils.

DISCUSSION

The 16 kDa antigen has been demonstrated in the cell wall of the pathogenic *S. hyodysenteriae* strain S80/5 in this study and in eleven other strains of *S. hyodysenteriae* by Sellwood *et al* (1989). It has been shown to be absent from *S. innocens* strains PWS/A and 4/71 and M1 by Sellwood *et al* (1989). The antigen has also been demonstrated in colonic washings from a pig infected with swine dysentery. Antibody against this antigen has also been found in convalescent pig serum. It therefore seems that antiserum against the 16 kDa antigen could prove a useful diagnostic tool in the

TABLE 14 RESULTS OF API ZYM TESTING OF ISOLATES

SAMPLE	ENZYME NUMBER																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
16029	-	+	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-
16665	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16666	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16667	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16669	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16670	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16671	-	+	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
16760	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-
16761	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	-
16763	-	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-
16780	-	+	-	+	-	-	-	-	-	-	+	+	-	+	-	+	+	-	-	-
17452	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
17594	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	-	-
17607	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	-	-
17608	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	-	-
B78	-	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	+	-	-	-
S80/5	-	+	+	+	-	-	-	-	-	-	+	-	-	+	-	+	+	-	-	-
PWS/A	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-

+=POSITIVE; -=NEGATIVE

TABLE 15 API ZYM RESULTS AND POSSESSION OF 16kDa ANTIGEN

SAMPLE	16kDaAg	API ZYM	HAEMOLYSIS
16029	+	?	WEAK
16667	+	NS	WEAK
16668	+	NS	WEAK
16669	+	NS	WEAK
16671	+	NS	WEAK
16761	-	NS	WEAK
16763	+	NS	WEAK
16780	+	S	STRONG
17452	+	S	STRONG
17594	+	S	STRONG
17607	+	S	STRONG
17608	+	S	STRONG
B78	+	S	STRONG
S80/5	+	S	STRONG
PWS/A	-	NS	WEAK

S=95% chance *S. hyo.* NS= non *S. hyo*

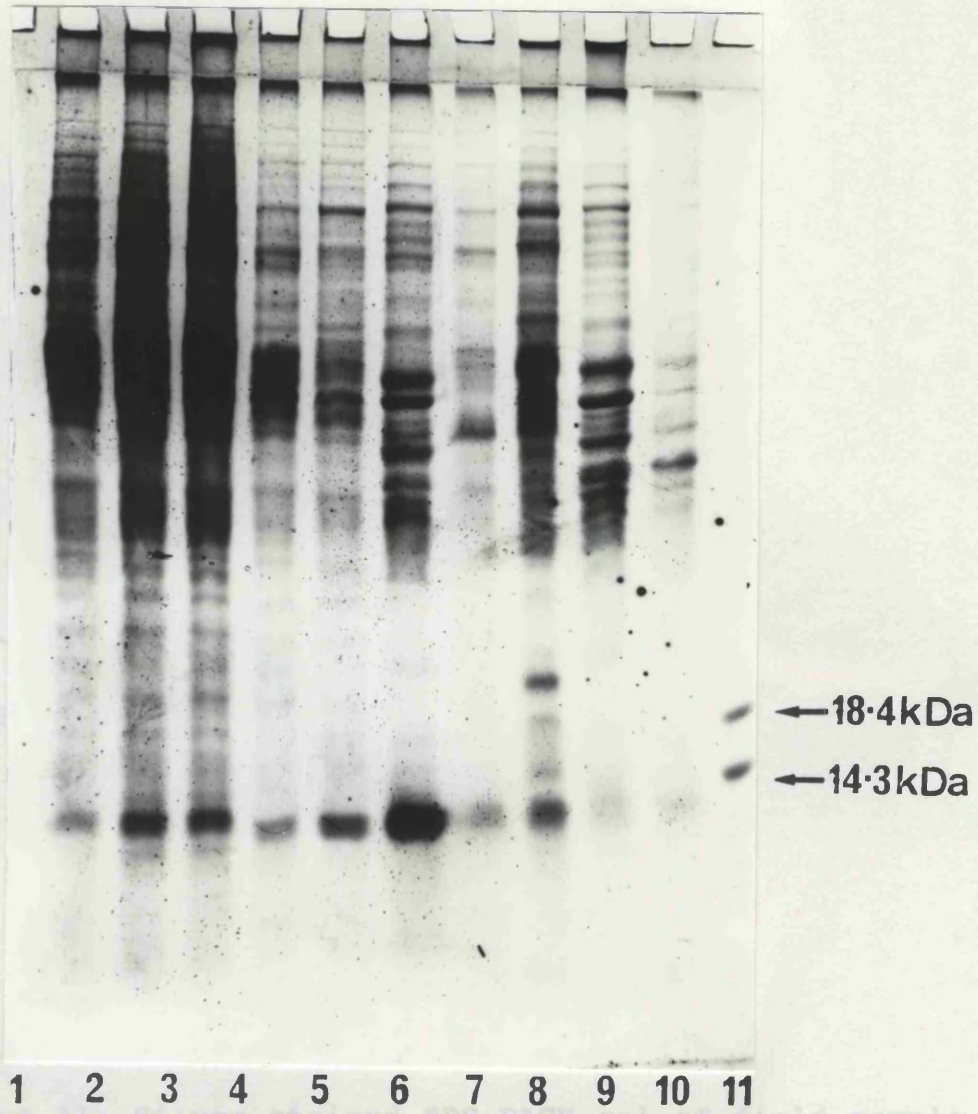

Figure 10: Coomassie blue stained SDS PAGE gel of field isolates. Lane 1, 16669; Lane 2, 16668; Lane 3, 16667; Lane 4, 16780; Lane 5, 16029; Lane 6, 16763; Lane 7, 16761; Lane 8, PWS/A; Lane 9, B78; Lane 10, S80/5; Lane 11, molecular weight markers. The 16kDa antigen of S80/5 and B78 is difficult to see on gels stained with Coomassie blue (see Figure 4).

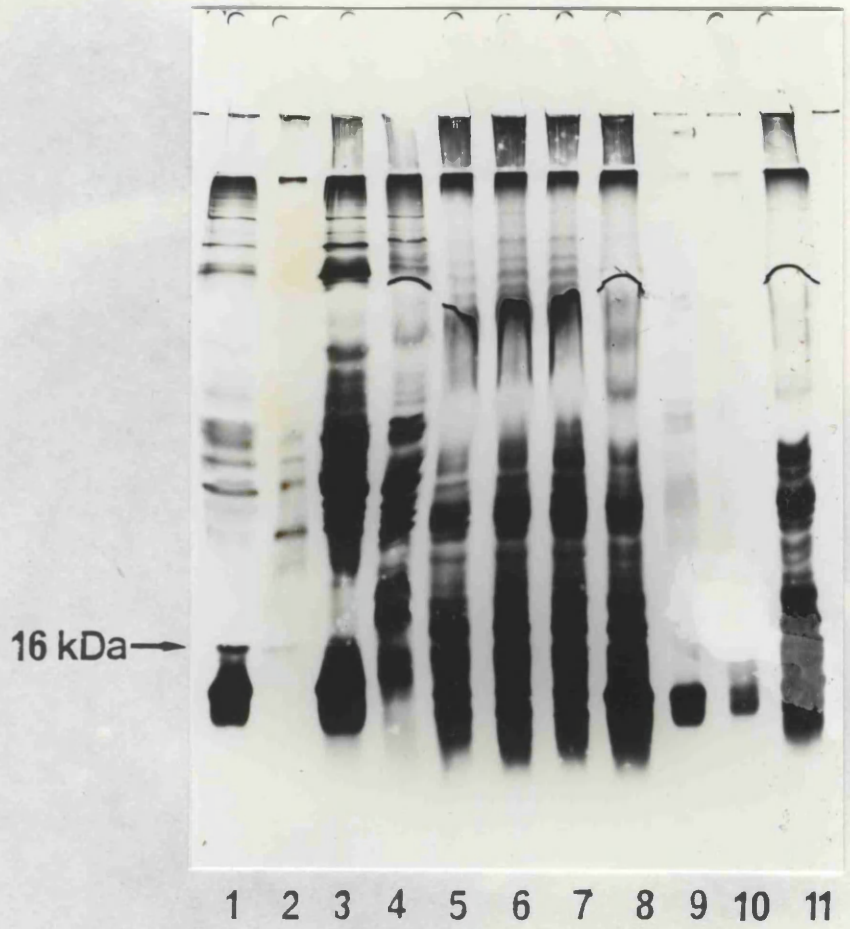

Figure 11: Silver stained SDS PAGE gel of field sample isolates. Lane 1, S80/5; Lane 2, outer membrane preparation of S80/5; Lane 3, B78; Lane 4, PWS/A; Lane 5, 16668; Lane 6, 16669; Lane 7, 16671; Lane 8, 16029; Lane 9, 17594; Lane 10, 17607; Lane 11, 17452.

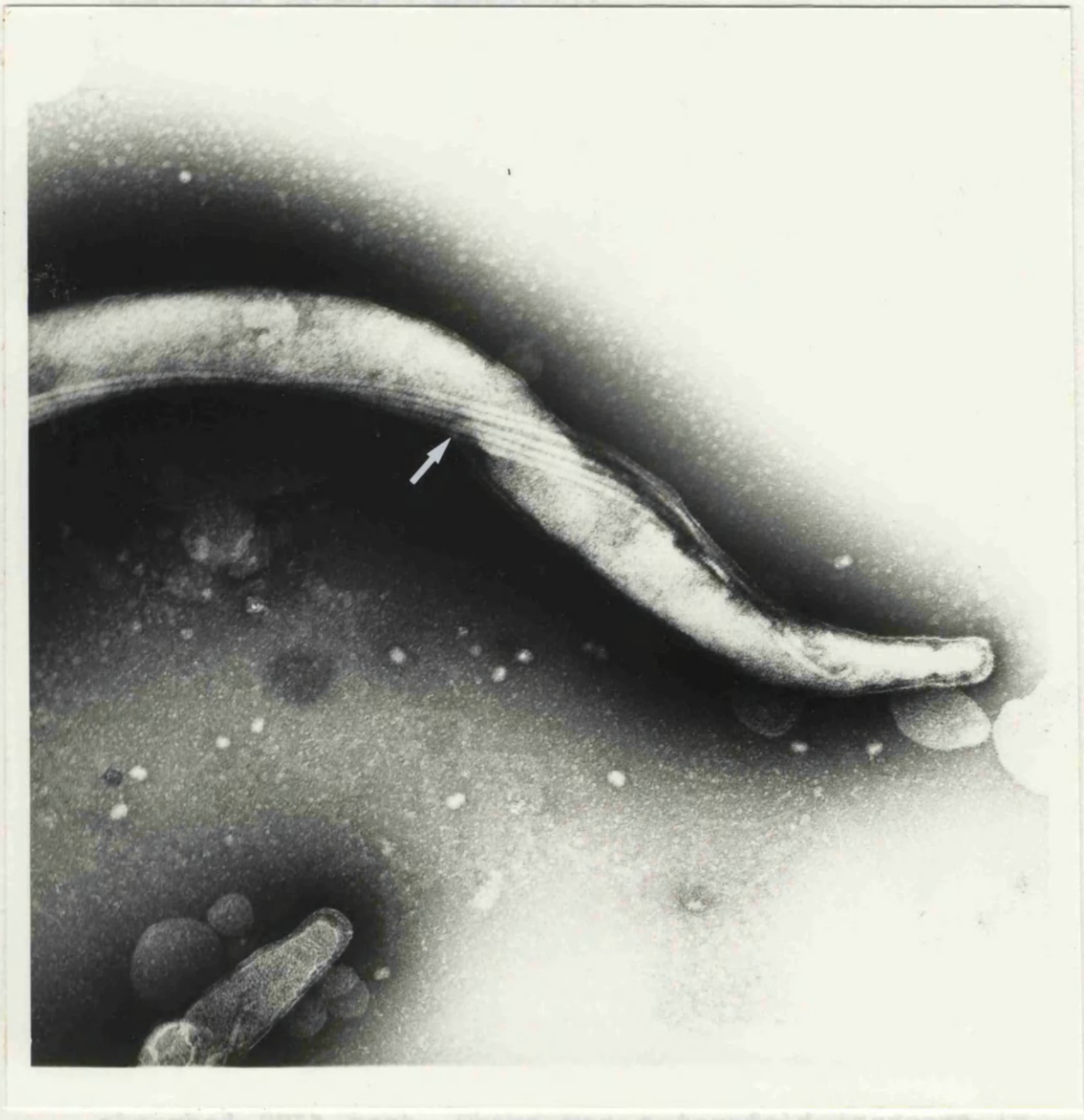

Figure 12: Electron micrograph of spirochaete bearing a 16kDa antigen isolated from field sample No.16669 (X83500). Five fibrils can be clearly seen (arrow).

diagnosis of swine dysentery.

The sensitivity and specificity experiments show that the anti-16kDa RPLA test is about 10-100 times more sensitive for the detection of S80/5 in culture than the absorbed RPLA and 10 times more sensitive for the detection of B78. The differences in the detection by RPLA of the two strains of *S. hyodysenteriae* may be due to the fact that they belong to different serotypes and do not possess equal amounts of the antigen (Sellwood *et al*, 1989). Alternatively these two serotypes may have few common epitopes on the 16kDa antigen (Sellwood *et al*, 1989). Both of these theories could explain the faint result obtained from the Western blot of B78. It must also be said that there was no reaction on the Western blots of the non-*S. hyodysenteriae* spirochaetes.

There were also differences in the detection of PWS/A and 4/71 by the anti-16kDa RPLA test compared with the absorbed RPLA test. There was a ten-fold increase in the numbers of organisms of PWS/A and 4/71 before a positive RPLA result was obtained. There had to be 10 times more of the 4/71 strain than the PWS/A strain before a positive RPLA result would be obtained. This may be due to some sort of prozone effect as this is only seen at concentrations of approximately 10^{10} organisms.

The differences in the numbers of S80/5 that could be detected in culture and in faeces may be explained by the fact that some of the organisms may have been trapped and perhaps soluble antigen produced by the organism throughout the course of the disease and antibody produced by the pig may have a part to play in the agglutination reaction. Both PWS/A and 4/71 remained detectable at the same level.

However when the anti-16kDa RPLA was used to determine whether or not field cases were cases of swine dysentery, the test was found to be almost as non-specific as the absorbed serum RPLA.

Sample numbers 17445, 17452, 17453 and 17456 were found to contain spirochaetes which were very delicate and difficult to grow. They also proved difficult to isolate and maintain in culture. The only one that was successfully maintained was 17452 which proved to be *S. hyodysenteriae* related on testing with API ZYM but for which the initial RPLA test was negative. This may be explained by either a low number of organisms or these organisms being of a different serotype to S80/5 and having few epitopes in common.

The fact that there are both negative and positive cases by RPLA indicates that the lack of specificity in the test is not due to the fact that there are unfilled sites on the beads which would react non-

specifically with any protein i.e. there is enough antibody there to cover all the sites.

Another possibility for the lack of specificity is that the variety of epitopes presented by the 16kDa antigen includes ones that are similar to epitopes on other antigens thus giving rise to the cross-reactions. It may also indicate that the 16kDa antigen is not as unique to *S. hyodysenteriae* as previously thought and may perhaps be a requirement for pathogenesis.

Hunter and Wood, (1979) stated that a spirochaete lacking ß galactosidase had a 95% chance of being *S. hyodysenteriae*. Sample numbers 16029 and 16763 were both shown to possess a 16kDa antigen although they were weakly ß haemolytic did not possess alpha galactosidase. Sample numbers 16667, 16668, 16669 and 16671 were all shown to possess a 16kDa antigen but were shown to be non-*S. hyodysenteriae* related by API ZYM testing. Further evidence in favour of this is that they were also weakly ß haemolytic and their ultrastructure which clearly showed that they were not *S. hyodysenteriae*.

Therefore it seems that the next stage in the development of an RPLA test for swine dysentery should be to raise monoclonal antibodies against those epitopes of the 16kDa antigen which are present only

on *S. hyodysenteriae* isolates. Alternatively polyclonal antisera could be raised against the antigen belonging to swine dysentery causing spirochaetes from different serotypes. These antisera could then be pooled and absorbed with the antigen extracted from non *S. hyodysenteriae* spirochaetes.

CHAPTER SIX

GENERAL DISCUSSION

Throughout the course of these experiments, one of the main points of value was found to be the speed with which the RPLA tests could be carried out. The turn-around time of the diagnosis could be reduced from over a week to minutes. The test is also user-friendly so that very little instruction and no special skills are required before using RPLA tests. From these results it can be seen that the actual RPLA test system has the potential to work well for swine dysentery diagnosis. The only thing holding back the test is its lack of specificity.

Many investigations have been carried out into *S. hyodysenteriae* and *S. innocens* in order to examine the differences between them and establish the identity of the pathogenic determinants of *S. hyodysenteriae*. Possible differences have been pointed out in the surface membrane (Chatfield *et al*, 1988), axial filaments (Kent *et al*, 1989), haemolysin production, (Lysons *et al*, 1991) and lipopolysaccharide differences (Nuessen *et al*, 1983; Halter and Joens, 1988). However there is much evidence in favour of a species specific pathogenic determinant being a 16kDa antigen (Sellwood *et al*, 1989; Joens and Marquez, 1986). Wannemuehler *et al* (1988) also recognised that there was a major pathogenic determinant in the 14-19kDa range. For the purposes of reverse passive latex agglutination tests the most useful differences would probably be surface related antigens present only on

swine dysentery related organisms such as the 16 kDa antigen or possibly those identified by Chatfield *et al* (1988).

It remains to be seen whether or not every single strain of swine dysentery causing spirochaete possesses this antigen, or indeed whether it is entirely species specific.

Variations have been noted in the quantities of the antigen produced by different strains (Sellwood *et al*, 1989). These variations may also be explained by the fact that the antiserum raised against one specific strain may have reacted only slightly with others suggesting that there is only partial conservation of epitopes in the strains involved. It may also suggest that there could be changes in the presentation of the antigen by some organisms when they are grown *in vitro*. However this theory does not account for organisms such as 16665-16671 which typed with API ZYM as being non-*S. hyodysenteriae* and were weakly haemolytic although they apparently possess a 16kDa antigen. The nature of the pathogenicity of *S. hyodysenteriae* therefore remains to be discovered.

It may be that the 16kDa antigen is a pathogenic determinant found on strains of spirochaetes causing dysentery or spirochaetal diarrhoea not just solely on *S. hyodysenteriae*. Variations in the amount of

cytotoxin or haemolysin produced combined with the presence of the 16kDa antigen accounting for the variations in the severity of the disease. Other factors that may contribute to the pathogenicity of *S. hyodysenteriae* include its ability to move faster than any other organism present and to burrow into the mucus layer and colonise it (Kennedy *et al*, 1988). Kennedy *et al* (1988) also demonstrated a significant chemotactic response of *S. hyodysenteriae* to the pig gut mucosa. However direct attachment of the organism to the epithelium or invasion of healthy cells are not thought to be important for lesion production or initiation of the disease (Glock *et al*, 1974; Kennedy *et al*, 1988; Albassam *et al*, 1985).

Lysons *et al* (1991) observed the effects of the haemolysin in ligated ileal and colonic loops in germ-free pigs. Their studies revealed that in the colon the cells in and around the crypts were unaffected by the haemolysin but those epithelial enterocytes which were far from the openings of the crypts were severely damaged and many sloughed off. In the ileum the goblet cells remained intact and accumulated but the enterocytes again were badly damaged and sloughed off. The spirochaetes colonise the mucus (Glock *et al*, 1974) and so the haemolysin must diffuse through the mucus layer to the cells but it may be inactivated by both ileal and colonic contents accounting for the sometimes patchy distribution of the lesions. The

exposure of the cells to the haemolysin for thirty minutes results in cytotoxic effects being seen. This did not result in membrane lysis of epithelial cells but caused disruption of internal organelles before the swelling and shedding of affected cells.

Pigs have been shown to be protected from swine dysentery by inoculation with killed organisms but this protection is limited to the serotype used in the inoculum and the animal still remains susceptible to other serotypes (Glock *et al*, 1974). This is due to the serotype specific LPS but there is thought to be a species specific response to the 16kDa antigen. The fact that there is only a very small response to the 16kDa antigen is probably due to the fact that it is present in only very small quantities whereas the LPS antigens are present in much larger amounts.

The nature of the 16kDa antigen has yet to be confirmed. SDS PAGE gels of whole cell isolates of *S. hyodysenteriae* show very little when stained with Coomassie blue although gels of the cell membrane preparation clearly show that protein is present in a small amount. The outer membrane preparation was made using Triton X-100. The fact that the 16 kDa antigen was found in the detergent fraction could suggest that there is a covalently attached fatty acid which may lead to the suggestion that 16kDa antigen might be a lipoprotein.

Related organisms such as *Treponema pallidum* and *Borrelia burgdorferi* are also thought to possess major lipoprotein antigens (Brandt *et al*, 1990; Swancutt *et al*, 1990).

Treponema pallidum possess a major 47kDa liporotein antigen. The limited antigenicity of *T. pallidum* has been shown to be due to the intracellular location of this lipoprotein (Cox *et al*, 1992). Cox *et al* (1992) suggest that these major membrane immunogens are anchored by lipids to the periplasmic leaflet of the cytoplasmic membrane. This would explain the ability of *T. pallidum* to evade the humoral immune reponse of the host. This type of mechanism may also explain why there is so little anti-16kDa antibody found in pigs infected with *S. hyodysenteriae*. Cox *et al* (1992) showed that if the outer membrane of *T. pallidum* was removed using a much gentler detergent then the 47kDa antigen can be shown to remain anchored to the periplasmic leaflet. The same may be true for *S. hyodysenteriae*.

Borrelia burgdorferi is similar to *S. hyodysenteriae* in that immunisation with whole cells produces protection against only that serotype (Johnstone *et al*, 1988) regardless of the fact that all strains of *B. burgdorferi* possess a 34kDa lipoprotein and *S. hyodysenteriae* strains possess a corresponding 16kDa

antigen. Fikrig *et al* (1990; 1992) suggest that a recombinant form of the *B. burgdorferi* 34kDa lipoprotein may be suitable for a vaccine and indeed the same may be true for *S. hyodysenteriae*.

Given that the 16kDa lipoprotein of *S. hyodysenteriae* is species specific then it seems reasonable to expect that a reverse passive latex agglutination test using either absorbed antiserum to whole *S. hyodysenteriae* cells or either polyclonal or monoclonal antibodies raised against the 16kDa antigen would produce a specific test.

However this appears not to be the case although the specificity could be further improved by using pooled monoclonal antibodies developed from the 16kDa antigen. These antigens would need to be from different serotypes in order to encompass the variations in epitopes that would be found in different strains of *S. hyodysenteriae*.

The problems of cross reaction with the RPLA test may be due to the presence of LPS in the original inoculum for the generation of hyperimmune antiserum - thereby unintentionally raising antibodies to the LPS as well. These antibodies may then be able to cross-react with non-*S. hyodysenteriae* spirochaetes.

This problem could be solved by the separation of LPS from the protein before raising the antiserum. The method used for raising antibody to the 16kDa antigen involved blotting the antigen onto nitrocellulose which was then able to serve as an adjuvant. It could therefore be possible to use an alternative membrane such as PVDF which does not bind LPS. Another approach would be to absorb the antiserum with purified LPS in order to leave only the antibody against the 16kDa antigen. This could be done by first of all making a hot phenol/water extract (Baum and Joens, 1979). Then to ensure complete removal of all protein, the LPS preparation would be digested with either trypsinase or proteinase K. SDS PAGE would then be carried out on the resulting preparation. The LPS would then be transferred to nitrocellulose by western blotting. After blocking the nitrocellulose, it could then be used to absorb the antiserum.

The problems of lack of sensitivity may be due to the means by which the antibody is removed from the serum. In removing the antibody by ammonium sulphate precipitation, there may still be a lot of residual non-specific serum proteins that are blocking sites on the beads that could be occupied by anti-16kDa antibodies. The removal of antigen specific antibodies from a preparation of polyclonal antibodies can be done by immunoaffinity purification on an antigen column. Although this does separate the specific from

the non-specific, it does have several drawbacks. The process requires large amounts of pure antigen and the elution of the specific antibody needs conditions that may lead to some loss of activity.

Should the 16kDa prove not to be specific to *S. hyodysenteriae* then it may be possible to develop a second latex test to use in conjunction. This would require further investigation into the work done by Chatfield *et al* (1988) in order to determine whether all strains of *S. hyodysenteriae* possessed antigens with molecular weights of 68, 36 or 31kDa.

Another possibility is that a battery of tests could be developed against the lipopolysaccharide antigens. This type of test would also be useful in determining the spread of the disease and the relative importance of each serotype which may be different in different countries. This type of test may however run into problems with the presence of multiple major LPS antigens on some serotypes and the loss of LPS antigens from others under certain conditions (Hampson *et al*, 1989).

S. hyodysenteriae produces a soluble antigen and it is possible that this could interfere with the agglutination of the RPLA test. The affect of the soluble antigen on the RPLA test could be assessed by using cell-free faecal filtrates.

Lemcke and Burrows (1981) carried out an investigation into the spirochaetes found in the porcine alimentary tract. They concluded that the diversity observed amongst those spirochaetes not associated with swine dysentery suggested that their inclusion in a single species may not be correct. This view is supported by the discovery of non-*S. hyodysenteriae* organisms which are not *S. innocens* either as they possess a 16 kDa antigen. Further investigation into non-*S. hyodysenteriae* porcine spirochaetes is needed before they can be separated into different species.

Should a specific RPLA test be devised for swine dysentery, then the next stage would be to develop test kits and distribute them to various farms and veterinary practices in order to sort out any difficulties that may arise. These kits would include the *S. hyodysenteriae* specific latex suspension, a negative control, a positive control, diluting buffer, a black tile and disposable syringes and positive filters.

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