THE EFFECT OF CATTLE CLEANLINESS SCORES ON BACTERIAL CONTAMINATION OF CARCASSES.

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Abstract

The control of inadvertent microbiological contamination of red meat carcasses has emerged in the last decade as the most important aspect of meat inspection and meat hygiene. The hide is recognised as the main sources of human pathogen contamination such as *Campylobacter spp.* or *Mycobacterium avium subsp paratuberculosis*. Introducing only clean cattle to the slaughter line partially minimises the potential for meat-borne zoonoses.

Following the outbreaks of *Escherichia coli O157* infection in Scotland in 1996, British abattoirs have imposed a scoring system called the Clean Livestock Policy (CLP), to assess the cleanliness of animals submitted for slaughter. The policy provides categories of a range of scores of dirtiness from 1 to 5.

In this study, 93 over thirty month (OTM) cattle classified in all 5 different CLP categories, were assessed. Swab samples were taken from the flank of the carcass and using the wet and dry method as advised by the Meat Hygiene Service (MHS), the Total Viable Count (TVC), and *Enterobacteriaceae* count were determined. This study showed that the level of contamination on carcasses increased with an increase in the CLP score. Dirty carcasses with CLP score 3, 4 and 5, as defined by MHS, were found to carry up to 30,000 times more micro-organisms, defined by TVC, than clean carcasses with CLP sore 1 and 2.

Different *ante-mortem* features of cattle were also studied and coat length, coat moisture, type of contamination, and site of contamination was shown to influence carcass contamination.

The classification of dirty or clean cattle during the *ante-mortem* inspection in an abattoir lairage sometimes creates difficulties for the Official Veterinary Surgeon (OVS), as the MHS CLP score provides only descriptive details and pictures of cattle in the five different categories. In this study, three different decision trees were designed; two for each of the outcome variables, TVC and *Enterobacteriaceae*, and a third one for the CLP, with the goal to minimize subjective decisions.

The association between carcass contamination and prevalence of potential zoonotic pathogens isolated from the cattle such as *Campylobacter spp.* and *Mycobacterium avium subsp. paratuberculosis* was studied. Different culture methods for isolation of *Campylobacter spp.* were employed. No colonies of *Campylobacter spp.* were found on direct swab samples from carcasses however *Campylobacter spp.* were isolated in faeces from 5 cattle using the combination Skirrow and Bluster medium.

Of the 93 cattle sampled, eight cattle were found to carry *Mycobacterium avium subsp. Paratuberculosis* (MAP) by direct smear using the Ziehl Neelsen staining method. In five cattle MAP were isolated from faeces, in one cattle from the lymph node and in two cattle MAP was isolated from both the lymph node and from faeces.

List of contents

List of figures

List of tables

Table 14. Explanatory variables for carcass contamination with *Enterobacteriaceae* and TVC with altered classification.

List of Pictures

Dedication

For Mercedes, mum, dad and sister.

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Author's declaration

Other than the help acknowledged, all the work presented in his thesis was carried out by the author. It has not been submitted, in full or in part, for consideration for another degree or professional qualification

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List of abbreviations

AGID	Agar-gel-immuno-diffusion test
BCS	Body Condition Score
CAT	Amphotericin Teicoplanin Agar
CCP	Critical Control Point
CFU	Colony Forming Unit
CLP	Clean Livestock Policy
DEFRA	Department of the Environment and Rural Affairs
EC	European Community
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
HACCP	Hazard Analysis Critical Control Point
HAS	Hygiene Assessment System
HCL	Hydrochloric acid
ISO	International Standard Organization
MAP	*Mycobacterium avium subsp. paratuberculosis*
MHS	Meat Hygiene Service
OTMS	Over Thirty Months Scheme
OVS	Official Veterinary Surgeon
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
SRM	Specific Risk Material
TVC	Total Viable Count
VRBGA	Violet Red Bile Glucose Agar

1. Review of the literature

1.1. Introduction

The control of inadvertent microbiological contamination of red meat carcasses has emerged in the last decade as the most important aspect of meat inspection and meat hygiene in modern meat production systems (Biss and Hathaway, 1996). The edible tissues of healthy livestock before slaughter are sterile with the exception of the tongue and the gastrointestinal tract (Hadley and others, 1997). The hide and the gastrointestinal tract are recognised as the main sources of human pathogen contamination. Activities on the slaughter floor, in particular those associated with the removal of the pelt and the gastrointestinal tract, are the main methods by which contamination is transferred to previously sterile tissues (Lawrie, 1985; Biss and Hathaway, 1996; Hadley and others, 1997; Ayres, 1995, Gill, 2004). The extent of contamination depends on the prevalence of the organism in the cattle slaughtered, in addition to the hygienic standards during slaughter and dressing (Hudson and others, 1996).

The ability of bacteria to establish themselves in cattle, and thus pass to the food chain from dam to offspring, is important. Total elimination of bacteria from the food chain will only be possible when the bacteria in question are eliminated from breeding stock. Introducing only clean cattle to the slaughter line will partially minimise the potential for meat-borne zoonoses resulting from unapparent contamination with enteric pathogens, many of which have high asymptomatic carriage rates (Mullen, 1997). Later intervention in the food chain may reduce the magnitude of the problem, but it does not prevent the risk (Skovgaard, 1996).

In the UK, the hygiene status of abattoirs is monitored regularly by the Hygiene Assessment System (HAS) developed by DEFRA, and recently from June 2002, in full throughput plants, and from June 2003, in low throughput plants, by the implementation of the hazard analysis critical control points system (HACCP) (The Fresh Meat Regulations, 2002). The HAS score, which is based on risk assessment, which still, is to some extent, subjective, seeks to facilitate the recognition of good hygiene and management practices and focuses attention on those parts of the operation that are

particularly significant in hygiene control. The HAS performance is in five main categories: *ante-mortem*, slaughter and dressing, personnel and practices, maintenance and hygiene of premises, and general conditions and management. The HAS score ranges from 0 to 100 (Hudson and others, 1996; MHS 1995).

1.2. Clean livestock policy in the UK

In the UK, it is mandatory to deliver cattle to the slaughterhouse in a clean condition and to reduce faecal cross-contamination, which can be an important source of pathogens (MHS 1995). Dirty cattle are a serious problem in the beef and tanning industries and the problem arises mainly during the winter months when cattle are housed (Davies and others, 2000).

Dirty cattle pose a problem for meat hygiene in the abattoir as slaughterhouse staff have to cut through dirty hides during the slaughtering and dressing process. A dirty coat has the capacity to contaminate a clean carcass during dressing (Davies and others, 2000). This contamination can be transferred either directly from the skin to the carcass or indirectly from the knives, hands, arms and clothing of slaughterhouse staff (Hadley and others, 1997; Davies and others, 2000). Dirt from dry coats can flake off onto the carcasses causing contamination. This capacity to contaminate increases when dirty coats become wet (MHS, 1997; Hadley and others, 1997). Water provides a ready means of transport for microbes to be transferred from one area to another and it also aids the spread of microbes over a wider area allowing a greater degree of contamination. Not only does water provide a means of transport, it also provides microbes with an increased ability to adhere to the surface of the carcass (Biss and Hathaway, 1996; MHS 1997).

The production and slaughter of beef cattle involves a number of interactive procedures from the farm, to the lairage, to the abattoir environment, to the carcass at the end of the line and the further processing of by-products (MHS, 1997; Davies and others, 2000). Transporters can take measures to ensure that cattle that are in a satisfactory condition at their point of origin are delivered to the abattoir in the same satisfactory state. Hygienic

transport conditions also reduce the levels of stress (Davies and others, 2000). Lairage facilities and lairage staff should be able to maintain the hygiene of the cattle that have been delivered in satisfactory hygiene status (MHS, 1997).

As a result of the outbreak of human infection with *Escherichia coli* O157 in Scotland in 1996, the MHS introduced and implemented in 1997 the "Clean Livestock Policy" (CLP) covering cattle and sheep. The policy provided categories of a range of scores of dirtiness, from 1 to 5 where score 1 is the cleanest and score 5 is the dirtiest (Davies and others, 2000; Hadley and others, 1997). In 1997, Hadley and others developed a study to assess the Total Viable Count (TVC) and *Enterobacteriaceae* count on sheep carcasses, using sheep with different cleanliness scores. They found that there were no significant differences between carcass contamination in scores 1 and 2 and scores 4 and 5 but there was different level of carcass contamination between scores 2 and 4. They also found that, in general, the proportion of carcass sampling sites contaminated with *Enterobacteriaceae* increased as the CLP score increased (Hadley and others, 1997). The results of this study showed that the degree of soiling of the live animal significantly affected the microbial load of the dressed carcass, as carcasses derived from sheep with dirtier fleeces carried up to 1000 times more micro-organisms, and a higher proportion of such carcasses were contaminated with *Enterobacteriaceae* (Hadley and others, 1997). Cattle from CLP 3 to CLP 5 are considered by the MHS to be too dirty for hygienic slaughter because they are potential carriers of food-borne pathogens (MHS, 1997).

Other authors developed a similar scoring system, with a scale of 1 to 5, to assess the cleanliness scoring of cattle in the abattoir. Different parts of the animal, including flanks, hind legs, udder and tail were assessed. Both sides of the animal were examined and if different, the higher score was recorded (Hughes, 2001). Separate anatomical sites on the animal were chosen because it was sometimes necessary to assign an overall animal score and because the site scores gave some indication of the sources of contamination, i.e. dirty flanks may reflect the state of the bed; dirty tails suggest loose faeces or overhanging in the passageway (Hughes, 2001). By numerically defining the cleanliness of each site and calculating the mean of the scores of the four sites, it is possible to give an overall assessment of the cleanliness of a cow (Hughes, 2001).

The sex, breed, floor type, dirtiness of the lorry and housing before transport were significantly related to the MHS score (Davies and others, 2000). The main factors identified by Davies and others were: age, coat length, journey time and distance, clipping, feed and abattoir. All these factors need to be fully understood to help producers meet the MHS requirements for animal cleanliness (Davies and others, 2000). In this study, 97.2% of the cattle were cleaner than MHS score 3 (2.8% in category 3 and 4). This figure is higher than in another study, which gave figures of 0.48% dirty cattle (categories 3, 4 and 5) between 1997 and 1998 (Davies and others, 2000). Recently a MHS report indicated that during 2002-2003, 0.4% cattle and 0.65% sheep of total animals slaughtered in the UK were initially rejected as unfit for slaughter under the CLP, (MHS, 2002). Feed is an important factor in producing clean livestock. Silage-based diets with a low dry matter content were associated with dirtier hides (Ingram, 1972; Davies and others, 2000). Stock finished on a diet with a high dry matter content should be cleaner. However, changing the diets of cattle close to slaughter should be approached with caution since this can produce loose faeces (Davies and others, 2000). Kudva and others studied the shedding of *E. coli* O157 by sheep and found that high nutrient, low fibre, intake led to the steady shedding of *E. coli* over a prolonged period (Davies and others, 2000). Long or medium coated cattle are dirtier than short coated cattle, hence clipped cattle have lower MHS scores than unclipped cattle, but there is less evidence that it reduces microbial contamination in the abattoir (Davies and others, 2000). Damage to the hide caused by clipping may significantly reduce its value to the tanner (Pearson, 1998). Davies and others found that journey distance appeared to have little effect on MHS score up to 150 miles, with an evident trend that cattle travelling more than 150 miles appeared to be more dirty. They also found that cattle less than 20 months of age were noticeably cleaner than older cattle (David and others, 2000).

Several techniques have been used to reduce the visible contamination on carcasses on the slaughter floor. One of these techniques is pre-slaughter washing but research in New Zealand has demonstrated a detrimental effect of pre-slaughter washing on the microbiological contamination of ovine carcasses at the completion of pelting, with total aerobic plate counts rising from 4.30 log/cm^2 on carcasses derived from dirty, woolly, unwashed, lambs to 4.63 log/cm^2 on washed lambs. This effect occurred in parallel with an improvement in the levels of visible contamination. Pre-slaughter washing has also

been shown to increase the pH of lamb carcasses if performed repeatedly or in combination with other pre-slaughter stressors, thus contributing to more favourable condition for the growth of microbiological contaminants of the carcass (Biss and Hathaway, 1996). Another New Zealand study on washed beef carcasses shows that carcass contamination associated with clean hide contact was significantly lower than that following contact with pre-slaughter washed, faecally-soiled hides. The latter was comparable to that resulting from direct contact with fresh faeces. This suggests that washing immediately pre-slaughter may not be the most efficacious time to address the problem of slaughter cattle cleanliness (Roberts, 1980; Bell, 1997). Under some circumstances it may be beneficial to wash cattle but only if they are washed and dried prior to going for slaughter (MHS, 1997). The use of alternative slaughtering techniques on excessively dirty cattle in Finland was unable to compensate for the dirty condition of the cattle. Other work in the European Community (EC) has also failed to find a correlation between factors suggestive of good manufacturing practice and reduction in the microbiological contamination of bovine carcasses (Roberts, 1980; Biss and Hathaway, 1996).

1.3. Microbiology techniques to assess carcasses contamination

The traditional system of *post-mortem* meat inspection was designed to ensure the safety and wholesomeness of meat products entering the human food chain. In the light of modern knowledge and experience, it is clear that the system does not deal adequately with the problem of microbial contamination of meat during slaughter and dressing operations, and its consequences for food-borne human disease (Lawrie, 1985; Hudson and others, 1996).

Several different sampling methods such as contact agar techniques, swab techniques, the destructive method can be used to provide information on total microbial numbers (TVC) and *Enterobacteriaceae* on carcasses (Stephan, 1996). Most of the methods only provide information on total colony counts and this is a disadvantage because the composition of the microbial flora is an essential factor for the full interpretation of results. In fact, specific pathogens cannot be identified using TVC, although their presence or absence is of significance to public health (Stephan, 1996).

Both destructive and non-destructive methods can be used for sampling the surface of meat, with the excision technique being the most accurate, as it is considered the reference method by many authors (Lazarus and others, 1977; Anderson and others, 1987). The destructive method, where samples are obtained by punching a sterile cork borer into the meat surface or by cutting a thin slice off the carcass with a sterile instrument enables differentiation of the microbial flora as well as the assay of specific pathogens. However, sample preparation may be laborious and involve homogenisation and filtration. A major disadvantage is that it may lower the carcass value, which is an important consideration for a routine method (Stephan, 1996). Non-destructive methods such as wet and dry swab, or wet and double dry swab techniques both consist of swabbing carcasses initially with a moist swab, then, swabbing the same place with a dry swab. These techniques do not require extensive preparatory steps. However they provide an incomplete and inconsistent removal of surface bacteria with recovery rates of 1 to 89 % being described (Stephan, 1996). Incomplete removal might be the result of incomplete release of bacteria from the swab during shaking in the diluent, inconsistency in the pressure applied to the swab and its moisture content, the length of swabbing time and the fat content and texture of the surface (Stephan, 1996). Although the excision sampling technique gives more precise results than swab sampling the latter is preferable for routine microbiological monitoring programme since it takes less time to sample and process (Stephan, 1996). The variation between the methods of wet-dry swab and excision sampling techniques is of minor importance compared to the enormous variation in colony counts that are encountered for different sites on the carcasses. The objective of sampling is not to provide the most precise results for one particular site, but rather to indicate the distribution of bacteria, and their numbers on different sites on the carcass (Stephan, 1996; Untermann and others, 1996).

Another non destructive method is the agar contact plate method, which consists of 5 cm diameter plastic dishes with lids filled with plate count agar, and dishes filled with violet red bile glucose agar (VRBG). These are pressed on to each sampling site and subsequently incubated (Enzenauer and others, 1984). The advantages of swab techniques, in contrast to agar contact plate techniques, include the accessibility of more sites. When a template is used, quantitative results can be obtained and when selective

media are used, specific pathogens can be differentiated from the normal flora (Stephan, 1996).

Dorsa and others (1996) evaluated six bacterial sampling methods that might be used for rapid sampling of beef carcasses. They found that the excision method was most consistently effective for faeces-inoculated carcasses and that cotton-tipped wooden swabs were the least effective of the methods used. However, in the case of carcasses contaminated with bovine faeces there was no significant difference between all six methods, hence cotton-tipped wooden swabs are indicated in plant process monitoring to detect faecal contamination (Dorsa and others, 1996). Nevertheless, the swab sample recovery rate under optimal wet carcass surfaces condition did not exceed 20% (Ingram and Simonsen, 1980; Dorsa and others, 1996).

Different parts of the same carcass can have different values for carcass contamination. Thirty sampling series from different parts of the carcass were examined (Untermann and others, 1996). The forearm and brisket showed the highest bacterial counts in 21 series, the abdomen in six, the lateral neck and round (the North American beef cutting name for the hip) (Swatland, 2000) in four, and the back in one, respectively. The results confirm that forearm and brisket are the most contaminated parts of the carcass (Untermann and others, 1996). The lowest bacterial counts were recorded for the cranial pelvis in 21, the pleura in 22 and the top round, (the North American beef cutting name that involve the *abductor* and *semimembranosus* muscles (Swatland, 2000), in ten out of the 30 sampling series (Untermann and others, 1996). Although the brisket and forearm are the places with highest microbiological contamination, contamination of the flank is due mostly to hide dirtiness, and not to slaughter practice (Kitchell and others, 1973; Hughes, 2001).

Currently, the UK meat inspection legislation dictates that between 5 and 10 carcasses should be sampled on a single day during each week for microbiological testing in full throughput slaughterhouses (MHS, 1995). A sample from four sites from each carcass should be taken after dressing and before chilling. The samples sites for cattle are neck, brisket, flank and rump; however, alternative sites may be used following consultation with the OVS, where, because of the slaughter technology at a particular plant, other

sites are more likely to carry higher levels of microbiological contamination (MHS, 1995; European Commission, 2001). The method indicated for microbiological examination of carcasses is wet and dry swabbing that comprises swabbing 100 cm^2 of each of four sites; brisket, flank, neck and rump, initially with a moist swab, and then, subsequently, swabbing the same place with a dry swab. All swabs are placed aseptically in the same container (MHS, 1995).

Post-mortem inspection procedures are labour intensive and mainly target grossly detectable abnormalities, which are now considered to be a minor source of food-borne hazards to the consumer (Pointon and others, 2000). An assessment of pig meat inspection in Australia demonstrated that the exposure of consumers of Australian pork to microbiological hazards is unlikely to be significantly reduced by the detection of removal of gross abnormalities. In addition, the traditional inspection process can increase the potential for carcass cross-contamination, by incising apparently normal lymph nodes that may be contaminated. Normal lymph nodes were found to be contaminated at virtually the same rate as lymph nodes which were judged grossly abnormal (Pointon and others, 2000; Hamilton and others, 2002). Microorganisms such as *Campylobacter spp.* or *Mycobacterium avium subsp. paratuberculosis* (MAP) may be transmitted horizontally in the food chain and can result in zoonotic infection (Pointon and others, 2000).

1.4. Total Viable Count

Total viable cell count, also called plate count, involves spreading a sample of a culture on a nutrient agar surface. The sample is diluted in a non-toxic diluent such as water or saline before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a colony forming unit (cfu) and the number of cfu is related to the viable number of bacteria in the sample (Patrick, 1978; Todar, 2001). The advantages of the technique are its sensitivity because theoretically, a single cell can be detected, and it allows for inspection and positive identification of the organism counted. There are also some disadvantages because only living cells develop colonies, clumps or chains of cells develop into a single colony, and the colonies

develop only from those organisms for which the cultural conditions are suitable for growth. This last disadvantage makes the technique virtually useless for characterization or counting of total number of bacteria in complex microbial ecosystems such as soil, the animal rumen or gastrointestinal tract, but the technique is indicated in the enumeration of bacteria in food because it is very sensitive if plating conditions are optimal (Todar, 2001). The optimal condition for culturing TVC plating is 30°C incubation over 48 hours (Guerra-Flores and others, 1997; Hadley, 1997; Balestra and Misaghi, 1997). Comparison between total viable count by spread plating and AquaPlak® for enumeration of bacteria in water from a shrimp farm resulted in no significant difference between the two techniques, suggesting that both techniques could be used to identified microbiological carcasses contamination (Guerra-Florres and others, 1997).

1.5. Enterobacteriaceae

Members of the *Enterobacteriaceae* family form a very large group of morphologically and physiologically similar bacteria. These are considered to be characteristic intestinal bacteria (Cowan, 1993), although they are not a major component of the intestinal flora in cattle (Finney and others, 2003). Other members of the group include *Citrobacter spp., Enterobacter spp.* and *Klebsiella spp.* that form part of the facultatively anaerobic bacteria flora, although the majority are non-pathogenic (Salminen and others, 1995).

The EU legislation indicates that analysis for TVC and *Enterobacteriaceae* should be performed on cattle carcasses. The basis for examination of samples should be provided by the International Standard Organization (ISO). The frequency of sampling is indicated by the MHS, e.g. between five and ten carcasses on a single day each week, although the frequency may be reduced to fortnightly if satisfactory results are obtained on six consecutive weeks (MHS, 1997).

1.6. Campylobacter spp.

1.6.1. Microbiology of *Campylobacter spp.*

The first species identified in the genus Campylobacter was *C. jejuni* (Skirrow, 1977). The genus Campylobacter was reclassified in 1994 and now contains seventeen defined species. The main pathogenic campylobacters are *C. jejuni*, *C. coli* and *C. lari* with *C. jejuni* being the most common in the UK, accounting for 90 to 95% of human cases of Campylobacter enteritis (Rollins and Colwell, 1986; Phillips, 1998).

Campylobacter jejuni is further divided into two subspecies: *C. jejuni jejuni* which is a human pathogen and *C. jejuni doylei* that is rarely associated with human illness. Campylobacteriosis due to *C. jejuni* is now regarded as the most common form of acute bacterial gastroenteritis in human. The infection is also important in farmed cattle, and although its exact role in disease is not always clear, campylobacteriosis has the potential to be zoonotic (Gracey and others, 1999).

Campylobacter upsaliensis is another Campylobacter which has been linked with human infection and which is isolated at a high rate from dog faeces (Phillips, 1998). *C. hyointestinalis*, which is present in pigs, has occasionally been isolated from patients with diarrhoea, although the importance of this species is uncertain (Edmunds and others, 1987; Skovgaard, 1996).

Campylobacter jejuni is Gram-negative, motile by a single flagellum at both poles, microacrophilic, slender, spirally curved rods with a tendency to form chains. As with *C. coli and C. lari, C. jejuni* is classified as a thermophilic campylobacter because it grows well at temperatures in the range 42-45°C, and does not grow below 25°C. It is sensitive to freezing and heat. Samples for testing should be held at 4°C (Hodge and Terro, 1984). It is catalase-positive and oxidase positive and the ideal atmosphere for growth is 3-15% O_2, 3-10% CO_2 and 85% N_2 (On, 1996; Gracey and others, 1999). Colonies appear moist, spreading, translucent, round, raised or flat. *Campylobacter spp.* are sensitive to levels of pH below 5.0, particularly organic acids, drying except during refrigeration,

salt concentration exceeding 2% and long periods at temperatures between 10 and 30°C (Doyle and Roman, 1981; Skovgaard, 1996). Counts of campylobacter from pig skin were reduced from $\log_{10}$ 3.5 cfu/cm^2 to $\log_{10}$ 1.0 cfu/cm^2 in 24 hours by chilling to 4°C with ventilation (Skovgaard, 1996).

Under adverse environmental conditions such as prolonged exposure to water or high oxygen concentrations, *Campylobacter spp.* change from their spiral rod-like shapes to a coccoid form (Butzler and Skirrow, 1979; Skovgaard, 1996).

1.6.2. Diagnosis of *Campylobacter spp.*

There are a number of methods for isolating *Campylobacter spp.* from faeces and food. For veterinary and other environmental specimens, direct plating is an effective technique for isolation and enumeration of *Campylobacter spp.* from a variety of sample types. However, distinguishing Campylobacter from non-Campylobacter contaminants that frequently grow on many existing agars is difficult. Skirrow developed the first selective media for *Campylobacter spp.* in 1977, and subsequently, many different supplements were added to the agar base to avoid non-campylobacter growth. Cycloheximide, amphotericin or nystatin are used to inhibit fungal competition, rifampicin is active against both Gram positive and Gram-negative bacteria, while Gram-positive bacteria are commonly inhibited by vancomycin or bacitracin in combination with cefoperazone. Polymyxin B and polymyxin E (colistin) inhibit most Gram-negative rod-shaped bacteria except *Proteus spp.*, for which trimethoprim is added (Corry, 1996).

Butzler and others (1983) developed various media. The first one contained bacitracin and novobiocin with polymyxin B or colistin. Later, bacitracin and novobiocin were replaced by rifampicin and cefoperazone and then novobiocin was replaced by vancomycin and cephazolin (Butzler and others, 1983). These media contain sheep blood with the exception of Goossens' medium, which is semi-solid and uses only cefoperazone in combination with a high level of trimethoprim as a selective agents (Corry, 1996). Skirrow's medium contains lysed horse blood and replaces some of the

polymyxin B used in the Butzler media with trimethoprim and vancomycin. Campy-BAP is basically Skirrow's medium with cephalothin (Corry, 1996). Preston media contains charcoal, cephazolin that was replaced later by cefoperazone deoxycholate agar and amphotericin teicoplanin agar (CAT agar) (Bolton and Robertson, 1982). Preston medium allows detection of low numbers of organisms present in food. This media is similar to Skirrow medium containing lysed horse blood, but has double the concentration of trimethoprim and polymyxin B, rifampicin instead of vancomycin due to its wider spectrum of antibacterial activity, and amphotericin to suppress fungi (Corry, 1996). The inclusion of amphotericin enables the media to be incubated at 37°C rather than 42°C (Corry, 1996).

In a preliminary report, Plumer and others (1962) described the use of Millipore filters in isolation of *Vibrio fetus* from preputial and semen samples. Later on, Steele and McDermott (1984) used the membrane filtration method for the isolation of *Campylobacter spp.* This method avoids the use of selective media; therefore, *Campylobacter spp.* sensitive to the inhibitors in selective isolation media can be cultured. The membrane filtration method consists of a 47 mm 0.45 or 0.6 μm pore cellulose triacetate membrane filter laid on the surface of an agar plate. A small volume of faecal, or other suspension, is dispensed onto the filter and the plate is incubated aerobically face up for 30-60 min before the filter is removed and incubation continued under microaerobic atmosphere. *Campylobacter spp.* appear to be able to penetrate through the membrane while other bacteria cannot, however this technique is useless if the samples contain fewer than 10^5 *Campylobacter spp.* per ml (Corry, 1996).

In a comparison of Butzler media and the membrane filtration method to avoid growth of non-Campylobacter bacteria in the selective medium, Butzler selective medium resulted in more efficient detection of total positive samples (36%) than the filtration technique, which detected just 19% of total positive samples. Forty five per cent of samples were detected using a combination of both techniques (Modolo, 2000).

Many of the solid media have been adapted for use as enrichment liquid media. Enrichment media are sometimes used to isolate *Campylobacter spp* from faeces, but

more often for examining food or water where the organisms are likely to be present in low numbers (Corry, 1996). Other methods of enrichment for organisms derived from foods involve incubating at 37°C for 4 to 6 h or even 4 h at 31-32°C, followed by 37 °C for 2 h before transfer to 42°C (Corry, 1996).

The ISO method for isolating thermophilic campylobacters advises enrichment of the sample in Preston broth for 18 h at 42°C, using a microaerobic atmosphere and then solid media such as Modified Butzler, Skirrow or Preston at 42°C with inspection after 48 h, 72 h and if necessary up to 5 days (ISO 102272, 1995).

In a comparison of three solid media: Skirrow, Butzler and Preston, for primary isolation of *C. jejuni* and *C. coli* from specimens of faeces and intestinal contents, the Butzler medium was superior to both the Skirrow and to the Preston medium. Using only Butzler's supplement, 85.2% of all thermophilic *Campylobacter spp* were recovered, with Skirrow's supplement alone 46.6%, and with Preston's supplement 25.6%. Parallel use of two selective media is recommended; the best combination was the simultaneous use of Butzler's and Skirrow's supplement, which identified 97.7% of all *Campylobacter spp.* (Rubsamen, 1986).

Merino and collaborator in 1986 studied seven selective media: Butzler, Blaser, Skirrow, Preston, Preston blood-free, modified Butzler, and modified Preston medium (2 mg/litre of amphotericin B) for isolation of *Campylobacter spp.* in human patients. A similar number of *C. jejuni* strains were isolated with all media studied. Preston Campylobacter blood-free medium with cefoperazone yielded the greatest number of *C. jejuni* isolations, and contamination with competing faecal flora occurred in only 9% of the plates showing *C. jejuni* growth; all the other media allowed the abundant growth of other faecal flora, regardless of whether *C. jejuni* was isolated from them or not (Merino and others, 1986).

Despite the number of culture media, detection of very low numbers of *Campylobacter spp* may require specific techniques such as the Latex Agglutination Test (LAT) or Polymerase Chain Reaction (PCR) (Phillips, 1998).

1.6.3 Epidemiology of *Campylobacter spp.*

Since 1983, *Campylobacter spp* has been the commonest enteric infection of human reported by laboratories in England and Wales. The number of reported food-poisoning cases in England and Wales was 94,382 in 1997; 50,247 of these cases were due to *Campylobacter spp* (Phillips, 1998). In 1989-1991, the average rate was 65 cases per 100,000 population with the highest rate being in the North West region, at 139 per 100,000 in 1989, and 231 in 1990 (Phillips, 1998).

This zoonosis is important for its public health significance, but also for its economic importance because each case of food poisoning caused by *Campylobacter spp* has been estimated to cost the country more than £500 (Phillips, 1998).

The most important feature of Campylobacter epidemiology is the fact that human campylobacteriosis always occurs as sporadic cases with no secondary spread (Tauxe and others, 1987; Phillips, 1998). Reports of Campylobacter infection tend to peak in June (Phillips, 1998) and the reasons for seasonal variation in Campylobacter infection remain controversial. One factor suggested is the drinking of milk from doorstep delivered bottles that have been pecked by birds. Campylobacter are not able to survive proper pasteurisation procedures but raw milk may be a source of infection and there have been several reports of cases linked to the consumption of raw milk by children after educational visits to farms. Other possible sources of Campylobacter infection are the handling and preparation of raw meat and exposure to pets with diarrhoea (Phillips, 1998).

Campylobacter jejuni and *C. coli* were isolated from rectal swabs of dairy cows in New Zealand and the prevalence was 24%, 31% and 12% in summer, autumn and winter respectively (Patton and others 1991). The organism may be present in about 15% of cattle at the time of slaughter (Warner, 1986.), and 60% of the specimens of healthy slaughter pigs may yield *C. jejuni* (Radostits and others, 1994). Faecal contamination, rather than udder infection, is considered to be the means by which *Campylobacter spp.* enter milk and thereby infect humans (Radostits and others, 1994). Abortions in beef cattle herds have also been attributed to *C. jejuni*.

Transmission of *Campylobacter spp* to stock is predominantly horizontal from wild and domestic cattle with further horizontal transmission down the food chain (Skovgaard, 1996). The epidemiology of human campylobacteriosis shows that both vertical and horizontal transmission are involved (Skovgaard, 1996)

1.6.4. Campylobacter spp. in humans

The main source of the bacteria is poultry, but cattle and pigs are also of importance in the epidemiology of human Campylobacter infection (Skovgaard, 1996). The number of organisms required for disease is thought to be as few as 500 (Phillips, 1998).

The symptoms in humans vary between individuals. The incubation period is generally between one and seven days and in the majority of cases, recovery is complete within one week. The symptoms range from those of an asymptomatic illness, characterised fleetingly by loose faeces, to abdominal pain. Bloody diarrhoea with inflammation of the gut tends to be common in young adults. In developing countries where infection is endemic, watery diarrhoea is a more common symptom and young children are at greater risk (Advisory Committee on the Microbiological Safety of Food, 1993).

Motility is required both to enable the organism to reach attachment sites in the intestinal mucosa and to enter epithelial cells. Campylobacter probably produces enterotoxins and these are responsible for the classic symptoms of gastrointestinal disease (Phillips, 1998).

1.6.5. Campylobacter spp. in cattle

Several species of the genus Campylobacter are known to cause disease in farm cattle; some are potentially zoonotic while the role of some others is uncertain. *Campylobacter fetus* and *C. venerealis* can cause sporadic abortion in cattle and sheep and diarrhoea and dysentery in calves (Radostits and others, 1994; Gracey and others, 1999). They can be found in the bovine reproductive tract particularly in the mucosa of the penis and prepuce in bulls. In these cases, the infection can pass from bull to cow during service.

Newly infected cows may show embryonic death after 17 days post-service or in some cases remain pregnant and abort after 5-6 months of pregnancy (Taylor, 2002)

Campylobacter jejuni, C. coli and C. hyointestinalis can be isolated from the intestines and rumens of healthy farm cattle, poultry, pets, and wild birds (Gracey and others, 1999). The role of *C. jejuni* as a primary pathogen in farm cattle is uncertain. The organism was originally though to be the causative agent of winter dysentery in cattle but evidence for this relationship has not been found (Warner and Bryner, 1984). The disease may be so mild as to be in apparent, without fever, and may be manifested only by mild depression and soft faeces with occasional strands of mucus (Al-Mashat and Taylor, 1980).

1.6.6. *Campylobacter spp.* in raw meat for human consumption

Campylobacter spp. are normal inhabitants of the intestinal tract of a wide range of birds and mammals, therefore contamination of meat originates from the gut during processing at the abattoir. Although the organisms are not able to grow during the post-processing period because of efficient chilling, they are able to survive (Phillips, 1998). At the abattoir there are many stages in the process such as slaughter, dressing and preparation of raw meat at which contamination can occur. The skins and intestinal contents of cattle may have high counts of pathogenic bacteria and intestinal contents may contaminate muscle during the gutting process (Skovgaard and Van Hoof, 1998). Sometimes, the contamination level can exceed $\log_{10} 7$ cfu/carcass (Phillips, 1998).

Contaminated poultry meat represents the most important source of human *Campylobacter spp.* infections. Surveys of poultry suggest that between 30% and 100% of all broilers on retail sale may be contaminated with *Campylobacter spp.* (Phillips, 1998). Ten to twenty per cent of poultry flocks are reported to be infected in some countries. Most carcasses of birds from infected flocks will be infected as the result of contamination with intestinal contents during slaughter (Skovgaard, 1996), therefore, cross-contamination has been shown to be an important way for the organism to enter the human food chain (Phillips, 1998). Other foods that have been documented to be

contaminated with *Campylobacter spp.* include raw seafood, fish and raw milk (Potter and others, 1983; Doyle and Schoeni, 1986). Even water can be a *Campylobacter spp.* source for humans and cattle (Blaser and others 1983; Hernandez and others, 1996).

It is not possible to completely eliminate *Campylobacter spp* from a cattle herd, therefore, intervention in cattle should focus on improved hygiene when delivering the cattle to the abattoir and during slaughter and dressing. The measures will be equally applicable to reducing the risk from other human pathogens as well as for improving the storage quality of the meat (Skovgaard, 1996).

1.7. *Mycobacterium avium subsp. paratuberculosis*

1.7.1. Microbiology of *Mycobacterium avium subsp. paratuberculosis*.

Mycobacterium avium subsp. paratuberculosis (MAP) belongs to the family *Mycobacteriaceae*. The main attributes of these bacteria are their slow growth rate and resistance to treatments with acid and alcoholic compounds, due to their strong cellular walls (Thorel and others, 1990). *Mycobacterium avium* forms a large group of closely related *Mycobacteria,* which can be subclassifed into *Mycobacterium avium subspecies avium, Mycobacterium avium subsp. silvaticum,* and *Mycobacterium avium subsp. paratuberculosis* (Thorel and others, 1990)

On microscopic examination, MAP is a small (0.5x1.5μm), Gram positive and acid fast aerobic bacillus that tends to form clumps. It has a thick, waxy cell wall that gives it its acid-fast staining properties and renders it more resistant to physical and chemical agents than many other species of bacteria. *Mycobacterium avium subsp. paratuberculosis* is extremely slow growing and may take several months to produce visible colonies in the laboratory. The long incubation times required to isolate MAP mean that culturing the organism from faeces or tissue samples requires a decontamination stage to remove the faster-growing species that would otherwise overgrow the culture medium (Hirsh and Chung Zee, 1999; Jones, 2001).

Like most bacteria, *Mycobacterium spp.* require iron for growth. Most mycobacterial species produce cell wall associated compounds called mycobactins that have a strong affinity for iron. As MAP does not produce mycobactin, the addition of mycobactin to culture media is essential. The inability of MAP to produce mycobactin suggests that the organism is unable to multiply outside the host, however, the organism remains viable for long periods in the environment, surviving for up to nine months in faeces and soil, but it is susceptible to sunlight and drying. Furthermore, the waxy cell wall renders the organism resistant to many disinfectants commonly used in agriculture. As a result, even if all infected cattle are removed from a herd, MAP is likely to remain viable in the environment for many months (Gracey and others, 1999; Jones, 2001).

1.7.2. *Mycobacterium avium subsp. paratuberculosis* in cattle

Paratuberculosis, also called Johne's Disease, is a chronic wasting disease of ruminants that was first described in cattle in 1895 by Johne and Frothingham (Johne and Frothingham, 1895). The authors demonstrated the presence of acid-fast bacilli in affected cattle and believed that the disease was an atypical form of tuberculosis. The causative organism was isolated by Twort in 1910, and was named *Mycobacterium enteriditis chronicae pseudotuberculosae bovie johne.* The disease was later known as paratuberculosis or Johne's Disease and it is generally accepted that *Mycobacterum avium subspecies paratuberculosis* (MAP) is the causal agent, although many of the details regarding the mechanisms of pathogenesis remain unknown (Johne and Frothingham, 1895; Jones, 2001).

Paratuberculosis is of principal economic importance in cattle, sheep and goats (Withers, 1959; Juste 1997; Jones, 2001; Royal Society, 2002). The net cost to farmers of subclinical infection with MAP in their cattle was estimated in Europe in 1987 to be £209 per infected dairy cow, mostly due to reduced milk production. Recent work in the US has estimated that paratuberculosis costs dairy farmers around $100 per cow in moderately infected herds, rising to over $200 per cow in heavily infected herds (Ott and others, 1987). In Spain, losses in sheep have been estimated to be 120 euros and 60 euros for clinical cases in dairy and meat sheep, respectively (Juste and others, 1999).

Infection with MAP has been reported in sheep, cattle, primates, other ruminant species including deer, South American camelids, bison, rabbit, foxes and stoats (Hirsh and Chung Zee, 1999; Jones 2001). Some breeds of cattle such as Jersey, Guernsey, Limousin and Shorthorn in Great Britain and Ayrshire cattle and Blackface sheep in Northern Ireland are commonly regarded as being particularly susceptible to MAP but there are no detailed studies to confirm this (Clarke, 1997; Gracey and others, 1999).

Johne's Disease is a worldwide disease, however, it is more common in certain countries, being enzootic in cattle in the UK, France, Denmark, Colombia, Ecuador, Mexico, Bangladesh, Australia and New Zealand (Gracey and others, 1999).

Infection takes place early in life, usually under one month of age in cattle. It is generally thought that cattle are infected via the faecal-oral route, although transplacental infection may also occur. Indeed, MAP has been recovered from the uterus and placenta of infected cows, however, classical lesions of paratuberculosis have not been recognised in infected foetuses (Rankin, 1961; Hirsh and Chung Zee, 1999;). *Mycobacterum avium subspecies paratuberculosis* has been also isolated from semen of infected bulls, nevertheless venereal transmission by artificial insemination or natural service is unlikely (Larsen and Kopecky, 1970).

Development of clinical disease often follows a period of stress such as calving, peak lactation, transportation to market or slaughterhouse, or even immunosuppression by agents such as bovine virus diarrhoea virus (Jones, 2001; Cranwell, 1997; Clarke, 1997).

Since Johne's disease has a long incubation period of not less than 2 years, it is almost always seen clinically in adult cattle (Jones, 2001; Cranwell, 1997). The long incubation period of the disease allows mycobacterial shedding in faeces by cattle for up to 18 months before clinical signs become apparent, but shedding is particularly high, up to $5x10^2$ MAP per day during clinical expression of the infection (Clarke, 1997).

Clinical signs include diarrhoea that tends to be intermittent at the beginning, accompanied by a slight weight loss and a slight decrease in milk yield. During these

early stages, cattle are generally bright and their appetite remains good. Gradually, the diarrhoea becomes persistent with a dramatic drop in milk yield, and a marked loss of skeletal muscle, especially from the hindquarters. As the disease progresses, affected cattle may develop submandibular oedema due to protein-losing enteropathy (Cranwell, 1997; Jones, 2001). An associated loss of coat pigmentation is also said to occur in a proportion of cases. Throughout the disease, the animal remains bright and continues to eat well (Cranwell, 1997; Gracey and others, 1999; Jones 2001). Decreased serum concentrations of calcium, total protein and albumin have been reported in both cattle and sheep with clinical Johne's Disease (Jones and others, 1996).

1.7.3. Epidemiology of *Mycobacterium avium subsp. Paratuberculosis* infection

Infected cattle with MAP have been recognised world-wide, however, the exact incidence of the disease is not well known because its non-specific clinical signs means that most farmers will note loss in production and may cull the animal without requesting further diagnosis (Chiodini and Van Kruiningen Merkal, 1984).

1.7.3.1. Subclinical Disease

Slaughterhouse surveys can provide information about the incidence of subclinical infection by examination of mesenteric lymph nodes, lengths of intestine and occasionally other tissues. Such surveys in the 1950s produced prevalences that varied from 5.8% to 17.0% with a mean of 11.2% of the total of 2,345 asymptomatic cattle using histological examination of the mesenteric lymph nodes. The infection was 15-20 times more common than was the clinical disease (Cranwell, 1997). Taylor and others in 1949 found 15% subclinically infected cattle in a group of 243 cattle. All those cattle were found to harbour the organism without showing the clinical signs associated with the disease. The diagnostic method in the mentioned study was histological examination and culture of the mesenteric lymph nodes was employed, using the method described by Coper and Ugei in 1930 (Taylor, 1949). Between 1952 and 1953, lymph nodes from 552 cattle were cultured by the method that Taylor described in 1949 and on 33

occasions *M. paratuberculosis* was recovered, giving a prevalence of 6 % (Rankin, 1954). Peck (1957) cultured mesenteric lymph nodes from knacker cattle and found an incidence of subclinical disease of 24 % in 1954. Cetinkaya and others (1996) studied 1553 cattle in three different slaughterhouses in the South-west of England. Polymerase chain reaction (PCR) based on IS900 was used to detect MAP in intestinal lymph nodes and positive samples were also cultured. The prevalence of subclinical disease in adult cattle was 3.5% by PCR and 2.6 % by culture (Cetinkaya and others, 1996). No differences were detected between different months, different abattoirs, or different groups of ages, and subclinical prevalence (Cetinkaya and others, 1996).

In other European countries such as Spain, the prevalence of subclinical disease in cattle was estimated to be 1% in adult cows in a slaughterhouse bacteriological survey, and 67% of herds using ELISA as a diagnostic technique (Juste and others, 1999).

1.7.3.2. Clinical Disease

Centinkaya and others developed a study based on telephone interviews with veterinary practitioners between September 1991 and January 1992 to assess the prevalence of Johne's Disease in herd in the South-west of England. The prevalence was 1.3 %; 28 of these had clinical disease during the five years from 1987 to 1991, giving an incidence of 1 % (Cetinkaya, and others, 1994). In dairy and beef herds the cumulative incidences were 1.7% and 2% respectively (Cetinkaya and others, 1994). The authors asked about methods of diagnosis and they did not find any significant difference between the incidence of disease measured by faecal culture, *post-mortem* examination or histology or using clinical signs and serological test as diagnostic procedures (Cetinkaya and others, 1994). In 3772 dairy farms in England, 17.4% of the farms had a case of Johne's Disease on at least one occasion between 1985 and 1994. The incidence was reported as 4.9% during this period but the incidence found in 1993 and 1994 was 1.5 and 1.3%, respectively. The highest prevalence was in the south of England. The incidence rate of clinical disease was estimated at 3/10,000 cow years in all herds in 1993 and 1994 (Cetinkaya and others, 1998). The differences between the surveys during this 50 year period may be due to differences in sample selection, changes in the breed structure of

the national herd, or due to improvements in husbandry and nutrition (Cetinkaya and others, 1994).

The incidence of clinical cases of Johne's Disease is always much lower than the overall incidence of infection, being less than 0.2%. In affected herds the incidence of clinical cases may be as high as 5%, due to the disease being sporadic and in affected herds usually just one or two cows showing clinical signs (Cranwell, 1997).

1.7.4. *Mycobacterium avium subsp. paratuberculosis* in humans

It has been recognized that MAP may play a role in the aetiology of Crohn's Disease in humans (Withers, 1959; Jones, 2001; Royal Society, 2002). The aetiology of this disease is still unclear: immunological factors, bacteria and viruses have been believed to be responsible for Crohn's Disease. The disease in human and animals are similar (see Table 1), and after isolation of MAP from some Crohn's patients, there has been speculation that Crohn's disease may also be linked to Johne's Disease (Hirsh and Chung Zee 1999; Jones 2001; Haining, 2001). The use of culture techniques to isolate MAP from patients with Crohn's Disease, however is problematic as a routine diagnostic method since some cultures take up to five years to produce visible colonies (Jones, 2001).

	Crohn's Disease	**Johne's Disease**
Preclinical Stage		
Symptoms and signs	Not known	Decreased milk yield
Incubation period	Not known	Minimum 6 months
Clinical Stage		
Presenting symptoms and signs	Chronic diarrhoea, abdominal pain, weight loss	Chronic diarrhoea, dull hair, weight loss, decrease in lactation
Gastrointestinal symptoms and signs		
Diarrhoea	Chronic	Chronic
Blood in stools	Rare	Rare
Vomiting	Rare	No
Abdominal pain	Yes	No evidence
Obstruction	Yes	No
Extraintestinal manifestation		
Polyarthritis	Yes	No
Uveitis	Yes	No
Skin lesions	Yes	No
Amyloidosis	Yes	No
Hepatic granulomatosis	Yes	Yes
Renal involvement	Yes	No
Clinical Course		
Remission and relapse	Yes	Yes

Table 1: Clinical features in Crohn's Disease and Johne's Disease (European Commission, 2000).

Crohn's Disease was first recognised by Dalziel in 1913 at the Western Infirmary in Glasgow. He described a condition observed in patients, which was a form of chronic enteritis very similar in some aspects to that of intestinal tuberculosis (Dalziel, 1913; Jones, 2001; Haining, 2001). In fact, the condition was recorded as intestinal tuberculosis until 1932 when Crohn and others described the disease as a chronic, low-grade inflammation of the terminal ileum with no visible acid-fast bacilli on histopathology (Haining, 2001).

Crohn's Disease is a chronic inflammatory bowel disease that primarily affects the ileum of young humans. Symptoms may include abdominal pain, diarrhoea or constipation and may mimic appendicitis or bowel obstruction. Patients with the disease may suffer from inflammation of the bowel over the long term, resulting in weakness and weight loss. It is a life-long disease and although periods of remission may occur, the disease is not curable and quality of life is always compromised (Hirsh and Chung Zee, 1999).

A European study has calculated an incidence rate for Crohn's Disease of 5.6 per 100,000 per year. It is estimated that over 200,000 people in the European Union (EU) are affected by this disease (Shivananda and others, 1996). It is believed that there were considerable local differences: Crohn's Disease is more prevalent in Western populations with Northern European and Anglo-Saxon ethnic derivations than in populations from Southern Europe, Asia and Africa. Nevertheless; when those people migrate to Western Europe, they become as susceptible to the disease as the population of the West European countries, suggesting environmental factors may play a role in the aetiology of Crohn's Disease (Jayanthi and others, 1992).

1.7.6. Laboratory tests for the diagnosis of *Mycobacterium avium subsp. paratuberculosis*

Many tests are available for diagnosis of paratuberculosis. These tests either identify the causal agent or measure the host's response to the organism. Most of the tests have reasonably high specificity but none of those available has high sensitivity (Jones, 2001; Haining, 2001). Detection of the infection of the host during the subclinical stage is

complicated by the fact that the bacterium does not multiply rapidly and, therefore, is difficult to detect in faeces. In addition, there is little or no immune response at this stage. Hence, no current control program is able to prevent further spread of the disease by detection of the disease in young cattle with the currently diagnostic available diagnostic techniques (Ridge and others, 1991).

The cell wall composition of MAP allows staining by heating with carbol fuschin and, following staining, decolourisation by acidified alcohol. This property is used to distinguish *Mycobacterium spp.* from other organisms and Ziehl-Neelsen is the stain used to examine faeces samples (Jones, 2001; Haining 2001). Clumps of acid-fast organisms in faecal smears are significant in confirming MAP since it has not been reported in the faeces of cattle other than clinical cases of Johne's Disease. Unfortunately MAP are only found in third of clinical cases which means that faecal smears are of limited value to the clinician (Cranwell, 1997; Jones, 2001).

A more sensitive method of identifying the organism is culturing faecal samples but the culture techniques require specialised media such Merckel's egg yolk medium and Dorset's glycerol egg medium that also require addition of growth promoting mycobactins. The optimum temperature for growth is 38-39°C. Colonies require a minimum of 4-6 weeks to appear and cultures should not be discarded as negative until they have been incubated for at least 3 months (Cranwell, 1997; De Lisle and others, 1980; Jones, 2001). An alternative to solid medium is the radiomimetic medium BACTEC. This medium is a liquid broth containing egg yolk with vancomycin, amphotericin B, nalidixic acid and Mycobactin J. The indicator of growth is the release of $^{14}CO_2$ from metabolised radiolabelled substrate. The advantage of this method is that mycobacteria can be detected within seven weeks of inoculation (Sockett and others, 1992; Cranwell, 1997; Jones, 2001).

Genetic techniques are also indicated in MAP diagnostics and PCR is the technique indicated. It is based on the identification of an insertion sequence (IS900) that is specific to MAP. These techniques have proved to be extremely useful for identifying MAP in tissue samples but the reaction is inhibited by faeces, hence, the technique

cannot be used to identify the organism in faecal samples without purifying the mycobacterial DNA (Jones, 2001; Cranwell, 1997).

Alternatively, detection of the immune response to MAP is used to diagnose the disease. Detection of cell-mediated reactions, complement fixation, detection of serum MAP antibodies or Agar-gel-immuno-diffusion test (AGID) are diagnostic techniques that have been used. Cell-mediated immune reactions can be detected *in vivo* using intradermal or intravenous inoculation, or *in vitro* using a leukocyte migration agarose test, and lymphocyte transformation tests. Both *in vitro* tests are of little practical value for diagnosis or detection of preclinical cases (Cranwell, 1997).

The intradermal test consists of an inoculation of Antigen Johnin Purified Protein Derivative (PPD). This antigen is administered, and in a sensitised animal, a localised cellular response occurs with an increase in skin thickness at the infection site, with the test being read 72 hours after inoculation. A high number of infected cattle react to the test but more than 50% of negative cattle also react. This test is useful for a herd control programme but has limited value at the individual animal level (Jones, 2001; Cranwell, 1997). If johnin PPD is inoculated by intravenous injection, positive cattle increase their rectal temperature by 0.83°C within 6 hours after the injection (Jones, 2001; Cranwell, 1997).

Between 70 and 90% of clinical cases of Johne's Disease have a positive complement fixation test, but in herds where clinical cases occur, 30-50% of normal cattle also have a positive reaction. It also has been shown that a proportion of the false positive complement fixation tests are due to cross-reactions as a result of infection with *Corynebacterium renale*, resulting in the test having limited value for diagnosis of MAP (Cranwell, 1997; Jones, 2001).

The AGID test is based on the detection of antibodies against MAP. It has a relatively low sensitivity in the early stages of the disease, but becomes more useful in later stages. Its sensitivity is lower than the ELISA test and is closely related to the immunopathological forms of the infection. Cattle with non-clinical infection such as

focal and tuberculoid forms are usually negative to AGID, however, those cattle with multibacillary forms are often positive (Sherman and others, 1985).

The absorbed ELISA test is sensitive and specific for serum antibody to MAP. Sera are absorbed during dilution with a buffer containing soluble *M. phlei* antigens to reduce non-specific reactions. The specificity is 99.8% and sensitivity in clinical cases is 88.3% in clinical and 48.8% in subclinical cases (Cranwell, 1997; Jones, 2001).

1.7.8. Mycobacterium avium subsp. paratuberculosis in meat inspection and animal products

Relatively little is known about the incidence of MAP and its survival characteristics in dairy processes. There is some evidence that the organism might survive the pasteurisation process applied to milk. However, the potential for its presence in raw milk and its survival in dairy products exists, especially those products made from unpasteurised milk (Millar and others, 1996; Holsinger and others, 1997; Grant and others, 2000). In a 1996 survey of retail pasteurised milk where milk was heated at 72°C for 15 seconds, in England and Wales, the organism was detected in 7% of samples using the IS900-PCR method. Studies of heat resistance of MAP have yielded mixed results. Some studies have suggested that the organism may survive pasteurisation treatment if present in sufficiently high numbers (Millar and others, 1996), while other studies show that fast growing atypical mycobacteria such *M. borstelense, M diernhoferi or M fortuitum* are reliably destroyed by short time pasteurisation of raw milk at 71-74 °C of raw milk (Schliesser and Weber, 1972). The effectiveness of pasteurisation in killing bacteria and in preventing Crohn's Disease continues to be widely studied and there is still debate on the effectiveness of pasteurisation on *M. avium subs. paratuberculosis* bacteria (Schliesser and Weber, 1972; Millar and others, 1996 and Grant and others, 2000).

Johne's Disease is not a condition that specifically renders cattle unfit for human consumption under current UK legislation. However, emaciation, oedema or advanced

anaemia that can be manifested in cattle with advanced disease which do lead to condemnation of the carcass (MHS Regulation, 1995; Gracey and others, 1999).

In the UK, only cattle below the age of 30 months may currently enter the food chain, restricting the number of clinical cases of Johne's Disease passing through mainstream abattoirs, as the mean age for peak clinical disease is between 2 and 5 years (MHS Regulation, 1995). Nevertheless, Cetinkaya and others reported that 2% of young cattle, although the exact ages were not specified, were PCR-IS900 positive in intestinal lymph nodes (Cetinkaya and others, 1996).

1.8. Aim of the project

The aim of this project was to determine the influence of coat dirtiness on carcass contamination and to assess how different *ante mortem* features may influence the cleanliness of cattle and carcass contamination. Carcass contamination was assessed using TVC and *Enterobacteriaceae* counts as methods indicated as appropriate by MHS. The association between carcass contamination and prevalence of potential zoonotic pathogens isolated from the cattle such as *Campylobacter spp.* and *Mycobacterium avium subsp. paratuberculosis* was investigated. Associations between the MHS scoring system CLP, bacteria on carcasses, and the prevalence of *Campylobacter spp.* and *Mycobacterium avium subsp. paratuberculosis* in cattle was quantified.

2. Materials and Methods.

2.1. Abattoir

The study was conducted in an Over Thirty Months Scheme (OTMS) abattoir in Kilmarnock, Ayrshire, Scotland. Facilities at the abattoir include a powered overhead rail dressing system, with an upward-pulling head to tail and hide-stripping machine. The abattoir had a throughput of up to 70 carcasses per hour and there were four meat inspectors, one Official Veterinary Surgeon (OVS) and 25 slaughter-men. This abattoir functioned under the OTMS Meat Hygiene Service (MHS) regulations and the CLP policy was not implemented. These facts allowed a large proportion of dirty cattle, with CLP categories score 3, 4 and 5 to be slaughtered, and, in contrast to 'clean' abattoirs, therefore provides a unique opportunity to obtain samples from dirty cattle.

At the time of writing the vertebral column, including dorsal root ganglia, are considered specific risk material (SRM) under the Specified Risk Material Amendment (Scotland) Regulations 2000. Under these conditions, the whole carcass is considered as not fit for human consumption and is, therefore, disposed as SRM.

The Hygiene Assessment System (HAS) score at this abattoir during the sampling period was 66.

2.2. Animal selection and clinical inspection

Ninety-three cattle from different origins all around the country were examined in the lairage prior to the stunning point on five different days in a period from 21st January 2003 until 25th February 2003. Cattle were selected following the CLP system-score. Cattle with CLP scores of 3, 4 and 5 were all selected, and cattle with CLP scores of 1 and 2 were selected using a random number table, where the numbers occur, on average, the same number of times but with no discernible pattern. A questionnaire based on clinical examination (Appendix 1) was completed and each animal was marked with a red number on the left rump using a cattle labelling gum. To calculate the proportion of

the coat surface that was dirty, the coat was divided in to eight different areas; front leg, back leg, flank low, flank high, rump, tail and udder, and the underside. Dirty areas were marked with a pen on the bovine diagram included in the questionnaire (Appendix 1).

2.3. Carcass identification

Selected carcasses with a red number on the flank were followed from the lairage to the slaughter hall up to the point of dressing. At this stage of the line, the carcass was given a line number that was stuck on the right flank of the carcass. At the evisceration point the rumen was also identified by line number to allow the sampler in the gut room to identify the relevant carcass. The correlation between red market number and line number was maintained throughout the process.

2.4. Sampling procedures

2.4.1. Lymph nodes and faeces sample

In the gut room, one whole ileocaecocolic lymph node and a faecal sample were taken from the gastrointestinal tract of each marked animal. This lymph node was located by finding the caecum, a blind-ended pouch at the junction of the small and large intestines, situated below the ileocaecal valve. Approximately 3 grams of faeces were taken by gloved hand directly from the rectum. Labelled screw-top bottles were used to store the samples from each animal. The operator changed latex gloves between each carcass to avoid cross-contamination of samples.

2.4.2. Microbiological assessment of carcasses.

The right side of the carcass was sampled at the flank immediately before it was weighed (see Figure 1).

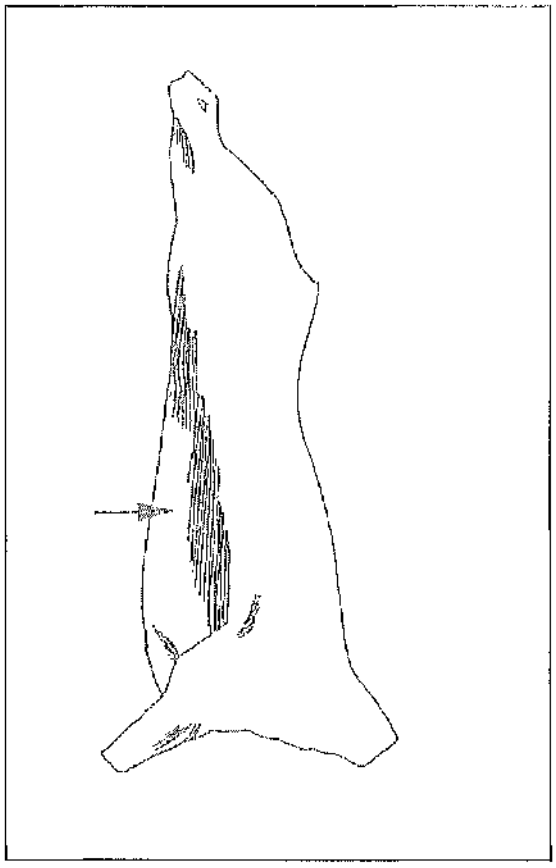


Figure 1: Illustration of the area that was swabbed. The arrow indicates the point where the template was placed.

For microbiological assessment, the wet and dry swab technique described in MHS Operations Manual, was used (MHS, 1995). This method consists of a combination of two swabs, one dry and the other moistened by a sterile peptone salt diluent that contains 1.0 g of enzymatic digest of casein, 8.5 g sodium chloride and 1,000 ml water (ISO

1999). An area of 100 cm^2 marked with a sterile template was swabbed with sterile cotton-tipped wooden swabs. The carcass was swabbed first with the moist swab and then with the dry one, and the two swabs were each then broken off into a single, labelled sterile screw-capped universal bottle containing 9 ml of the sterile peptone salt diluent. To reduce the error introduced by different individual sampling techniques, all swab samples were taken by the same person.

2.4.3. Transportation and maintenance of samples

All samples were transported to the laboratory on ice in an insulated container, stored at 4°C and examined within 24 hours.

2.5. Laboratory procedures

2.5.1. Total Viable Count and count of *Enterobacteriaceae*

In the laboratory the bottle with both swabs was shaken vigorously for 60 seconds on a vortex mixer. A five decimal dilution series was then prepared with a calibrated automatic pipette. A 0.01 ml volume of each dilution was spread on to the surface of a quadrant of a petri dish containing microbiological growth medium, using a calibrated automatic pipette. All of the inoculations were made in duplicate. Plate count agar (PCA; Lab M) incubated at 30°C for 48 hours, was used to enumerate the total viable count (TVC) and violet red bile glucose agar (VRBGA; Lab M) incubated at 37°C for 24 hours, was used to enumerate the *Enterobacteriaceae.*

Colony forming units (cfu) and dilutions were stored in a Microsoft Excel file. The highest value of cfu per cm^2 of carcass was transformed to $\log_{10}$ values. To avoid negative values the value one was added to the total cfu, therefore, the limit of detection was 1 ($\log_{10}$ (0.00+1)).

2.5.2. Demonstration of *Mycobacterium avium subsp. paratuberculosis*

The ileocaecocolic lymph node was cut through with a sterile scalpel and two impression smears were made on a labelled slide. A direct faecal smear, of approximately 2 cm^2 in area was made, with a previously flamed loop, onto another labelled slide.

The Ziehl Neelsen staining method was performed using hot carbol fuschsin for 5 minutes, followed by differentiation for 20 minutes under 3% HCl and finally counterstained for 30 seconds with Loeffler's methylene blue. The whole slide was examined systematically by microscopy from left to right, moving down the slide until a positive field was found.

Results were recorded as positive when clumps of acid-fast bacilli were detected, or negative if there were no acid-fast bacilli or where they were not in clumps. The results were stored in a Microsoft Excel file.

2.5.3. Demonstration of *Campylobacter spp.*

Campylobacter spp. were isolated on plates of blood agar base No.2 (Oxoid ® Code CM271), an improved blood agar base for the cultivation of *Campylobacter spp.* This medium has 15 g/litre of protease peptone, 2.5 g/litre liver digest, 5 g/l of yeast extract, 5 g/litre of sodium chloride and 12 g/litre of Agar, in a pH of to 7.4±0.2. Skirrow supplement (Oxoid® Code SR069E) containing vancomycin, polymyxin B and trimethoprim was added to this medium. In 27 samples, modified Butzler (Oxoid®. Code SR0214E) supplement containing amphotericin B, colistin, cefoperazone and rifampicin was also used as a supplement to avoid fungal and *Pseudomonas spp.* overgrowth, which is a common problem when culturing ruminant or equine faecal samples.

Two plates were used; one was inoculated with 0.2 ml from the initial water peptone diluent using a calibrated pipette and spread across the whole medium surface with a L-

shaped sterile spreader made from a Pasteur pipette. The second plate was inoculated with faeces using a previously flamed loop by streaking on the medium directly, so the bacterial colonies are separated. Both plates were incubated in a microaerophilic environment (5% O_2: 10% CO_2 atmosphere) at 37 °C for 48 hours. Not all Campylobacter colonies isolated with this medium are responsible for food borne disease although some colonies cultured might be identified as *C. jejuni, C. coli, C. hyointestinalis* and other campylobacters such as *C. fetus.*

Gram stain was necessary to confirm that the organisms isolated were indeed *Campylobacter spp.* Data on the campylobacters isolated were stored in a Microsoft Excel file.

2.6. Data analysis

A number of different outcome variables for isolation of *Enterobacteriaceae* and for TVC were considered for statistical analysis. First, the $\log_{10}$ counts were considered as a continuous variable, second using categorical classification (contamination/no contamination), and third, an altered classification rule defining values of less than 0.8 $\log_{10}$ cfu/cm^2 as an acceptable level for microbiological contamination on carcasses, thus giving the categorical outcome pass/fail. Explanatory variables included CLP score, breed, body condition score (BCS), diarrhoea, lameness, length of the coat, coat moisture, type of contamination, dirty underside, number of dirty areas, presence of MAP in faeces, presence of MAP in lymph nodes, and presence of *Campylobacter spp.* in faeces.

For analysis of the continuous outcome variables with categorical explanatory variables, ANOVA or Kruskal-Wallis, techniques were used as appropriate, with the exception of when there were only two categories when parametric and non-parametric two-sample tests were used. For the categorical outcome variables, in general, chi-square analysis was used, collapsing categories where biologically appropriate, to deal with sparse data. For all two-by-two contingency tables, Fishers Exact Test was used.

Two different regression models were used, for categorical outcome variables such as: infected, or not, and for CLP a binary/ordinal logistic regression was used and, secondly, a normal linear regression model (stepwise) for when counts of bacterial contamination were used as the outcome variable of interest. Stepwise regression was used for model selection.

All tests were two tailed and significance was set at the 5% level.

The computer software used was Minitab® for Windows.

The different types of explanatory variables are shown in Table 2.

VARIABLE	TYPE OF VARIABLE
Clean Livestock Policy	Categorical
Body condition score	Categorical
Diarrhoea	Binary
Lameness	Categorical
Mastitis	Binary
Number of clipped areas	Categorical
Coat moisture	Categorical
Coat contamination	Categorical
Number of dirty areas	Categorical
Dirty underside	Binary

Table 2: Classification of explanatory variables.

Finally, classification trees were used to find a useful discriminatory model for contamination. This method picks out the single best variable and then performs a binary split using the variable that gave the fewest misclassifications for the data (Angote and others, 1996). This process was repeated in order to reduce the misclassification rate and the final results are presented graphically.

3. Results

3.1. General analysis relating outcome to the different explanatory variables

The raw data for TVC and *Enterobacteriaceae* for all explanatory variables for the cattle included in the study are shown as cfu/cm^2 in Appendix 2. The summary of the results of the statistical analyses investigating the relationships between each explanatory variable and the different outcome variables is shown in Table 3.

Explanatory variable	*Enterobacteriaceae* on carcasses		TVC on carcasses	
	Cont.	Cat.	Cont.	Cat.
CLP	P= 0.05	ns	P=0.04	P=0.01
Breed	ns	ns	ns	ns
BCS	ns	ns	ns	ns
Diarrhoea	ns	ns	ns	ns
Lameness	ns	ns	ns	ns
Mastitis	ns	ns	ns	ns
Coat length	P=0.02	P=0.02	P=0.05	ns
Coat moisture	ns	P=0.05	P=0.05	ns
Type of contamination	ns	ns	P=0.02	P=0.01
Dirty underside	ns	ns	ns	P=0.01
No. of dirty areas	ns	ns	ns	P=0.01
MAP in Lymph node	ns	ns	ns	ns
MAP in faeces	ns	ns	ns	ns
Campylobacter spp. in faeces	ns	ns	ns	ns

Table 3: Explanatory variables for carcass contamination with *Enterobacteriaceae* and TVC. Key: (Cont.) Continuous outcome variable; (Cat.) Categorical outcome variable; (CLP) Clean livestock policy; (BCS) Body condition score; (MAP) *Mycobacterium avium subsp. paratuberculosis*; ns=P>0.05.

The individual results are considered in more detail in later sections.

The cattle studied consisted of 11 different breeds, and the predominant breeds were Holstein Friesan, 31.2%, Aberdeen Angus, 17.2%, Hereford, 15% and Limousin, 14%. The other breeds together accounted for less than 10% of the cattle studied.

Statistical analysis showed that breed, BCS, presence of diarrhoea, lameness, mastitis and presence of MAP in lymph node and faeces or *Campylobacter spp.* in faeces were not significantly related to carcass contamination measured as either *Enterobacteriaceae* or TVC (Table 3).

The presence or absence of diarrhoea, lameness and mastitis were studied as categorical variables and the results are show in Tables 4.

	Yes	No
Diarrhoea present	13.0%	87.0%
Lameness present	10.7%	89.3%
Mastitis present	14.0 %	86.0%

Table 4: Proportion of cattle with diarrhoea, lameness and mastitis of 93 cattle studied.

Of the 93 cattle examined in the lairage, 51.6% cattle were found to be "clean" with CLP scores of 1 and 2 while 48.4% were classified as "dirty" with CLP scores of 3, 4 or 5 following MHS criteria for cleanliness in cattle. The proportions for each of the CLP scores are shown in Table 5.

CLP (MHS score system)	%
1	24.7
2	26.9
3	24.7
4	18.3
5	5.4

Table 5: Proportion of each CLP score of 93 cattle studied.

The microbiological effects of a dirty coat, as measured by the CLP scoring system, on carcass contamination with *Enterobacteriaceae* and TVC are shown in Table 6.

	Enterobacteriaceae		TVC	
CLP	Mean $\log_{10}$ cfu/cm^2	Range	Mean $\log_{10}$ cfu/cm^2	Range
1	0.07	0-0.72	0.56	0-2.13
2	0.11	0-0.74	0.64	0-1.37
3	0.14	0-1.66	0.86	0-2.65
4	0.40	0-1.96	1.40	0-2.65
5	0.76	0-1.96	1.62	0-5.43

Table 6: Mean and range $\log_{10}$ *Enterobacteriaceae* cfu/cm^2 and TVC cfu/cm^2 from 93 cattle with different CLP scores.

In the case of *Enterobacteriaceae,* the mean $\log_{10}$ cfu/cm^2 for each carcass ranged from 0 to 1.96 $\log_{10}$ cfu/cm^2. In case of TVC, the mean cfu/cm^2 ranged between 0 and 5.43 $\log_{10}$ cfu/cm^2. In both TVC and *Enterobacteriaceae*, the highest mean $\log_{10}$ cfu/cm^2 was in cattle with CLP score of 5 and the lowest in cattle with a CLP score of 1, and with both measurements; TVC and *Enterobacteriaceae*, the mean $\log_{10}$ cfu/cm^2 increased as the CLP score increased. These results are summarised in Figure 2.

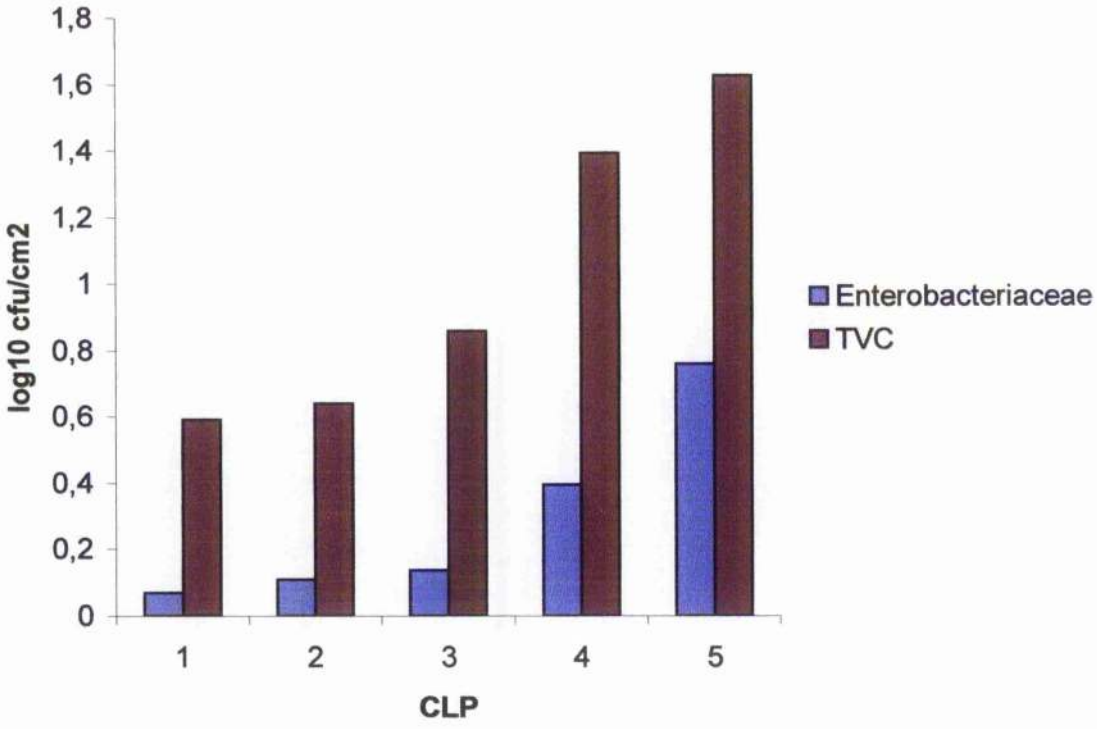


Figure 2: Mean log cfu/cm^2 of *Enterobacteriaceae* and TVC for each different CLP score of 93 cattle studied.

Kruskal-Wallis comparison of the 5 categories revealed borderline significant differences in mean $\log_{10}$ cfu/cm^2 (P=0.05) when considered for *Enterobacteriaceae,* and significant differences in mean $\log_{10}$ cfu/cm^2 (P=0.04) when considered for TVC. In the case of TVC, there were also significant differences (P=0.01) using TVC cfu/cm^2 as a categorical variable defined as "clean cattle" (CLP score of 1 and 2) or "dirty cattle" (CLP score of 3, 4 and 5), following MHS criteria. This difference was not found for *Enterobacteriaceae* using the same methods.

The majority of the cattle (84.9%) had short coats and only a small proportion had medium or long coat. The proportion of cattle with the different categories of coat length are shown in Table 7.

Length of cows coat	%
Short	84.9
Medium length	11.8
Long	3.3

Table 7: Proportion of cattle with the indicated coat length of 93 cattle studied.

One-way ANOVA showed that there was a statistically significant difference between mean $\log_{10}$ cfu/cm^2 for *Enterobacteriaceae* (P= 0.02) and mean $\log_{10}$ cfu/cm^2 for TVC on carcass (P=0.05), for each category of coat length (Figure 3).

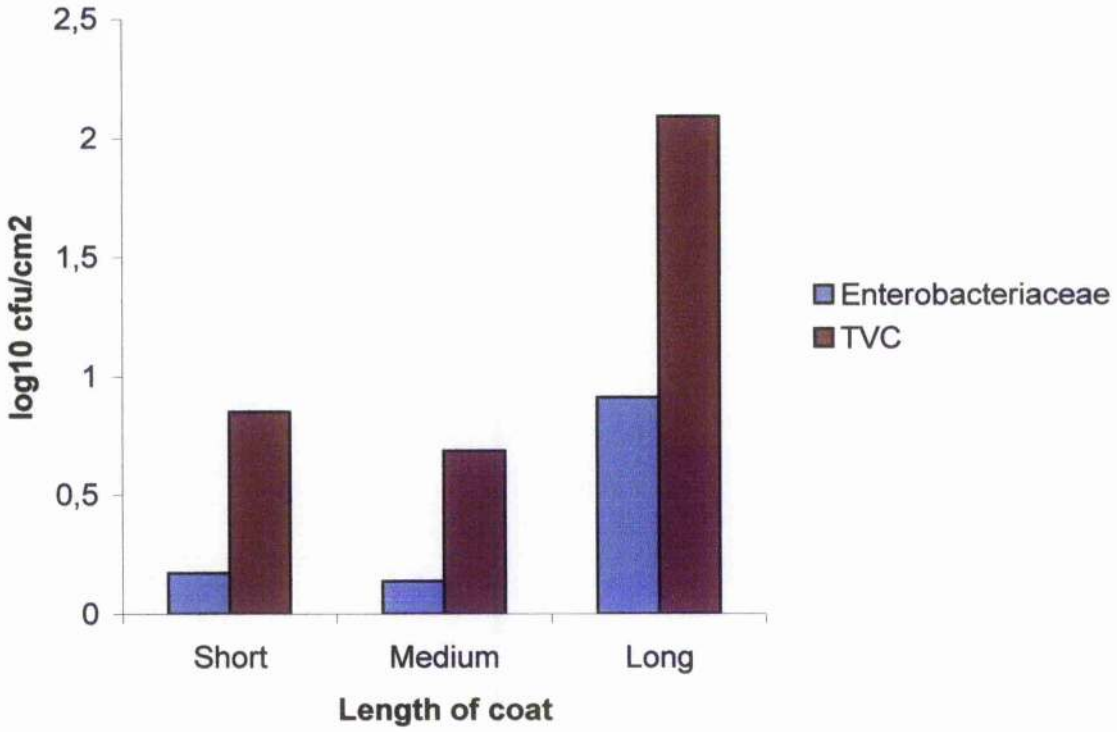


Figure 3: Mean of $\log_{10}$ cfu/cm^2 of *Enterobacteriaceae* and TVC and different categories of coat length of 93 cattle studied.

Cattle with longer coats were likely to have more high level of *Enterobacteriaceae* (P=0.02) or high TVC values (P=0.05) than cattle with short coats. Cattle with short or medium length coats are more likely to have no contamination at all than cattle with

long coats (P=0.01). The microbiological effects of length of coat on carcass contamination with *Enterobacteriaceae* and TVC are shown in Table 8.

	Enterobacteriaceae		TVC	
Length	Mean $\log_{10}$ cfu/cm^2	Range	Mean $\log_{10}$ cfu/cm^2	Range
Short	0.17	0-1.96	0.85	0-5.43
Medium	0.14	0-1.16	0.69	0-2.43
Long	0.91	0.72-1.28	2.09	1.66-2.65

Table 8: Mean and range $\log_{10}$ cfu/cm^2 *Enterobacteriaceae* and TVC of 93 cattle with different categories of coat length.

Most of the cattle had a dry coat and only a few had damp, or wet, coats. The proportion of cattle with different categories of coat moisture are shown in Table 9.

Coat moisture	%
Dry	77.4
Damp	16.1
Wet	6.5

Table 9: Proportion of cattle with different categories of coat moisture of 93 cattle studied.

Carcasses from cattle with dry or damp coats were less likely to be contaminated with *Enterobacteriaceae* with a borderline statistic significance (P=0.05), and also to have lower TVC values (P=0.05), than carcasses from cattle with wet coats. No significant differences in carcass contamination were found between the group of cattle with dry and damp coats. The microbiological effects of coat moisture on carcass contamination with *Enterobacteriaceae* and TVC are shown in Table 10 and in Figure 4.

Coat moisture	*Enterobacteriaceae*		TVC	
	Mean $\log_{10}$ cfu/cm^2	Range	Mean $\log_{10}$ cfu/cm^2	Range
Dry	0.15	0-1.96	0.81	0-2.65
Damp	0.27	0-1.66	0.86	0-1.96
Wet	0.48	0-1.96	1.64	0-5.43

Table 10: Mean and range $\log_{10}$ cfu/cm^2 *Enterobacteriaceae* and TVC of 93 cattle with different categories of coat moisture.

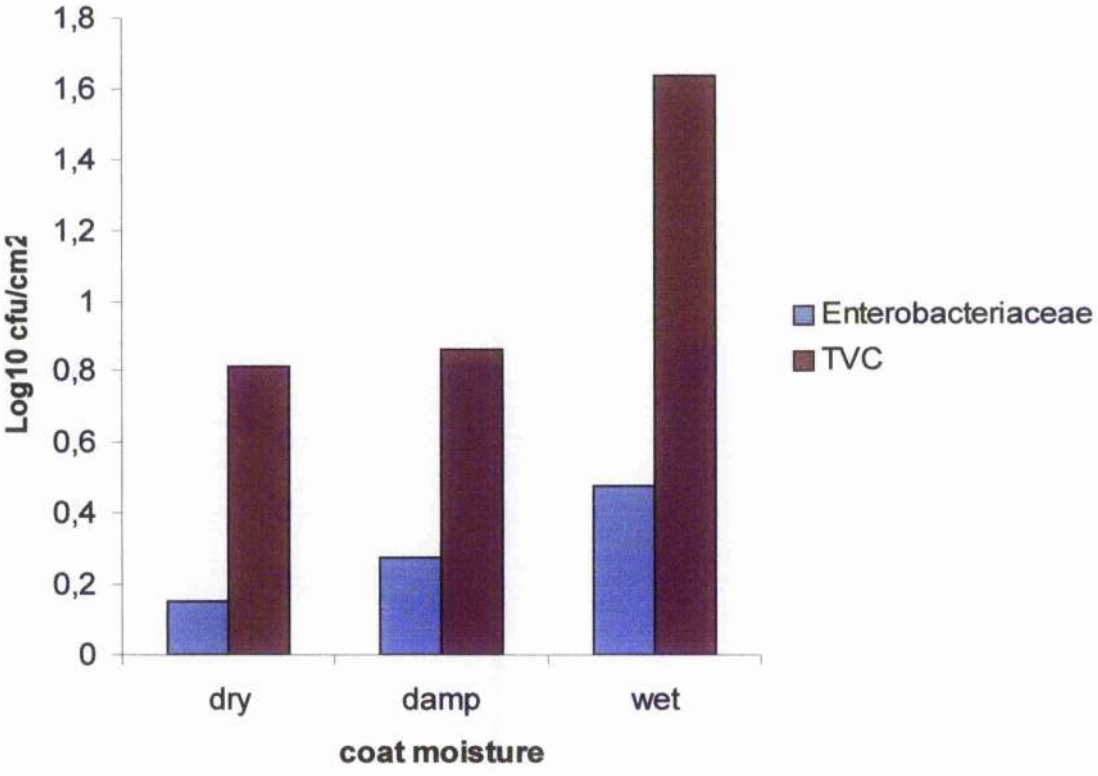


Figure 4: Mean of $\log_{10}$ cfu/cm^2 of *Enterobacteriaceae* and TVC for different categories of coat moisture of 93 cattle studied.

Only a small proportion, 18.3 %, of cattle had no coat contamination at all, with the remainder, 64.5 %, having either one of, or a mixture of, faeces, urine and bedding on their coats. The proportion of cattle with different types of coat contamination is shown in Table 11.

Type of contamination	%
None	18.3
Faeces	26.9
Bedding	4.3
Mixed	33.3
Matted	17.2

Table 11: Proportion of 93 cattle with different types of coat contamination.

ANOVA analysis showed that the type of contamination was associated with the TVC values on carcasses. Cattle with coats contaminated with only faeces had lower carcass TVC values than cattle with coats contaminated with bedding, and which in turn had lower TVC counts on their carcasses than those with a mixed contamination on their coats (P=0.02). The highest TVC values were found in cattle that had matted coats (Table 12).

	Enterobacteriaceae		TVC	
Type of contamination	Mean $\log_{10}$ cfu/cm^2	Range	Mean $\log_{10}$ cfu/cm^2	Range
None	0.04	0-0.16	0.55	0-2.13
Faeces	0.14	0-1.96	0.70	0-5.43
Bedding	0.18	0-0.72	0.87	0-1.66
Mixed	0.21	0-1.66	1.01	0-2.05
Matted	0.40	0-1.96	1.21	0-3.13

Table 12: Mean and range $\log_{10}$ cfu/cm^2 *Enterobacteriaceae* and TVC of 93 cattle with different types of coat contamination.

The type of coat contamination was also significant (P=0.01) on carcass contamination when TVC was used as a categorical variable. Significant associations were not found with *Enterobacteriaceae* either as a continuous, or as a categorical, variable. The effect of the type of contamination on carcass contamination are shown in Figure 5.

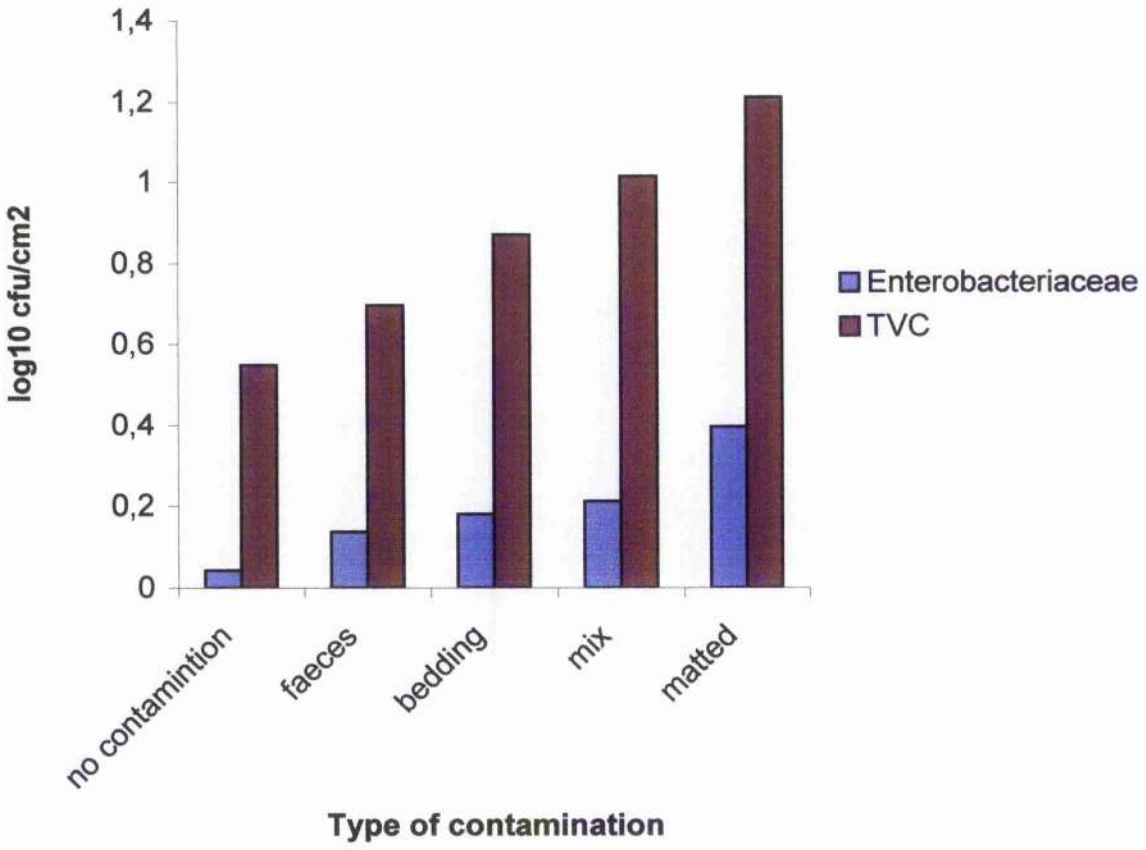


Figure 5: Mean of $\log_{10}$ cfu/cm^2 of *Enterobacteriaceae* and TVC for different types of coat contamination of 93 cattle studied.

Two thirds of the cattle had a dirty underside, the area where the hide is cut for removal. The proportion of cattle with dirty underside is shown in Table 13.

Dirty underside	%
No	33.3
Yes	66.7

Table 13: Proportion of 93 cattle with dirty undersides of 93 cattle studied.

Chi-squared test showed that cattle with dirty undersides are more likely to have contiamination with value of TVC (P=0.01) than cattle that had no dirt in this area. This association was not present in the case of contamination with *Enterobacteriaceae*. The number of dirty areas associated with contamination with TVC were also significant (P=0.01) using chi-squared test.

3.2. Altered classification statistical analysis

A certain level of microbial contamination is unavoidable during the slaugter process The MHS acept carcasses with minimal levels of bacterial contamination present rather than none at all, and this level of contamination is considered a parameter of process hygiene and performance in the abattoir. In this study the quantity of microorganisms at which the carcass was considered as contaminated were greater than 0.8 $\log_{10}$ cfu/cm^2, and less than this level was considered as uncontaminated This value was an arbitrary figure selected for this study, and was not related either to legislation or previous published studies. The level was selected due to the fact that only one sampling site was used rather than all four sites as indicated in the legislation. It should also be acknowledged that the same threshold value was used in this study for TVC and Enterobacteriaceae, however, in the legislation two different acceptable levels are provided for TVC and Enterobacteriaceae.

The summary of statistical results obtained with this altered classification using the new level of contamination/no contamination with the two outcome variables, *Enterobacteriaceae* and TVC as categorical variables is shown in Table 14.

	Enterobacteriaceae on carcasses	TVC on carcasses
CLP	P=0.02	ns
BCS	ns	ns
Diarrhoea	ns	ns
Lameness	ns	ns
Mastitis	ns	ns
Coat length	ns	ns
Coat moisture	ns	ns
Type of contamination	ns	ns
No. of dirty areas	ns	ns
Dirty underside	ns	ns

Table 14: Explanatory variables for carcass contamination with *Enterobacteriaceae* and TVC with altered classification.

With this altered classification there were no variables associated with TVC and only CLP was found to be associated with contamination by *Enterobacteriaceae* (P=0.02).

3.3. Classification trees

Each animal was classified as infected or not infected based on sequential splitting of relevant explanatory variables in turn. Three different decision trees were designed, two for each one of the outcome variables and one for the CLP.

The variables used in the original classification tree with the outcome variable *Enterobacteriaceae* were CLP, diarrhoea, number of dirty areas, length of coat, BCS, dirty underside, number of clipped areas and type of contamination. Using a cross validation method to reduce the complexity and over-fitting of the data, the results suggested fitting the classification tree using four nodes (Figure 6).

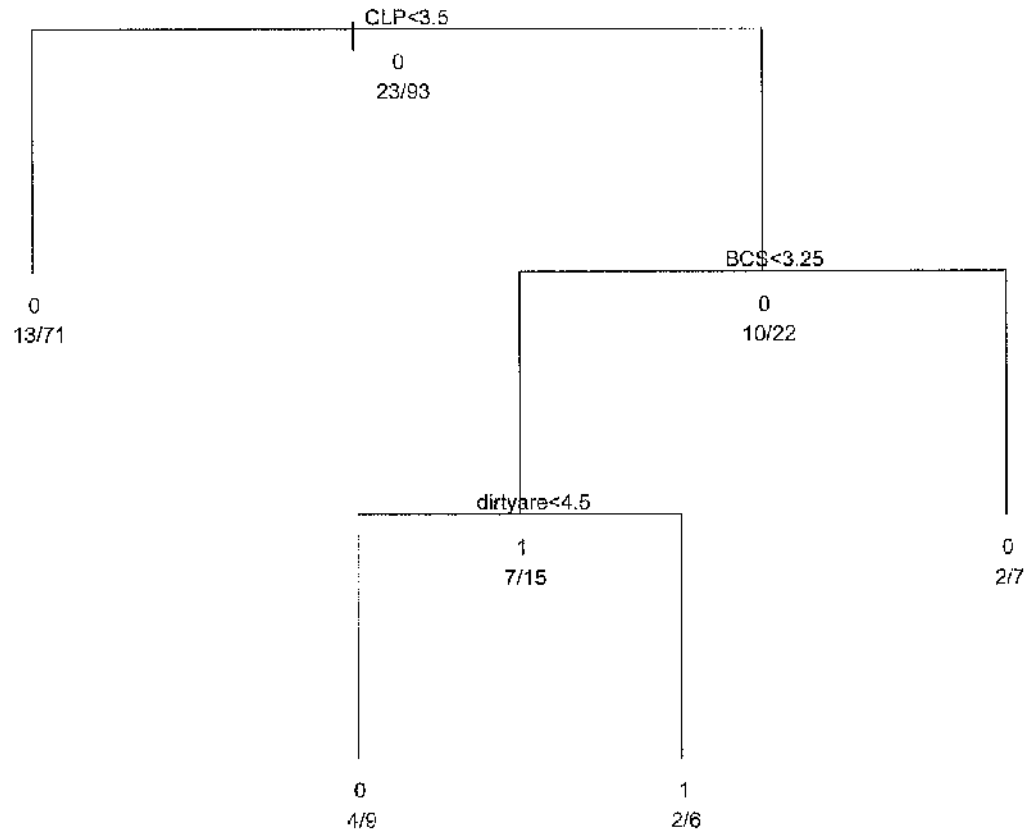


Figure 6: Pruned decision tree based on the outcome variable *Enterobacteriaceae*.
Key: Clean livestock policy (CLP); Body condition score (BCS); Number of dirty areas (Dirtyare).

This pruned tree used the variables CLP, BCS and the number of dirty areas with a misclassification rate of 21/93 (22.6%). For the same misclassification rate, therefore, there were fewer variables to deal with than in the original decision tree.

The variables used in the second classification tree with the outcome variable TVC were number of dirty areas, BCS and CLP. There were 12 nodes in the original classification tree and a misclassification rate of 19/93 (20.4%). Using cross validation five nodes were identified (Figure 7).

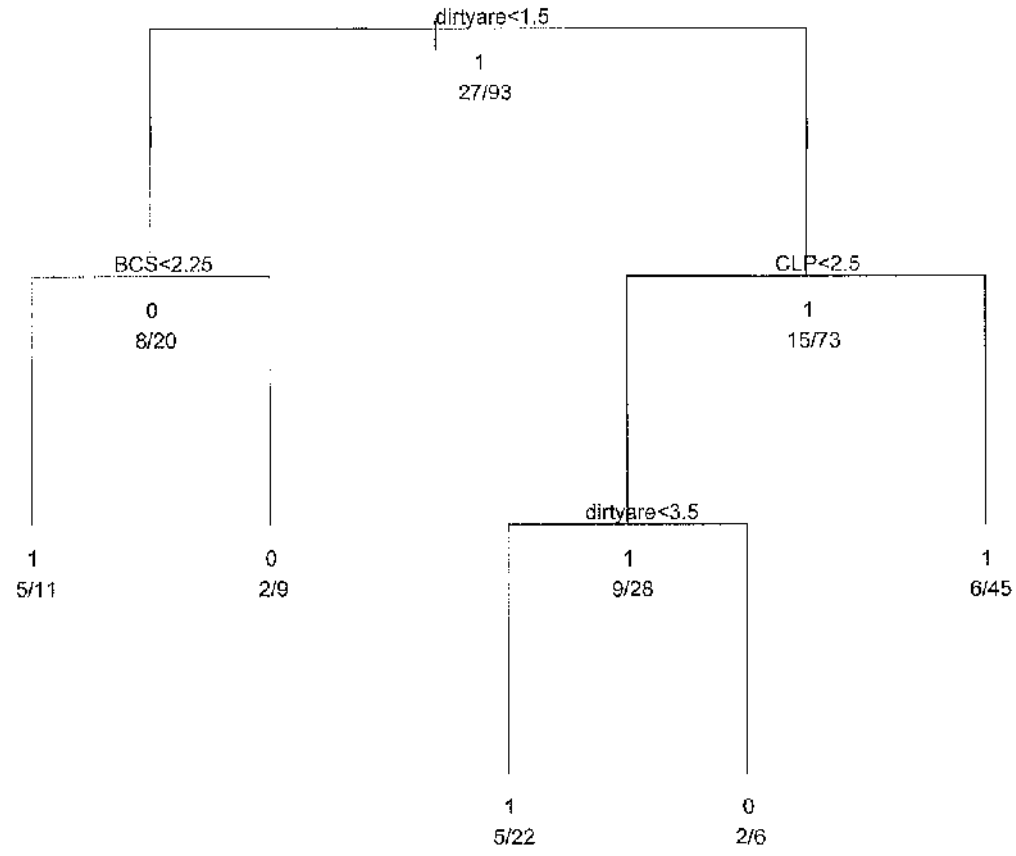


Figure 7: Pruned decision tree based in the outcome variable TVC. Key: Number of dirty areas (dirtyare); Clean livestock policy (CLP); Body condition score (BCS).

This pruned tree used the variables number of dirty areas, BCS and CLP with a misclassification rate of 20/93 (21.5%) which was higher than the original misclassification value.

Finally, the last decision tree was designed based on the actual CLP. The variables used in the original classification tree were type of contamination, number of dirty areas, BCS and coat moisture (Figure 8).

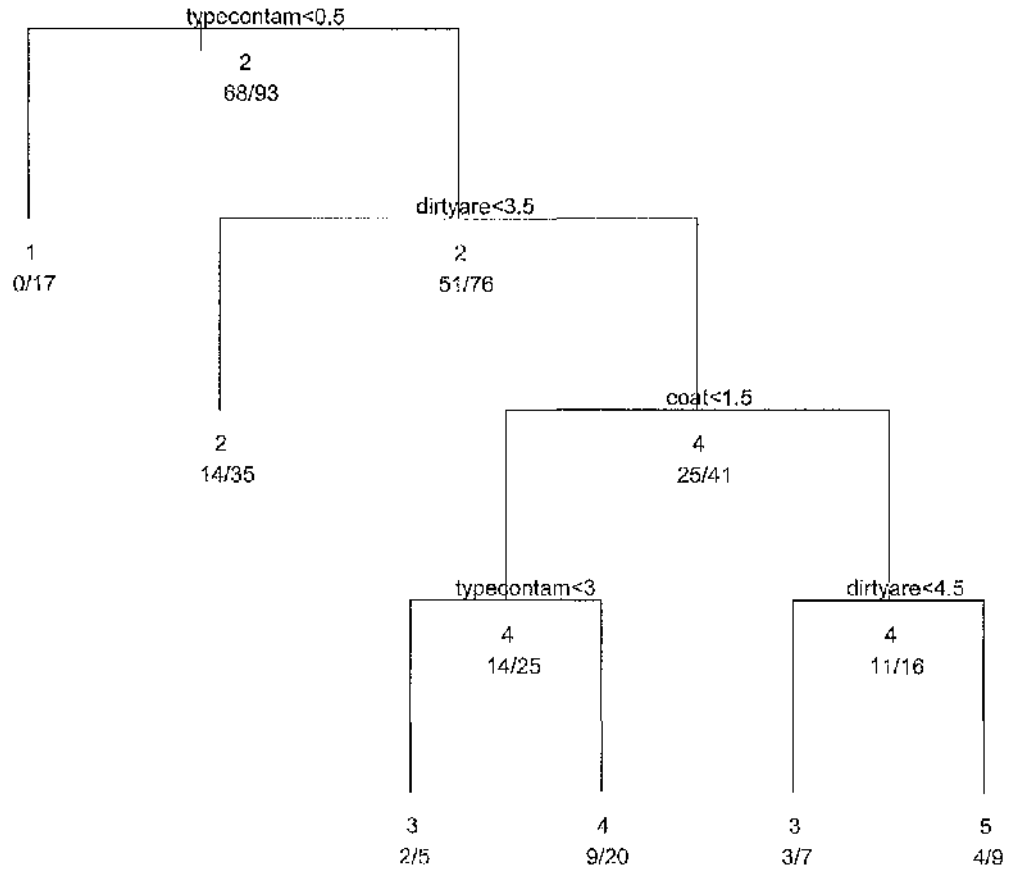


Figure 8. Pruned decision tree based in the actual CLP. Key: Number of dirty areas (dirtyare); type of contamination (typecontam); coat moisture (Coat).

The original tree had 12 nodes and a misclassification rate of 28/93 (30.1%). The cross-validation suggested pruning to 6 nodes. The pruned tree used type of contamination, number of dirty areas and coat condition with a misclassification rate of 32/93 (34.4%) an increase in misclassification, but a reduction from 4 to 3 variables and the number of nodes halved.

3.4. Epidemiology of *Mycobacterium avium subsp. Paratuberculosis* infection

Mycobacterium avium subsp. paratuberculosis was isolated only from the faeces of five cattle (figure 1), and in one case MAP was found only in the lymph node (figure 2), whilst in two cattle, MAP was isolated from both the lymph node and from faeces. The presence of MAP was not associated with carcass contamination. Of the 93 cattle sampled, eight cattle were found to carry MAP, indicating the prevalence of Johne's Disease was 8.6% in the study population.

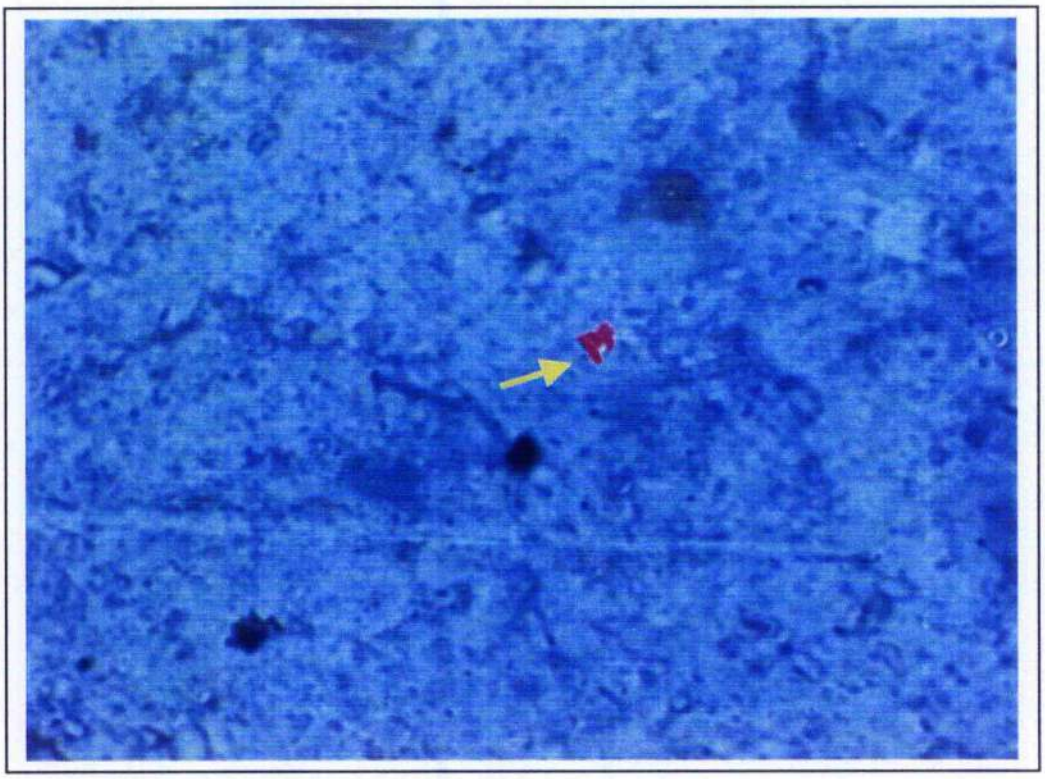


Figure 1: Faecal smears stained with Ziehl Neelsen stain and magnification X 1000. Arrow indicates an acid-fast clumps corresponding with *Mycobacterium avium subsp. paratuberculosis*

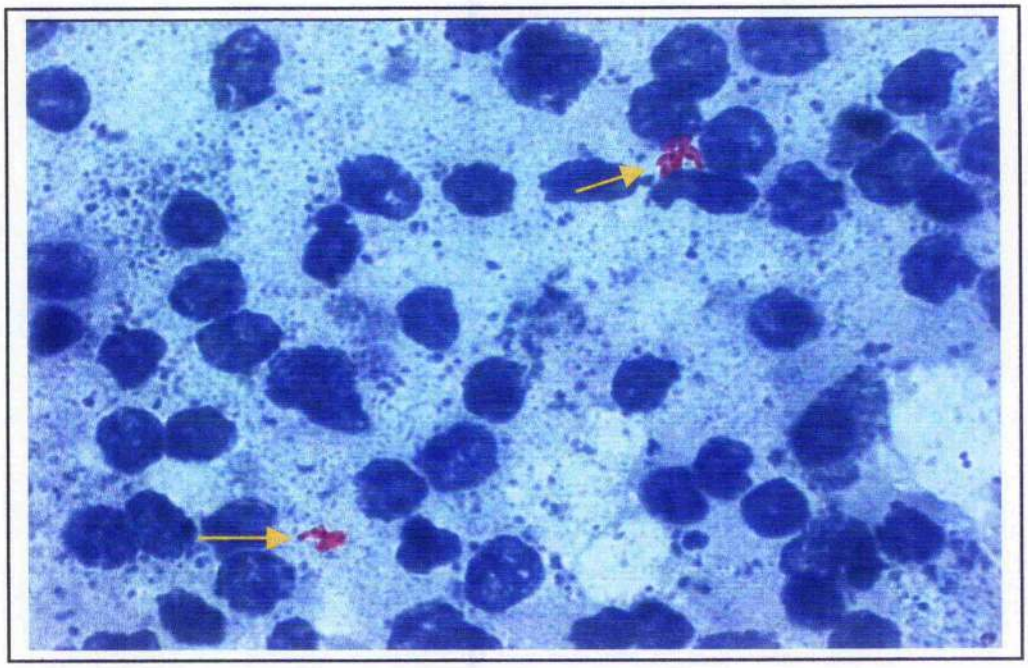


Figure 2: Ileocaecocolic lymph node impression smears stained with Ziehl Neelsen stain and magnification X 1000. Arrows indicate two acid-fast clumps corresponding with *Mycobacterium avium subsp. paratuberculosis*.

3.5. Epidemiology of *Campylobacter spp.*

Campylobacter spp. did not have a significant effect on carcass contamination as it was not isolated in any swab samples from carcasses. Nevertheless, 5 faecal samples were positive for *Campylobacter spp.*, all of which were isolated using the modified Butzler's and Skirrow's enriched medium (Figure 3). As only 27 samples were cultured with this enriched medium, the prevalence of *Campylobacter spp.* amongst these cattle was 18.5%.

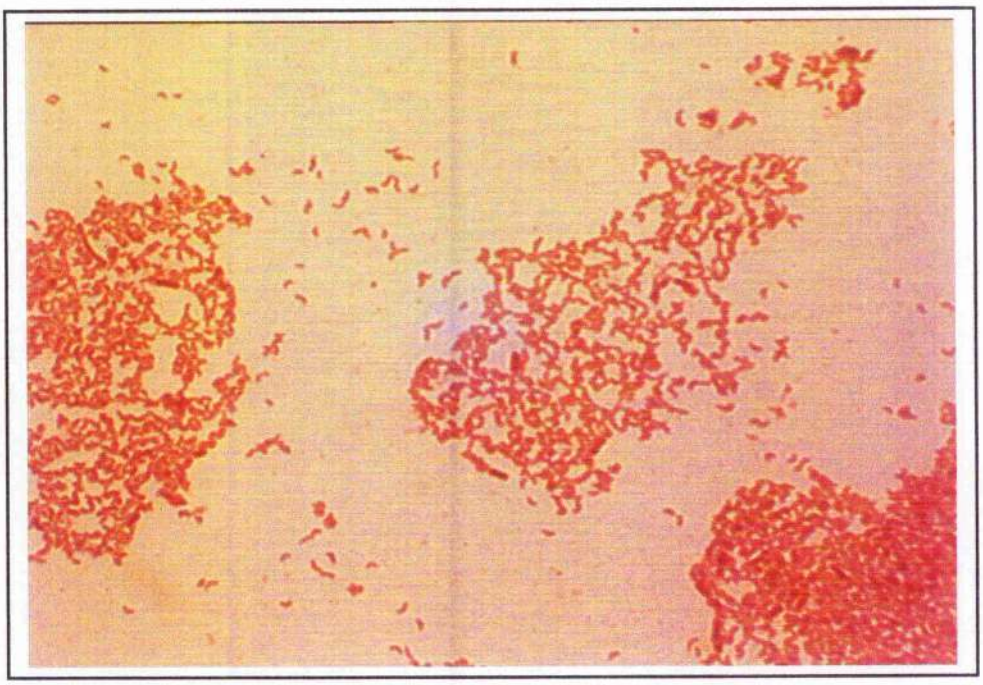


Plate 3: Direct smears obteined from faeces culture in a modified Butzler medium, magnification X1000, stained with Gram stain. Gram negative colonies correspond with *Campylobacter spp.*

4. Discussion

4.1 Introduction

TThe traditional risk-based method of *post mortem* meat inspection was designed to ensure the safety of meat products entering the human food chain. However, this method does not deal adequately with the problem of microbial contamination of meat during slaughter and dressing operations, and the associated consequences of food borne human disease (Newton and others, 1978; Hathaway and McKenzie, 1991; Ayres, 1995; Hudson and others, 1996; Elder and others, 2000; Pointon and others, 2000). Contact between carcass and hide allows a mixture of microorganisms to be introduced to the carcass, which are derived from the animal's preslaughter environment, and these might may be of faecal, soil, urine, water or feed origin (Bell, 1997).

4.2 Microbiological sampling procedure

Microbiological sampling procedures for carcasses have been described in the literature over many years (Dorsa and others, 1996; Stephan, 1996; Balestra and Misaghi, 1997). In the case of carcass contamination with bovine faeces, cotton-tipped wooden swabs are indicated in plant process monitoring to detect faecal contamination (Dorsa and others, 1995). However, this method might not be the most appropriate if identification of the composition of the microbial flora is the goal of the study (Stephan, 1996). The MHS advises the use either of a non-destructive method such as the wet and dry method using cotton-tipped wooden swabs, or the destructive method using a sterile cork borer, as a national procedure for conducting microbiological checks on carcass surfaces (MHS, 1997). The UK legislation indicates that neck, brisket, flank and rump are the sites that are appropriate for microbiological control on carcasses. Alternative sites can be used in cases where it has been demonstrated that other sites are more likely to carry higher levels of contamination (MHS, 1997). Samples taken from the four recommended sites, defined by legislation on the tested carcass, should be pooled in the same container, although in cases where unacceptable results are obtained, samples should not be pooled in order to identify particular microbiological problems during slaughter (MHS, 1997). The brisket and forearm have been identified as sites with the highest microbiological

contamination on the carcass (Kitchell and others, 1973; Untermann and others, 1996; Hughes, 2001), whilst the flank has been shown to carry lower levels of microbiological contamination is due mainly to coat dirtiness (Dorsa and others, 1995). Between five to ten cattle carcasses should be sampled weekly in the abattoir and analysis for TVC and *Enterobacteriaceae* should be performed in the laboratory (MHS, 1995) .

In the current study, the non-destructive wet and dry method was used as this is the most popular method used in the majority of UK abattoirs. A single site on the carcass, the flank, approximately 20 cm from the ventral midline, was selected for sampling as flank contamination has been shown to be mostly due to hide dirtiness rather than slaughter practice (Kitchel and others, 1973; Hughes, 2001). The swab sample recovery rate using the wet and dry method under optimal wet carcass surface conditions has been shown previously to be 20% (Bell, 1997). Bell (1997) expected a value of log 5.13 for TVC from an indirect faecal contamination resulting from hide-carcass contact, assuming 20% population recovery by swab sampling. In the current study, an area of $100cm^2$ on the flank was used and, therefore, lower TVC and *Enterobacteriaceae* values were expected (Bell, 1997).

The study abattoir during the sampling period had a HAS score of 66, therefore, a TVC mean of $\log_{10}$ 0.42 cfu/cm^2 would have been expected (Hudson and others, 1996). In the current study, the TVC mean $\log_{10}$ cfu/cm^2 was double (log 0.87 cfu/cm^2) the expected value which can be explained because samples from carcasses from cattle with a CLP score of 3, 4 and 5 that are considered by the MHS too dirty to be slaughter, were also included in the calculations. In an abattoir producing meat for human consumption, these cattle would not be slaughtered (MHS, 1997). If only clean cattle, with a CLP score of 1 and 2 are included in the calculation, the TVC value is $\log_{10}$ 0.6 cfu/cm^2, which is closer to figures obtained by Hudson and others (1996). This value would correlate with a HAS score of 55 (Hudson and others, 1996). Hence, carcasses with the highest TVC mean $\log_{10}$ cfu/cm^2 on the flank might be caused by hide dirtiness rather than slaughter practice in the study abattoir. This agrees with Kitchell and others (1973), and Hughes (2001), who identified that flank contamination was due to hide dirtiness.

4.3 Factors influencing carcass contamination.

The current study shows that carcass contamination was influenced by many different factors, including the length of the coat, coat moisture, number of dirty areas, dirty underside and type of coat contamination. All these factors showed statistically significant associations with carcass contamination measured by TVC and *Enterobacteriaceae*. Davies and others (2000) also showed that other factors influencing the cleanliness of cattle included time and distance of journey, feed, and type of abattoir. Scott and Kelly (1989) also showed that the housing of cattle influenced the dirtiness of cattle. The sampling period in the current study occurred during the winter months and it is likely that all cattle included in the study were housed prior to transportation to the abattoir. In addition, it is likely that the diet of most of the animals in the current study prior to slaughter would have been silage based, a factor which has also been associated with dirtier hides (Ingram, 1972; Lowman and others, 1988).

Other authors found absence of any strong relationship between the visual cleanliness of animals and the microbiological condition of carcasses, therefore it might be preferable for visible contamination to be controlled largely by superior skinning and eviscerating practices rather than by animal or carcass cleaning treatments (Gill, 2004).

4.3.1. Coat length.

Coat length showed significant associations with carcass contamination. Carcasses from cattle with long coats had higher levels of *Enterobacteriaceae* and TVC than animals with short or medium coats. These results concur with results obtained by Davies and others (2000) who found that carcasses from cattle with long coats had the highest levels of microbiological contamination, whilst those carcasses from cattle with short coats had the lowest levels of microbiological contamination. Several authors have also demonstrated a relationship between the length of fleece and subsequent contamination of the carcass in sheep (Ellerbroek and others 1993; French and Morgan, 1996; Biss and Hathaway, 1996; Hadley and others, 1997). These findings suggest that breeds with characteristically long coats are at a greater risk of producing contaminated carcasses.

The current study, however, showed that breed had no significant effect on carcass contamination, perhaps due to the fact that almost 20% of cattle included in the study had been clipped prior to slaughter.

4.3.2. Coat moisture

Coat moisture showed significant associations with carcass contamination. Carcasses from cattle with wet coats had higher TVC values and they were more likely to be contaminated with *Enterobacteriaceae* than those from animals with dry or damp coats. This result is in agreement with the findings of Leech (1971), and Patterson and Gibbs (1978), who showed sheep with wet fleeces to be a greater hygienic risk than those with dry fleeces. Other studies show that the microbiological population of a dry hide may increase by five to ten times following the addition of sufficient moisture, as the wet environment helps to improve the bacterial recovery rate and, therefore, also increases the chance of cross contamination between hide and carcass (Empey and Scott, 1939). It is, therefore, recommended that following arrival at the abattoir, cattle should be allowed time to dry before being slaughtered with the goal of reducing the risk of microbiological contamination of their carcasses (Newton and others, 1978; Patterson and Gibbs, 1978).

4.3.3. Type of contamination

The type of contamination showed statistically significant associations with carcass contamination. Carcasses from cattle with matted coats were shown to have higher TVC values than those from cattle with a mixed contamination such as faeces, urine and bedding on their coats. The TVC values of carcasses from cattle with matted coats (mean $\log_{10}$ 1.21 cfu/cm^2) was almost double that of carcasses from cattle with only faecal contamination ($\log_{10}$ 0.70 cfu/cm^2), or mixed contamination ($\log_{10}$ 1.01 cfu/cm^2). This finding could be explained because matted coats may provide an ideal microclimate for micro-organism.

VanDonkersgoed and others (1997) found no correlation between type of contamination defined by mud, bedding and faeces on hides and bacterial contamination on carcasses,

but identified association between type of contamination on hides and visual demerit given to the cattle on ante mortem inspection by industry personnel (Kain and others, 2001).

The type of contamination has not been considered in the literature before, and the current study suggests that this factor should be considered in future studies.

4.3.4. Dirty underside and number of dirty areas

At the study abattoir, the hide was removed by cutting distally along the mid-line of the abdomen. Presence or absence of dirtiness in the mid-line was investigated because dirt in this area might increase carcass contamination. The factor dirty underside showed a statistically significant association with carcass contamination, demonstrating that cattle with dirty undersides had higher chance to carry TVC values than cattle that were clean.

Each coat was also divided into seven different areas in the *ante mortem* inspection and the number of dirty areas was recorded on the cattle diagrams of the questionnaire with the goal of calculating the total dirty surface area of each animal. The number of dirty areas and, therefore, the proportion of dirty coat surface showed statistically significant associations with carcass contamination measured by TVC.

There is no data in the literature to confirm that the dirty underside and proportion of the dirty coat surface are significant factors in carcass contamination. Nevertheless, the legislation indicates that special attention must be paid during the dressing process when the knife cut through the hide (MHS, 1997). The MHS also advises using two different knives, one to cut through hide and a different one to cut through flesh to avoid carcass contamination. For this reason, the dressing process is considered the main factor in transfer of micro-organisms from hide to carcass (MHS, 1997; Hadley and others, 1997; Davies and others, 2000) and, therefore, dirty underside should be included as a factor to determinate the dirtiness of a life animal on *ante-mortem* inspection.

4.4. Microbiological study of the meat hygiene service "clean livestock policy" score system

The degree of coat contamination of the live animal was significantly associated with the microbial load of the dressed carcass. Carcass from dirty cattle, as defined by the CLP, were found to carry up to 30,000 times more micro-organisms (TVC) than clean carcasses. Significant differences were found between cattle with differing CLP scores for *Enterobacteriaceae* and TVC contamination, however, differences between "clean" cattle and "dirty" cattle as defined by MHS criteria were found only for TVC contamination, and not for *Enterobacteriaceae*. The proportion of carcasses contaminated with *Enterobacteriaceae* and TVC was found to increase as the coat score increased. These results agree with the findings of Hadley and others (1997) relating to sheep fleeces. Statistical analysis showed a high level of consistency between results for the explanatory variable CLP using different methods of analysis. Hence, it can be concluded that the MHS score system for assessing cattle dirtiness is a useful method of classification for predicting the bacterial contamination of carcasses.

4.5. Non-significant factors

Alterations of the normal health state of the cattle, could mean a deterioration of the cleanliness of the hide as they might spend more time liying down thus causing contamination of their hides. However, in the current study, statistical analysis showed that breed, BCS, presence of diarrhoea, lameness, mastitis and presence of MAP in lymph node an faeces or *Campylobacter spp.* in faeces, were not significantly related to carcass contamination with either *Enterobacteriaceae* or TVC and hence they did not affect the cleanliness of carcasses.

4.6. Altered classification

The MHS allows a maximum level of contamination per carcass of $\log_{10}$ 3.5 cfu/cm^2 of TVC and a maximum of $\log_{10}$ 1.5 cfu/cm^2 for *Enterobacteriaceae* from a pool of the four main sampling sites. As the current study was based on just one sampling site the

amount of bacteria isolated in a contaminated carcass was expected to be lower than those values (Bell, 1997). The statistical analysis of the current study also included an altered classification based on an empirical value of $\log_{10}$ 0.8 cfu/cm^2. Thus carcasses with more than $\log_{10}$ 0.8 cfu/cm^2 were considered as contaminated. Using this altered classification system, no variables were found to be significantly associated with TVC, and only the CLP classification was found significantly associated with *Enterobacteriaceae* contamination. This finding also suggests that the CLP is an appropriate method for the classification of dirty cattle, although different ranges of contamination should be considered if the investigation is based on a single sampling site.

4.7. Decision Trees.

Classification trees are tools used to help choice between several courses of action. They provide a highly effective structure, within which options can be demonstrated, and possible outcomes of such options can be investigated. Decision trees can help in forming a balanced picture of the risks and rewards associated with each possible course of action (www.mindtools.com/decisiontree.html, Angote and others, 1996). The classification of dirty or clean cattle during the *ante mortem* inspection in an abattoir lairage sometimes creates difficulties, as the MHS CLP score provides only descriptive and photographic details of cattle in the five different categories (MHS, 1997). This is a problem inherent in the use of any subjective score system. In the particular case of CLP the main concern is when the OVS must distinguish between scores 2 and 3, that would lead the cattle to be rejected for slaughter (Davis and others, 2000).

Decision trees can be a very useful tool of classification of dirty cattle as they help to minimise subjective decision. As a result of the current study, three different decision trees have been designed, two for each of the outcome variables, TVC and *Enterobacteriaceae* and a third one for the CLP. All three decision trees resulting from the current study do, however, have a high rate of misclassification most probably due to the sample size, particularly relating to the rarity of bacterially contaminated carcasses. A higher number of contaminated carcasses would be necessary in future studies to

obtain more accurate decision trees. The decision trees designed in the current study could, therefore, be used as an example for future investigations, and not as tool in the abattoir at this point in time.

4.8. Epidemiology of *Campylobacter spp.* and *Mycobacterium avium subsp. paratuberculosis.*

In the current study, no *Campylobacter spp.* colonies were found on carcasses. It can, however, be argued that the appropriated medium for culture of *Campylobacter spp.* was only used in 27 of the 93 carcasses, and that the microbiological sampling method used cotton-tipped wooden swabs, an inappropriate method if identification of the microbial flora composition is desired (Dorsa and others, 1996). Nevertheless, *Campylobacter spp.* was isolated from five of the 27 faecal samples using the combination Skirrow and Buster medium. Blood agar base supplemented with Skirrow supplement and Butzler supplement is recognised as being the most appropriate method for the isolation of *Campylobacter spp.* from cattle faeces (Dorsa and others, 1996). The prevalence of *Campylobacter spp.* in cattle slaughtered as part of the current study was 18.5 %, a figure which is slightly higher than that found in the study by Warner (1986) which showed a prevalence of 15% in cattle at the time of slaughter.

In relation to subclinical paratuberculosis, of the 93 cattle sampled, eight cattle were found to carry MAP. Only infected cattle can shed MAP in the faeces (Jones, 2001), therefore all isolations made in the study can be assumed to be true positive results. This suggests that the prevalence of the subclinical form of Johne's Disease was 8.6%, whilst if the 49 extra samples from the prior study were included the prevalence decreased to 7.0%. These figures are slightly lower than those obtained in a survey in the 1950s which used histological examination of the mesenteric lymph node for diagnosis, and found a prevalence of 11.2% (Cranwell, 1997). Figures given by other studies for the prevalence of Johne's Disease vary according to the different diagnostic method used. Prevalence figures range from 11.2% using histological examination (Cranwell, 1997), to 24.0% when lymph nodes were cultured (Peck, 1957). Other authors found a prevalence of subclinical Johne's Disease of 3.5% using PCR, and 2.6% by culture

(Cetinkaya and others, 1996). Polymerase chain reaction has the same specificity and similar sensitivity to faecal smears (Collins and others, 1993), therefore, a 3.5% prevalence of Johne's Disease was expected using faecal smears as a diagnostic method. *Mycobacterium avium subsp. paratuberculosis* is only found in a third of clinical cases using faecal smears; therefore, the use of this diagnostic method for Johne's Disease may be of limited value (Cranwell, 1997; Jones, 2001). Hence, considering the five cattle where MAP was demonstrated from faeces and the two thirds that are considered missed by diagnostic method, the prevalence of subclinical disease using faecal smears would be 16.2%, higher than figures obtained in studies prior this one. A figure of 16.2% confirms that Johne's Disease is a disease with a high incidence in Scotland (Daniels and others, 2002). Nevertheless, the different figures found in the literature about the prevalence of MAP in cattle suggests that faecal smear, direct smear from ileocaecocolic lymph node, or culture alone, may not be sufficient for a diagnosis of Johne's Disease (Lightfoot and others, 1989; Jadhav and others, 1993).

4.9. Conclusion

The data obtained in the current study confirms that factors studied in previous works such as coat moisture and length of the coat are significant risks for carcass contamination. Therefore under general hygiene measures, cattle that are presented damp or wet to slaughter should be separated in an isolation pen and allowed time to dry before the slaughter, and cattle with long coats must be clipped before transporting to the slaughter house. These two considerations should be considered for introduction to the Meat Hygiene Service (MHS) Clean Livestock Policy (CLP) as important factors. These data confirm that the current CLP policy is an appropriate method to assess coat dirtiness in the lairage. Nevertheless, this could be improved if factors such as type of contamination, dirty underside and number of dirty areas, which were identified for the first time as significant for carcass contamination in this study, were included as criteria for the CLP.

In the current study, dirty livestock have been demonstrated as a hygiene critical point that needs to be fully controlled to produce low levels of microbiologically contaminated

carcasses, poor control at this stage may lead to contaminated carcasses and, therefore, represents a risk of food borne disease. Hence the CLP must be included as a critical control point (CCP) in the HACCP plan in any abattoir.

Other techniques of discrimination such as decision trees have been trialled. With this method of classification, the subjective part of the evaluation would be reduced. However due to the small number of infected carcasses, the misclassification rate obtained was too high, hence, the decision trees designed in the current study should be considered as pilot study and should not be used at the abattoir at the current time.

The study of Johne's Disease, produces prevalence figures for Scotland that confirm this disease as of relevance in that country. This has importance for public health due to its role as a potential zoonosis and also for bovine medicine because it could be underdiagnosed in practice and be the cause of significant loss in the cattle industry.

The prevalence of *Campylobacter spp.* has not increased in recent years according to data from previous studies. However, the technique of isolation from bovine samples can cause problems due to contamination with other microorganisms. The trials with different culture media used in the current study, confirm blood agar bases supplemented with Skirrow and Butzler are useful for isolation of *Campylobacter spp.* from bovine faeces due their anti fungal action.

The high incidence of *Campylobacter spp.* and *Mycobacterium avium subsp. paratuberculosis* in the 93 cattle studied suggest that specific microbiological tests to assess carcass contamination in the slaughter hall might be performed on a regular basis as a monitoring or surveillance tool. Composition of the microbial flora is an essential factor for the full interpretation of the microbiological results. Total viable count and *Enterobacteriaceae* count provide information on total microbial number on carcass and therefore are indicate of the process hygiene and performance in the abattoir, but cannot be used to assess potential microorganism pathogens such as *Campylobacter spp.* and MAP.

Samples used in this study were collected in an Over Thirty Month Scheme (OTMS) abattoir where carcasses were not for human consumption. However, this abattoir allowed an unique screening of the current health state of cattle considering Johne's Disease and infection with *Campylobacter spp.* as the legislation concerning consumption of cattle of over thirty months in age is about to be revised, this will result in the resumption of older cattle entering the food chain. The results of the current study could be extended to further explore the measures by which contamination of cattle carcasses by bacteria may be minimised. Also this study focused on correlating visual cleanliness of cattle with carcass contamination, further studies will be necessary to evaluate the correlation between both visual cleanliness and hide microbial contamination, and hide contamination and carcass microbial contamination.

APPENDIX

Animal ID:

EXAMINATION OF CATTLE IN LAIRAGE.

Date of examination _____/_____/_____

1. INDIVIDUAL ANIMAL DETAILS

1.1. Breed: _____

1.2. Body condition score (1 to 5):

1 1.5 2 2.5 3 3.5 4 4.5 5

1.3. Have the animal been clipped: (**Y**)es /(**N**)o _____

a) If so, where? .(circle all that apply)

NECK/BACK/RUMP/ABDOMEN/FLANK/HIND LEG/TAIL/ OTHER_____

2. CLINICAL ABNORMALITIES.

2.1. Has this animal diarrhoea? (**Y**)es/(**N**)o _____

2.2. Does the animal have any obvious signs of lameness? (**Y**)es/(**N**)o _____

2.3. IN COWS OR HEIFERS. Does the cow have any obvious signs of mammary gland abnormalities? (**Y**)es/(**N**)o _____

2.3.a. IF YES, which quarter is/are affected? (Mark any that apply)

RF LF RH LH

2.3.b. What types of lesion does the animal have?_____

_____

_____

_____

2.4. Are there any other visible abnormalities?

_____

_____

_____

_____

3 CLEANLINESS OF THE ANIMAL

3.1.Clean livestock policy category (MHS): **1 2 3 4 5**

3.2. Length of the coat: **SHORT**(*Holstein*) / **MID-LENGTH/ LONG**(*Highland*)

3.3. The coat is (**D**)ry/D(**A**)mp/(**W**)et: _____

3.4. Is the coat contaminated, (**Y**)es/(**N**)o: _____

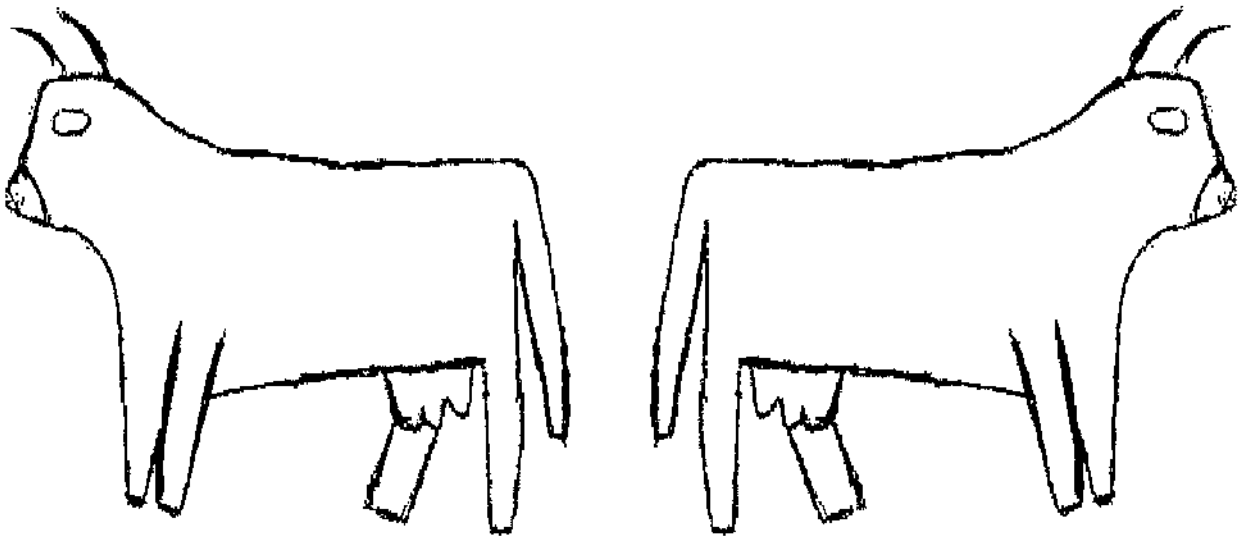
If it is contaminated:

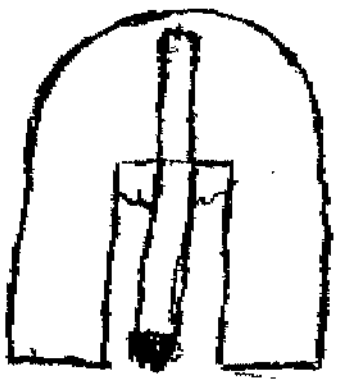
3.4.1 The contamination is (**U**)nilateral or (**B**)ilateral _____

3.4.2 What types of contamination are present? (Tick any that apply)

Faeces		**Urine**		**Bedding**		**Others**_____

3.4.3. MARK DIRTY AREAS ON DIAGRAMS BELOW.





Appendix 2. Raw data

No.	Entero	TVC	Campy Faeces	Campy Swab	Johnes Faeces	Johnes Lymph	CLP	Breed	BCS	Clipped	Diarrho.	Lameness	Mastitis	Lengt coat	Coat	Contami.	Dirty
1	0	0	0	0	0	0	1	2	3	0	0	0	0	1	1	0	0
2	0	0	0	0	0	0	1	2	3	0	0	0	0	1	1	0	0
3	0	1,662758	0	0	0	0	1	1	3,5	0	0	0	0	1	1	0	0
4	0	2,133539	0	0	0	0	1	1	2	0	0	2	0	1	1	0	0
5	0	0	0	0	0	0	1	2	3	0	0	0	0	1	1	0	0
6	0,161368002	0	0	0	0	0	1	1	2,5	0	0	0	0	1	1	0	0
7	0	1,662758	0	0	0	0	1	5	2,5	0	0	0	0	1	1	0	0
8	0,568201724	1	0	0	2	2	1	6	1	0	0	3	1	1	1	0	0
9	0	0	0	0	0	0	1	4	3,5	0	1	0	0	2	2	0	0
10	0	0	0	0	0	0	1	5	3,5	0	0	0	0	2	1	0	0
11	0	1,278754	0	0	0	0	1	1	2	0	0	2	1	1	1	0	0
12	0	0	0	0	0	0	1	7	2	0	0	0	0	1	1	0	0
13	0	0	0	0	1	0	1	3	2	0	1	0	0	1	1	0	0
14	0	0	0	0	0	0	1	1	4	0	0	0	0	1	3	4	1+2+3+7
15	0	0,278754	0	0	0	0	1	6	1,5	4+6	0	0	0	1	1	0	0
16	0	1,447158	0	0	0	0	1	3	4,5	0	0	4	0	1	1	2	9+2+6
17	0	0	0	0	0	0	1	3	3	0	0	0	0	1	2	2	1+3+2+6
18	0,720159303	1,662758	0	0	0	0	1	8	3,5	0	0	0	0	3	1	2	6+7+2
19	0,161368002	1,147676	0	0	0	0	1	3	3	0	0	0	0	1	1	1	9+6
20	0	0	0	0	1	0	1	2	3	5+6	0	3	0	1	1	4	6
21	0	1,318063	0	0	0	0	1	1	2	0	0	0	1	1	1	0	0
22	0	0	0	2	0	0	1	2	2	0	0	0	1	1	1	0	0
23	0	0	0	2	0	0	1	1	1,5	0	0	0	1	1	1	0	0
24	0,161368002	0,863323	0	3	0	0	2	1	3	0	0	0	0	1	1	1	3+2+7
25	0	0,278754	0	0	0	0	2	2	4	0	0	0	0	1	1	1	1+3
26	0	0	0	0	0	0	2	2	2,5	0	0	0	0	1	1	4	3+4++9+7
27	0	0	0	0	0	0	2	1	2	0	0	0	0	1	1	1	4+6
28	0	0	0	0	0	0	2	4	2	3+4+2	0	0	0	1	1	1	7+6+2
29	0	0	0	0	0	0	2	9	3,5	5+3+4+2+6	0	0	0	2	1	1	3+1+2+6
30	0	0	0	0	0	0	2	9	3	3+2+6	1	0	0	2	1	1	1+3
31	0	0	0	0	0	0	2	3	2,5	5+6	0	0	0	2	1	1	3+9+6
32	0	1,278754	0	0	0	0	2	2	2	3+6	0	0	0	1	2	1	1+2+9+6
33	0	0	0	0	1	0	2	1	1,5	0	1	0	0	1	1	1	5
34	0	0	0	0	0	0	2	5	3	0	0	0	0	1	1	1	5+6
35	0	0,740363	0	0	2	2	2	1	4	0	0	0	0	1	1	1	3+2+6
36	0	0,740363	0	0	0	0	2	2	2,5	0	0	0	1	1	1	1	7+3+2
37	0	1,371068	0	0	0	0	2	5	2,5	0	1	0	0	2	1	4	3+2+7+1
38	0	1,161368	0	0	0	0	2	5	3	0	0	0	0	1	1	1	3+7+2
39	0	0,278754	0	0	0	0	2	1	1,5	6	0	0	0	1	1	4	1+3+2+7
40	0,161368002	1	0	0	2	2	2	5	4,5	8+6	0	0	0	1	3	1	2+9+7
41	1	1,740363	0	0	1	0	2	1	1,5	6	0	0	0	1	1	1	3+9
42	0	0,889302	0	0	0	0	2	1	2	5+6	0	0	1	1	1	1	3+9+6
43	0	0,161368	0	0	1	1	2	3	1,5	6	1	0	0	1	1	4	9+6
44	0,740362689	1,662758	0	0	0	0	2	1	1,5	0	0	0	0	1	1	4	7+9+3
45	0	1,147676	0	0	0	0	2	1	2,5	0	0	0	0	1	1	4	3
46	0,662757832	1,037426	0	0	0	0	2	10	2,5	0	0	0	0	1	1	4	3+9+6
47	0	0,511883	0	0	0	0	2	3	3,5	0	0	0	0	1	2	4	3+9
48	0	1,161368	2	2	0	0	2	1	1,5	0	0	0	1	1	1	4	9+6+7
49	0	0,278754	0	0	0	0	3	2	1,5	0	0	0	0	1	1	1	3+1+2-4

50	0	0,371068	0	0	0	0	3	2	2	0	0	0	0	2	1	1	9+2+6
51	0	0	0	0	0	0	3	11	2	0	0	0	0	1	1	1	9+6
52	0	0,278754	0	0	0	0	3	10	3,5	0	0	0	0	1	1	4	1+3+6+9
53	0	0	0	0	2	2	3	6	1,5	0	0	0	0	1	1	4	7+9+2+6
54	0	0,618048	0	0	0	0	3	5	3	0	0	0	0	1	1	5	9+7+2+3+6
55	0,740362689	2,654177	0	0	0	0	3	4	3,5	0	0	0	0	3	3	5	3+9+2+7+6
56	0	0,371068	0	0	0	0	3	5	1,5	6	1	0	0	1	1	2	3+4+2+6+7
57	0,371067862	0,740363	0	0	0	0	3	3	3,5	0	0	0	0	2	1	5	9+2+3
58	0	1,662758	0	0	0	0	3	1	3,5	0	0	3	0	1	1	5	1+2+3+4+6+9
59	1,662757832	1,662758	0	0	0	0	3	1	3	5+6	0	0	0	1	2	4	3+6+7+9+2
60	0	0	0	0	1	0	3	1	2	0	0	0	1	1	2	1	5+6
61	0	0,511883	0	0	0	0	3	5	2,5	5+3+6	0	0	0	1	1	1	5+6
62	0	0,371068	0	0	0	0	3	2	3,5	0	0	0	0	1	1	4	1+2+3+9+7+6
63	0	0,371068	2	0	0	0	3	5	5	0	0	0	0	1	2	4	3+9+6
64	0	2,054996	1	0	0	0	3	5	4,5	0	0	0	0	1	1	4	3+9+6+2
65	0	1,662758	0	2	0	0	3	2	2,5	0	1	0	0	1	1	5	3+7+9+6
66	0	0,278754	0	0	0	0	3	2	2,5	0	0	0	0	1	1	1	3+9
67	0	1,298853	0	0	0	1	3	10	3,5	0	0	0	0	1	2	4	1+3+6+2
68	0	1,618048	0	0	0	0	3	1	3	0	0	0	1	1	1	4	3+7+2
69	0	0,913814	0	2	0	0	3	1	3	0	0	0	0	1	1	4	1+3+7
70	0	1	0	2	0	0	3	4	4	8+5+2+6	0	0	0	2	1	4	1+3+9+2+7
71	0,371067862	1,054996	2	2	0	0	3	1	1,5	0	0	0	1	1	1	4	1+3+6
72	1,161368002	2,432969	0	0	0	0	4	4	3	0	0	0	0	2	1	4	1+3+9
73	1,662757832	1,161368	0	0	0	0	4	2	3	0	0	1	0	1	1	5	1+3+7+9+6
74	0	0,703291	0	0	0	0	4	1	2,5	0	0	0	0	1	2	1	1+3+9+6+7
75	0	0,161368	0	0	0	0	4	1	2	0	0	0	0	1	2	5	3+6+2+9
76	0	0,511883	0	0	0	0	4	1	2,5	0	0	0	0	1	1	4	1+3+9+6
77	0	0	0	0	0	0	4	1	2,5	0	0	0	0	1	1	5	3+9+6+7
78	1,959041392	2,654177	0	0	0	0	4	3	2,5	0	1	0	0	1	1	5	1+9+7+6+3
79	0	1,133539	0	0	0	0	4	3	2,5	0	0	0	0	1	1	5	3+9+7+6
80	0	3,130655	1	0	0	0	4	5	3,5	0	1	0	0	1	1	5	1+3+9+6
81	0,447158031	2,133539	2	0	0	0	4	3	2	0	1	2	0	1	2	4	3+9+6+7
82	0	0,161368	1	0	0	0	4	2	4	0	0	0	0	1	1	5	1+3+9+6
83	0,161368002	1,863323	0	2	0	0	4	5	3,5	0	0	0	0	1	1	4	3+9
84	0	0,740363	2	0	0	0	4	10	5	8	0	0	0	1	3	4	1+3+4+6
85	0	0,447158	1	0	0	0	4	10	4	0	0	4	1	1	1	4	1+2+3+4+6+9
86	0,161368002	2,133539	1	0	0	0	4	1	2,5	0	0	0	0	1	3	4	3+9+6
87	1,161368002	2,654177	0	2	1	1	4	3	2,5	8+5+2+6	0	0	0	1	1	4	3+4+9
88	0	1,662758	0	0	0	0	4	4	3	0	0	0	0	2	1	4	3+9
89	0,568201724	0,740363	0	0	0	0	5	11	1,5	0	0	0	0	1	2	5	1+3+4+9+6+7
90	0	0	0	0	0	0	5	5	3	0	1	4	0	1	2	5	1+3+9+6+7
91	0	0	0	0	0	0	5	3	4	0	0	0	0	1	3	5	1+4+9
92	1,278753601	1,959041	0	0	0	0	5	9	3,5	0	0	0	0	3	2	5	1+3+9+7
93	1,959041392	5,431365	2	2	0	0	5	1	1,5	0	1	0	1	1	3	1	1+2+3+4+5+6+7+9

Key

Entero: *Enterobacteriaceae* log cfu/cm^2.
TVC: Total viable count log cfu/cm^2.
Campy faeces: *Campylobacter spp.* isolated from faeces. 0:no colonies isolated, 1:Isolated, 2:colonies isolated not corresponding with *Campylobacter sp*
Campy swab: *Campylobacter spp.* isolated from swabs. 0:no colonies isolated, 2:colonies isolated not corresponding with *Campylobacter spp.*

Johnes Faeces: *Mycobacterium avium subsp. paratuberculosis* isolated from faeces. 0:No isolated, 1:Isolated, 2:Not applicable.

Johnes Lymph: *Mycobacterium avium subsp. paratuberculosis* isolated from Lymph node 0:No isolated, 1:Isolated, 2:Not applicable.

CLP: Clean Livestock Policy.

Breed: 1: Holstein Fresian, 2:Aberdeen Angus, 3:Limousin, 4:Short horn, 5:Hereford, 6:Ayrshire, 7:Jersey, 8:Highland, 9:Galloway, 10:Belgiam Blue, 11:Charolais.

BCS: Body Condition Score.

Clipped: Areas Clipped. 0:Not clipped, 1:Front leg, 2:Hind leg, 3:Abdomen plus low flank, 4:Upper flank, 5:Rump, 6:Tail, 8:Back.

Diarrhoea: 0: no, 1: Yes.

Lameness: Lameness score (1-4).

Mastitis: 0:No mastitis, 1:Presence of mastitis.

Length coat: 1:Short coat, 2:Mild-length coat, 3:Long coat.

Coat: Coat moisture. 0:Dry, 1:Damp, 2:Wet.

Contamination: Type of contamination. 0:No contamination, 1:Faeces, 2:Urine, 3:Bedding, 4:Mixed, 5:Matted.

Dirty: Areas contaminated. 1:Front leg, 2:Hind leg, 3:Dirty underside, 4:Flank, 5:Rump, 6:Tail, 7:Udder, 8:Back, 9:Upper hind leg.

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