Zoonotic bacteria and antibiotic resistance in the GI tract of horses

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BY

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Abstract

The overall aims of the work described in this thesis were to assess the prevalence of potentially zoonotic pathogens and antibiotic resistance in horse faeces. Cross sectional surveys for *E. coli*, *Salmonella*, *Campylobacter* and vancomycin resistant enterococci showed that *E. coli* to be the most prevalent followed by very low prevalences of *Campylobacter* and vancomycin resistant enterococci, and no *Salmonella*.

E. coli isolates were tested for antibiotic sensitivity and resistance. Higher rates of antibiotic resistance were found in horses in a referral equine hospital than in two local stables. Moreover multi-drug resistant (MDR) *E. coli* were obtained mainly from the hospital, along with resistance to drugs not meant to be used for horse therapy (for example, chloramphenicol and florfenicol). The molecular basis of this resistance has been examined, and compared between horses from different environments. Furthermore, a small-scale longitudinal study of horses admitted to the referral hospital showed an increasing prevalence of resistance during hospitalisation, and then gradual loss of resistance once the horse had returned home.

Salmonella spp. were not obtained from any horse in this study, but archived *Salmonella* isolates were investigated and compared in terms of antibiotic resistance and relatedness (by PFGE). There was some evidence for an epidemic strain of *S. enterica* Typhimurium in horses.

Vancomycin resistant enterococci (VRE) were also isolated from horses, but PCR assays showed that the genes responsible were those usually associated with low-level, intrinsic resistance, and the species were not those usually associated with human infection.

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Chapter 1
General Introduction

1.1 Introduction

Zoonoses are seldom out of the headlines, not least because most new, or emerging diseases, of mankind are zoonoses (Woolhouse & Gowtage-Sequeria, 2005), and new and emerging diseases are intrinsically news-worthy.

Most research has been done on zoonoses acquired from food-animals, such as cattle, sheep, pigs and poultry, although SARS and avian influenza have recently highlighted a role also for wildlife. Relatively little attention, however, has been given to companion animals, which in the UK would include, in particular, cats, dogs and horses. This thesis describes an investigation of horses for zoonotic enteric bacteria, and for antibiotic resistance (which might contribute to zoonotic disease, and is an issue in veterinary medicine).

1.2 Aims

The overall aims of the work were therefore:-

1. To determine the prevalence of zoonotic bacteria (*Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., and vancomycin resistant enterococci (VRE)) in the faeces of horses, and the antibiotic susceptibility of these bacteria.
2. To identify some of the resistance genes responsible for antibiotic resistance, for comparison with data collected by others in human and food animal studies.
3. To investigate some of the risk factors underlying infection with zoonotic enteric bacteria, or the development of antibiotic resistance, in horses.

1.3 Important Zoonotic bacteria

1.3.1 *Escherichia coli*

Escherichia coli identified in 1885 (Escherich, 1885) is a facultative anaerobe, which colonises the gut soon after birth, becoming part of the normal gut flora of many mammals, including both man and horses (Bonten *et al.*, 1990; Neill *et al.*, 1994; Conway *et al.*, 1995). It can also cause disease if inoculated into sites away from the gut or if specific 'virulence' genes are present (Sunde *et al.*, 1998). Because large numbers of *E. coli* are excreted in faeces, its presence is considered to be an indicator of faecal contamination of water or food.

Pathogenicity depends on the presence of virulence factors (Donnaderg & Whittam, 2001) that disrupt normal host physiology. Strains pathogenic to humans are often considered as six groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Meng *et al.*, 1998), each pathogenic mechanism being associated with characteristic virulence genes.

In addition to gastrointestinal disease, *E. coli* often causes disease when outside of their normal gut environment. Thus, in humans, *E. coli* are the principal cause of urinary tract infection (Falagas *et al.*, 1995), and may cause neonatal meningitis and wound infections. Furthermore some EHEC, expressing the Shiga toxin (STEC), can cause a haemolytic uremic syndrome (HUS), hemorrhagic colitis and thrombocytopenic purpura (Riley *et al.*, 1983).

The normal gut flora *E. coli* are exposed to antibiotics given to treat other infections, and in some species have been found to be reservoirs of antibiotic resistance (discussed more later). Treatment of non-enteric *E. coli* might involve the use of trimethoprim-sulphamethoxazole for acute, uncomplicated cystitis, and ciprofloxacin in more complicated cases, or cefotaxime in cases of intra-abdominal infections.

1.3.1.1 *E. coli* and horses

In a study conducted between 1987 to 1989 in the UK and Ireland the prevalence of *E. coli* among horses was found to be similar in normal and diarrhoeic foals (Browning *et al.,* 1991). Also, in adult horses, the number of *E. coli* genotypes did not differ significantly between horses with and without diarrhoea (van Duijkeren *et al.,* 2000). However, *E. coli* isolated from diarrhoeic horses often express virulence factors or contain genes such as *STb, LT, STb* and *eae*, similar to those associated with diarrhoea in other animals and man (Ward *et al.,* 1986; van Duijkeren *et al.,* 2000). Whether or not horses can be a source of pathogenic *E. coli* to other animals or man is not known.

1.3.1.2 Antibiotic resistance and *E. coli*

Before the introduction of antibiotics, most bacteria were fully susceptible to antibiotics (Blazquez *et al.,* 2002). The prevalence of resistance has increased following the use of antibiotics in humans and animals, and, because it lives in both animals and in the environment, it has been suggested that resistance in *E. coli* can be a useful indicator of the level of resistance among bacteria populations in general (Yolanda *et al.,* 2004). Resistance in *E. coli* has been intensively researched and resistance to many different classes of antibiotics (multi drug resistance– MDR) has been recorded.

Data on antibiotic susceptibility in *E. coli* from horses, however, are scarce, most research having been done on farm animals and humans, although some work has recently been done on smaller pet species.

1.3.2 *Salmonella*

Salmonella was first identified in 1885 by Daniel Salmon, and since than, more than 2,500 serovars of non-typhoidal *Salmonella* have been described. Most are considered potential food-borne pathogens (Gorman & Adley, 2004; Gebreyes & Thakur, 2005). In humans, *S. enterica* Typhimurium and *S.* Enteriditis are the most common serovars associated with gastroenteritis. Complications of salmonellosis include headache,

chills, vomiting, diarrhoea, and fever. Moreover, multiple antibiotic resistance has been reported in several serovars, particularly *S.* Typhimurium (Threlfall *et al.,* 1998).

1.3.2.1 *Salmonella* infections in horses

Salmonellae remain major contributors to acute enteric disease, and are responsible for many cases of diarrhoea in horses (Traub-Dargatz *et al.,* 2000). Furthermore, horses can harbour salmonellae in their gastrointestinal tract with no clinical signs (Sanchez *et al.,* 2002). The prevalence of such healthy shedders can be as low as 2% or as high as 20%, and the carrier state may persist for up to 14 months after infection (Losinger *et al.,* 2002; Sanchez *et al.,* 2002). One study suggested that horses that travel for one hour or more may develop diarrhoea and are likely to shed salmonellae in their faeces (Kim *et al.,* 2001). Also, an association between salmonella shed in faeces and antibiotic intake has been reported (van Duijkeren et al., 1995; Kim *et al.,* 2001).

Most outbreaks of salmonellosis in horses can be attributed to a carrier animal within the group. Clinical salmonellosis in horses occurs most commonly after stress (Hird *et al.,* 1984). For example, animals that have been overfed before shipment and or have water withheld for the duration of a journey are predisposed to clinical disease. The prevalence of equine faecal salmonellae is particularly associated with hospitalized horses (Spier *et al.,* 1993). Shedding of *Salmonella* by hospitalized horses in their faeces is an important problem for large animal hospitals. One study of the prevalence of faecal *Salmonella* shedding in hospitalized horses with gastro-intestinal (GI) disease showed that 13 % of the admitted horses with GI signs were positive for *Salmonella*, that horses undergoing abdominal surgery were most likely to shed *Salmonella*, and suggested that it was a combination of stress and antibiotic administration that led to *Salmonella* shedding (Kim *et al.,* 2001).

1.3.2.2 Antibiotic resistance and *Salmonella*

As already mentioned, the clinical use of antibiotics has led to a huge increase in the prevalence of antibiotic resistance (Russell, 2000), and this resistance is often encoded within genetic cassettes that can be rapidly transferred between Gram-

negative organisms (Ferber *et al.*, 1998). *Salmonella* provide a particularly good example of how antimicrobial resistance is selected for and can disseminate between humans and food animals. Resistance to streptomycin and sulphonamides was amongst the first to appear, and the isolation in the United Kingdom of *Salmonella* strains resistant to one or more antibiotics rose consistently for many years (Low, 1997), followed between 1990 and 2000 by a dramatic rise in the prevalence of resistance to four or more drugs (Lawson *et al.*, 2004). In recent years the rise in MDR salmonellosis has been especially important owing to the emergence of the multi-resistant strain *S.* Typhimurium DT104 in both humans and animals (Briggs, 1999).

Salmonella enterica serovar Typhimurium is well documented as the serotype most likely to be MDR, and the definitive type 104 (DT104) is considered a major public health problem, as it is resistant to five antimicrobial agents: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (referred to as ACSSuT) (Glynn *et al.*, 1998), a phenotype often attributed to the use of antibiotics in livestock. *S.* Typhimurium DT104 has become a major cause of enteric infection in Britain, USA, and Canada (Besser *et al.*, 1997; Hollingworth *et al.*, 1997; Brenda *et al.*, 2002; Duc *et al.*, 2004) and has been documented worldwide (Wall *et al.*, 1994). It was first identified in the UK in 1984 (Threlfall *et al.*, 1996) and emerged in the early 1990s as the dominant type of *Salmonella* in the USA (Susan *et al.*, 2004). The resistance genes in DT104 appear to have come from plasmid-born integrons that have become chromosomal by integration, and this enables the resistance genes to persist even in the absence of antibiotic seletion (Threlfall *et al.*, 1994a).

Other *S.* Typhimurium strains have also been associated with multidrug resistance (Rabsch *et al.*, 2001), in particular DT193 (Threlfall *et al.*, 1978; Pontello *et al.*, 1998).

1.3.3 *Campylobacter*

The genus *Campylobacter* consists of Gram-negative, microaerophilic microorganisms. Human disease may include clinical signs such as fever, cramps, and bloody diarrhoea, and is usually the result of infection with *C. jejuni* (90-95%), and *C.*

coli (5-0%) (Walker *et al.*, 1986; Desenclos *et al.*, 2002). *C. jejuni* has also been identified as precipitating factor in Guillain-Barre syndrome (Mishu & Blaser, 1993). Other species, which are less commonly found as clinical isolates are *C. lari*, *C. upsaliensis*, and *C. fetus*.

Humans are thought to acquire the microorganism mainly by eating under-cooked meat, or drinking contaminated water or unpasteurized milk (Eberhart-Phillips *et al.*, 1997; Koenraad *et al.*, 1997). *Campylobacter* infections are believed to be the most common cause of food-borne disease in the UK and other industrial countries (Skirrow *et al.*, 1994, Richardson *et al.*, 2001; Friedman *et al.*, 2004; Moore *et al.*, 2005) and in developing countries (Coker *et al.*, 2002). The incidence of campylobacteriosis is higher than that of salmonellosis and shigellosis (Koenraad *et al.*, 1997; Frost, 2001). The incidence of campylobacteriosis has increased in many European countries over the past decade or so (Anonymous, 2000; Schlundt *et al.*, 2002). Under-cooked chicken has been particularly blamed as a source of infection (Eberhart-Phillips *et al.*, 1997). Many reports have estimated and reported infections among population caused by *Campylobacter* in the USA (Altekruse *et al.*, 1997; Samuel *et al.*, 2004).

1.3.3.1 *Campylobacter* and horses

Little research has been done on *Campylobacter* infections in horses. Horses have not been regarded as a reservoir of these microorganisms, unlike cattle, sheep and pigs (Prescott *et al.*, 1982; Manser *et al.*, 1985), and surveys have not found equine infection to be common (Prescott *et al.*, 1981; Hong *et al.*, 1989).

1.3.3.2 Antibiotic resistance and *Campylobacter*

Fluoroquinolones have been extensively used for treatment of *Campylobacter* infections and also frequently used as prophylaxis for traveller's diarrhoea. Erythromycin, gentamicin and tetracycline are also used. *C. jejuni* is intrinsically resistant to trimethoprim (Karmali *et al.*, 1981).

The increased recognition and treatment of campylobacteriosis in humans has led to a significant increase in the prevalence of resistance in *C. jejuni* and *C. coli* (Threlfall *et al.*, 2000), and resistance to fluoroquinolones increased worldwide during 1990s (Engberg *et al.*, 2001). In the USA, increasing resistance to ciprofloxacin is also evident among *Campylobacter* isolates (Nelson *et al.*, 2004); the prevalence of ciprofloxacin resistance among *Campylobacter* spp. found in the UK was the lowest in the European Union (Bywater *et al.*, 2004). *C. coli* appears to have higher rates of resistance to ciprofloxacin than *C. jejuni* (Beilei *et al.*, 2003).

Some studies have suggested that the emergence of resistance in *Campylobacter* spp is linked to the use of antimicrobials in veterinary medicine (Jimenez *et al.*, 1994; Luber *et al.*, 2003). Indeed, fluoroquinolone resistance in *Campylobacter* from food animals is now recognized as an emerging public health problem (Engberg *et al.*, 2001), selected for by the use of fluoroquinolones in food animals (WHO, 1997). A particular problem appears to be the emergence of resistant strains in poultry and their subsequent consumption (Van Looveren *et al.*, 2001).

1.4 Antibiotic resistance

The emergence of antibiotic resistance in bacteria has been well documented as a serious problem worldwide (Cohen *et al.*, 2000) and as the greatest threat to the use of antimicrobial agents for therapy (Aarestrup *et al.*, 1999; Aarestrup *et al.*, 2001). The emergence of resistance began soon after Fleming discovered penicillin in 1928; in the 1940s Abraham & Chain described penicillinase, an enzyme that inactivates penicillin in *E. coli* (Tenover *et al.*, 1996). Increasing resistance to each new antibiotic introduced has been seen since then (Levin *et al.*, 1998; Reinthaler *et al.*, 2003), selected for by the clinical use and abuse of antibiotics in human and veterinary medicine, and by the use of some antibiotics as growth promoters (Copenhagen, 1998; Braoudaki *et al.*, 2004).

While the subsequent drop in usage of some antibiotics has led to the suggestion that this may enable the selection of fitter 'wild type' bacteria, and so a drop in the prevalence of antibiotic resistant bacteria (Ballow & Schentag, 1992), other reports

show no effect of banning the use of drugs on the prevalence of resistance, for example in the case of choloramphenicol in the UK (Phillips *et al.*, 1998).

1.5 Antimicrobials and antimicrobial resistance in animals

Although some have divided the bacteria that make up the normal bacterial flora of animals into symbionts, which mutually benefit themselves and the host, commensals, which do not seem to be of any benefit to the host, and opportunists, which may harm the host and produce disease under certain circumstances (Sorum & Sunde, 2001), the complex relationship between host and bacterium, and the relationships between the bacteria, and indeed the environment outside of the host, together form a much more dynamic ecosystem than this.

Antibiotic resistance within environmental bacteria existed long before the use of antibiotic drugs in either humans or animals, and the use of these drugs has merely selected for the genes responsible (Dancer *et al.*, 1997). Domestic, and especially food animals are considered to be a reservoir for pathogenic bacteria such as *Salmonella*, *Campylobacter*, and pathogenic types of *E. coli* to humans, and the use of antimicrobials in animals will select for resistance in these, which will subsequently pose a risk to public health. Furthermore, the use of antimicrobials also selects for resistant non-pathogenic bacteria, which act as reservoirs for the horizontal transfer of resistance to pathogenic species. Therefore, the normal flora may play a key role as an acceptor and donor of transmissible antimicrobial resistance mechanisms (Yolanda Sa´enz, 2004).

Some antibiotics have (and still are in some countries) been used as growth promoters, and not just for the treatment of food animals (Franklin, 1999; Schwarz *et al.*, 2001), and this has led to the emergence of resistance to important drugs (Copenhagen, 1998). The use of tetracyclines, sulfa drugs, cephalosporins, and penicillins for a variety of bacterial conditions has been a major factor in the emergence and dissemination of antimicrobial-resistant *E. coli* (van den Bogaard & Stobberingh,1999), and Boatman, (1998) reported that some drugs, such as tetracyclines, are particularly overused in animals. Many studies have shown the role

of antimicrobial use in food animals in the selection of antimicrobial resistance (Corpet, 1989; Wegener *et al.*, 1999), even when used in small quantities (Van Houweling & Kingma, 1969) and the risk that resistance poses to public health (Huber *et al.*, 1970). As a result of this, the use of several antimicrobial growth promoters has been banned in the European Union (Prescott *et al.*, 2000). For example, avoparcin, which has been linked to the emergence of vancomycin resistance enterococci (VREs) in cattle, has been banned. Indeed antibiotic growth promoters will be banned from use in the European Union from December the 31st 2005.

Approximately 50% of all antibacterial agents used annually in the UK, and in the EU as a whole, are given to animals ((Veterinary Medicine Directorate, 2003); Ungemach, 2000), and the rate of antibiotic resistant infections in Europe has increased steadily (Harbarth *et al.*, 2001). In the 1990s in the UK, antimicrobials for therapy use in companion animals (including horses) represented approximately 6% of the total amount used in animals (VMD-Veterinary Medicine Directorate, 2003).
Apart from antibiotic use, another important factor involved in the dissemination of resistance appears to be exposure to stressors (Moro *et al.*, 1998; Moro *et al.*, 2000), which can increase shedding of resistant bacteria. The combination of stress with antibiotic therapy has been show to be particularly important in the dissemination of resistance (Levy *et al.*, 1998a). A further factor, returned to later, is that many genes for resistance are linked – often they are on the same integron or plasmid - and so selection pressure for resistance to one of these antibiotics is likely to select for resistance to the others also (Garau *et al.*, 1999; Braoudaki & Hilton, 2004).

1.6 Mechanisms of resistance

Resistance is often said to be either intrinsic or acquired. Intrinsic resistance is usually inherited vertically, and usually expressed by chromosomal genes, e.g. the β-lactamases of Gram-negative bacteria. Acquired resistance may occur by mutation in chromosomal genes, but is more likely to be transferred horizontally, for example by plasmids and transposons (Hall & Collins, 1995; Normark *et al.*, 2002).

The mechanisms by which bacteria can transfer genes are transformation, in which bacteria take up naked DNA from the environment, conjugation, in which DNA is transferred directly between bacteria through cell contact, and transduction, in which a bacteriophage acts as a vector for DNA between bacterial cells (Giraud *et al.*, 2002). Conjugation is probably the most rapid, efficient and common mechanism of transfer of resistance between bacteria, but transformation, for example is considered by some as the most likely mechanism for the transfer of antibiotic resistance genes from transgenic crops to bacteria (Stuart & Carlson, 1986; Nielsen *et al.*, 1998).

1.7 Resistance systems and mechanisms

Resistance genes are commonly present on plasmids, transposons, gene cassettes or mobile genetic elements (Recchia & Hall 1995), allowing the horizontal spread of resistance genes between strains, species and even between genera. Integrons are specialized genetic elements capable of integrating or mobilizing gene cassettes by site-specific recombination (Recchia & Hall, 1995). Many of the antibiotic resistance genes found in clinical isolates of Gram-negative microorganisms are part of a gene cassette inserted into an integron and many of these mobile elements are reported to be similar in *E. coli* in both humans and animals (Lanz *et al.*, 2003) suggesting transmission between the two.

Many environmental and genetic factors play a role in the spread of resistance (Zatyka *et al.*, 1998), but selection pressure by the use of antibiotics drugs is probably the greatest of these (Witte *et al.*, 1997; Witte *et al.*, 1998; Dzidic *et al.*, 2003; Perreten *et al.*, 2005). Novel resistance genes may be selected in the normal bacterial flora of animals as a direct consequence of antibiotic use (Lee *et al.*, 1999), and these bacteria and genes may be transferred from animals to humans (Mevius *et al.*, 1999, Kruse, 1999).

1.7.1 MDR (multi-drug resistance)

Multiple drug resistance is often defined as simultaneous resistance to at least four antimicrobials (Bywater *et al.*, 2004), although it is sometimes, and perhaps more

literally, defined simply as resistance to two or more antibiotics. The emergence of bacteria possessing multiple-antibiotic resistance genes has become a major concern in recent years (Briggs & Fratamico, 1999; Shojaee & Lees, 2000) and multiple resistance among *E. coli* has been reported as serious health treat (Ariza *et al.*, 1994; Maynard *et al.*, 2003; Navia *et al.*, 2003).

The multidrug resistance in bacteria is especially related to the acquisition of resistance genes through plasmids, transposons (Gold & Moellering 1996; Alekshun *et al.*, 2000) or through clonal selection of strains with mobile genetic elements (integrons), such as *S.* Typhimurium DT104 (Ridley & Threlfall, 1998) and *E. coli*. Mobile genetic elements and chromosomal systems (such as the *mar* locus) both contribute to the multi-drug resistance phenomenon (Alekshun *et al.*, 1997; Alekshun *et al.*, 2000).

1.7.2 Integrons

Integrons, were first identified in 1980s (Stokes *et al.*, 1989). They are genetic elements able to capture genes, including antibiotic resistance genes, using site-specific recombination at an integrase-specific recombination site (Recchia & Hall, 1995; Partridge *et al.*, 2000). Integrons have an important role in the increase of multi-drug resistance through the acquisition of multiple genes, which confer resistance to multiple drugs (Recchia *et al.*, 1997; Putman *et al.*, 2000; Carattoli *et al.*, 2001; Hanau-Bercot *et al.*, 2002; Leverstein-van Hall *et al.*, 2003) and are widely distributed among both nosocomial and community Gram-negative isolates (Hall *et al.*, 1997). Integrons not only acquire genes, but are mobile, and able to move between plasmids and chromosomes, they play a role not only in the increase of multi-drug resistance, but also in the dissemination of resistance genes (Shaohua *et al.*, 2001; Singh *et al.*, 2005).

To date, eight classes of integrons have been described (Nield *et al.*, 2001; Collis *et al.*, 2002; Barlow *et al.*, 2004). Class 1 integrons are the most common integrons among clinical isolates and resistant bacteria (Jones *et al.*, 1997; Hall *et al.*, 1998), and confer resistance to drugs such as β-lactams, aminoglycosides, chloramphenicol,

trimethoprim and also disinfectants (Laraki *et al.*, 1999). Class 4 integrons are a distinctive class found in *Vibrio cholerae,* and also associated with antibiotic resistance (Rowe-Magnus *et al.*, 1999). The remaining integrons also encode antibiotic resistance and may play role in resistance dissemination among the *Enterobacteriaceae* (Fluit & Schmitz, 1999; Lindstedt *et al.*, 2003).

1.7.3 Mar locus (*mar* locus)

Recently, there has been increased awareness of chromosomal multi-drug resistance systems such as the *Mar* locus, which is widely found amongst Gram-negative bacteria (Cohen, 1993a; Cohen, 1993b; Barbosa & Levy, 1999). It was first discovered in *E. coli* in 1980 (George & Levy, 1983), in which both *marA*, a transcriptional activator negatively regulated by *marR*, and *SoxS*, the regulator of the superoxide *SoxRS* regulon, confer increased resistance to chemically unrelated antibiotics by activating or depressing a number of genetic loci in *E. coli* that contribute to a multi-drug resistance phenotype (Miller *et al.*, 1996). Thus it can control expression of resistance to fluoroquinolones, chloramphenicol and tetracycline, and also organic solvent, oxidatives agents and household disinfectants (Ariza *et al.*, 1994; Conrad *et al.*, 1996; Alekshun & Levy, 1999). The complete sequence of this locus has been determined in *E. coli* (Sulavik *et al.*, 1997).

1.8 Antibiotic drugs and resistance important in humans and animals

1.8.1 Trimethoprim (TMP)

Trimethoprim is a synthetic antimicrobial agent used on its own or in combination with sulfamethoxazole (sulphonamides) in the treatment of infections, especially urinary tract infections, caused by Gram-negative organisms (Joyner, 1984; Houvinen, 1995). Sulphonamides compounds were first introduced into therapy in 1932, and trimethoprim was first introduced therapeutically in 1962 in England (Huovinen, 1987; Huovinen, 2001).

Trimethoprim is a structural analogue of folic acid, and acts on all living cells by inhibiting the reduction of dihydrofolate to tetrahyrofolate by dihyrofolate reductase

(DHFR). It inhibits bacterial cells preferentially, as its affinity for mammalian DHFR is low, and so the concentrations of drug needed to inhibit bacteria have little effect on the host.

1.8.1.1 Trimethoprim resistance

The most common mechanism of resistance to trimethoprim in enterobacteria is through the acquisition of a plasmid-mediated DHFR, which, unlike the chromosomal enzyme, is less sensitive to inhibition by trimethoprim. These DHFR's are encoded by *dfr* genes, of which *dfrA1* is the most commonly reported and the first identified (Sköld, 2001) among Gram-negative bacteria, and often found as part of a cassette in both class 1 and class 2 integrons (Sköld, 2001). At least seventeen types of trimethoprim-resistant *dfr* genes and nine *dfr* gene cassettes have been identified in Gram-negative bacteria: *dfr genes* include *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12*, *dfrA14*, *dfrA17*, *dfrB1*, *dfrB2* and *dfrB3*. Other resistance mechanisms have been reported, but appear to rarely encountered.

Higher levels of resistance to trimethoprim can be related to changes and mutations in the basic *dfr* genes, as reported in, for example *Haemophilus influenzae* (de Groot *et al.*, 1996) and *Streptococcus pneumoniae* (Adrian & Klugman, 1997), in which the resistance is chromosomal.

Reports suggest an increasing incidence of TMP resistance in *E. coli*. TMP resistance has been reported at a prevelance of 25 to 68% in South America, Asia, and Africa (Lester, 1990; Huovinen, 1995; Yu *et al.*, 2003).

1.8.2 Sulphonamides

The sulphonamides were the first non-overtly toxic antibacterial drugs developed for use in humans, and were first introduced in Europe in 1930s. Resistance had developed by the 1960s, as a result of which sulphonamides are often administered with trimethoprim. Sulfonamides also interfere with folic acid synthesis, but act by

inhibiting the bacterial enzyme dihydropteroate synthase. The co-administration of the two drugs was restricted in the UK in 1995 as a consequence of the emergence of resistance.

1.8.2.1 Sulphonamides resistance

Well documented genes responsible for sulphonamide resistance include *sulI, sulII* and *intI.* The *sulI* in *E. coli* is often part of a cassette of resistance genes within integrons, and is especially linked to genes like *strA, strB* which determine streptomycin resistance (Radstrom *et al.,* 1991).

1.8.3 Tetracyclines

Tetracyclines were discovered in the 1940s and entered clinical use in the 1950s. They are broad-spectrum agents, used against wide range of microorganisms including Gram-positive and Gram-negative bacteria, and they act by inhibiting protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra *et al.,* 2001; Chopra & Roberts, 2001). Because tetracyclines are broad spectrum drugs, with few major side effects and of low cost, they are reported to be the second most used antibacterial after penicillin for the treatment of animal and human infection (Col *et al.,* 1987). First generation drugs included oxytetracyline and chlortetracycline; second-generation drugs were semi-synthetic compounds such as doxycycline and more recently third-generation (glycyclines) include, for example, minocycline.

1.8.3.1 Tetracycline resistance

The resistance to tetracycline has emerged in many pathogenic and non-pathogenic bacteria due to aquisition of *tet* genes. The acquired tetracycline resistance genes often are part of mobile genetic elements - plasmids, transposons and/or integrons.

At least twenty nine different tetracycline resistance genes (*tet*-genes) and two oxytetracycline resistances (*otr*-genes) are reported, and are summarized in the table 1 (Chopra *et al.*, 2001). Three resistance mechanisms so far identified are: tetracycline efflux, ribosomal protection, and enzymatic-tetracycline modification. The efflux system is the most commonly found system.

Table 1. Summary of the different mechanisms of *tet genes*

Mechanism	Genes
Efflux	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(I), *tet*(J),*tet*(Z), *tet*(30)[b] *tet*(31)[b] *tet*(K), *tet*(L) *otr*(B), *tcr*3[c] *tet*P(A) *tet*(V) *tet*(Y)[d]
Ribosomal protection	*tet*(M), *tet*(O), *tet*(S), *tet*(W) *tet*(Q), *tet*(T) *otr*(A), *tet*P(B),[e] *tetc*
Enzymatic	*tet*(X)
Unknown	*tet*(U), *otr*(C)

Tetracycline resistance was first identified in 1953 from *Shigella dysenteriae* and two years later in 1955 multi-drug resistant isolates of the same species were isolated (Falkow, 1975). Prior to the mid-1950s, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines (Levy *et al.*, 1984): among 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, only 2% were resistant to tetracycline (Hughes *et al.*, 1983), but by the 1970s and mid 1980s increase in tetracycline resistant was documented among wide range of Gram-positive and Gram-negative bacteria (Chopra *et al*, 2001).

1.8.4 Aminoglycosides

The aminoglycosides (gentamicin, spectinomycin, streptomycin, amikacin, apramycin, - although this last is technically an aminocyclitol) are widely used antimicrobial agents against infections by both Gram-negative and Gram-positive organisms. They interfere with protein synthesis by binding to the ribosome, and resistance to these agents is mostly by the production of modifying enzymes that hydrolyze these agents.

1.8.4.1 Aminoglycoside resistance

Resistance to aminoglycosides can be by either targeting the accumulation of drug or the expression of enzymes, which is the most reported mechanism, that render the drugs unable to bind to the bacterial ribosome. Hydrolytic enzymes can be grouped into three main enzymes: acetyltransferases (AAC), nucleotidyltransferases (ANT) and phosphotransferases (APH) (Quintiliani *et al.*, 1995). Efflux systems or mutations in rRNA have also been described, but are uncommon (Schmitz *et al.*, 1999).

Some of the genes responsible are widely found to be inserted into transferable genetic elements and therefore linked to multidrug resistance.

1.8.4.2 Apramycin: Apramycin has been used extensively in animal husbandry since 1978. Although it has not been used in human medicine, apramycin resistance has been detected in human isolates of *Klebsiella pneumoniae* and *E. coli* (Hunter *et al.*, 1993; Johnson *et al.*, 1995), and animals are seen as the original source for apramycin resistance in human isolates (Mathew *et al.*, 1998).

1.8.5 Quinolones

The quinolones are synthetic, broad-spectrum antibiotics that are largely used for community-acquired and nosocomial infections. First described in 1962, quinolones are effective against members of the family *Enterobacteriacea*, and target the bacterial DNA gyrase and topoisomerase IV enzymes, which are essential for DNA replication and transcription and the fluoroquinolones are used to treat wide range of infections and have been used intensively by veterinarians since their introduction in the 1990s for veterinary use despite resistance from the medical community. Enrofloxacin, in particular, because of its broad spectrum of activity, and safety for use in a wide range of species, has been used intensively by veterinarians and the emergence of resistance among humans has been attributed to this use.

The quinolones constitute a group of synthetic antimicrobial agents that exert their bactericidal effect by targeting and inhibiting bacterial DNA gyrase (topo-isomerase II that catalyzes the negative supercoiling of DNA and the separation of interlocked

replicated daughter chromosomes) and topoisomerase IV enzymes, leading to lethal double-strand DNA breaks (Hooper *et al.,* 2001). These processes are vital for the replication of DNA, for the segregation of replicated chromosomes. In Gram-negative organisms, such as *Escherichia coli,* the primary target of fluoroquinolones is the topoisomerase II enzyme, DNA gyrase (Gellert, 1981).

First generation quinolones include nalidixic acid, oxolinic acid and cinoxacin, second generation fluoroquinolone drugs are ciprofloxacin, lomefloxacin, norfloxacin, and third generation include moxifloxacin gatifloxacin, sitafloxacin, clinafloxacin and trovafloxacin and also include enrofloxacin (Baytril).

1.8.5.1 Quinolone and fluoroquinolone resistance

In *E. coli* and *Campylobacter* spp., quinolone resistance has been attributed mainly to mutations in the quinolone resistance-determining region (QRDR) in the target topoisomerases of the drugs, DNA gyrase (e.g., in *gyrA*) and topoisomerase IV (e.g., in *parC*). Resistance in *E. coli* is most frequently associated with alterations in *gyrA* (Gootz *et al.,* 1991; Cambau *et al.,* 1993; Deguchi *et al.,* 1996; Conrad *et al.,* 1996), athough mutations in *gyrB* have also been found associated with quinolone resistance. Recently, plasmids and intergrons mediating resistance to quinolones have been reported associated with a high level of resistance (Martinez *et al.,* 1998).

Other possible mechanisms of resistance to fluoroquinolones include decreased intracellular drug accumulation as a result of alterations in the outer membrane proteins of the cell wall, or active efflux of the drug mediated by a number of efflux pumps. These kinds of resistance are often linked to the multi-drug resistance (Levy, 1992).

It has been reported that the introduction of fluoroquinolones for veterinary clinical use was followed by increased prevalences of resistance to these drugs in both *E. coli* and *Salmonella* from various animal species, including humans (Wiuff *et al.,* 2000). For instance, these agents are not used for children though a study of faecal samples from children has shown a prevalence of 26% of quinolone-resistant *Escherichia coli.* Furthermore, 99 % of *Campylobacter* isolates isolated from broilers and pigs and 72

% human isolates were resistant to ciprofloxacin in surveys in Spain (Garau *et* al., 1999).

1.8.6 Beta-lactam drugs

Beta-lactam drugs are frequently used for human and veterinary medicine. The most important groups of β-lactam antibiotics are the penicillins and cephalosporins (Mason & Kietzmann, 1999). Penicillin was one of the first antibiotics developed, and the cephalosporins were first discovered in 1948: subsequent generations of both groups of drugs, with broader spectums of activity and improved therapeutic properties have been developed since, with, not surprisingly, resistance evolving soon after each development (Fluit *et al.,* 2001).

They act by binding to penicillin binding proteins (PBPs), which are important in bacterial cell wall synthesis. PBP's are extra-cellular or periplasmic enzymes responsible for the final stages of peptidoglycan synthesis. The β-lactam ring of the antibiotic binds strongly to the transpeptidases (PBP) so they can no longer catalyze the cross-linking of peptidoglycans. The family includes the penicillins, methicillin, cephalosporins, monobactams, cephamycins and carbapenems.

1.8.6.1 β-lactam resistance

Resistance was reported soon after the introduction of penicillin in 1940, and the penicillinase or β-lactamase enzyme was quickly identified. Indeed, resistance to these agents was reported in *Escherichia coli* even prior to the release of penicillin for use clinically (Abraham & Chain, 1988). Selection for β-lactamases among Gram-negative bacteria is thought to have developed due to the selective pressure exerted by β-lactam-producing organisms found in the soil (Ghuysen *et al.,* 1991). Among *Enterobacteriaceae* the production of β-lactamases is the single most prevalent mechanism responsible for resistance to β-lactams drugs (Pitout *et al.,* 1998): β-lactamase hydrolyzes the β-lactam ring and thereby inactivates the antibiotic (Livermore, 1995).

Several classification systems for β-lactamases have been published (Ambler, 1980; Bush *et al.*, 1995), and many β-lactamases have been described and classified according to their functional and structural characteristics. Over 200 β-lactamases have been identified (Bush *et al.*, 1995) although only a few commonly occur. Most of the enzymes in Gram-negative microorganisms are the TEM and SHV types (Heritage *et al.*, 1999) and *Tem* genes are the most prevalent among resistant *E. coli* isolates (Petrosino *et al.*, 1999).

1.8.6.2 Extended spectrum β-lactamases

Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime, and the monobactam aztreonam (DuBois *et al.*, 1995; Jacoby & Medeiros, 1991). First reported among *Enterobacteriaceae* in Europe in 1983 and 1984, ESBLs have now been reported almost worldwide (Huletsky *et al.*, 1990). The prevalence ESBL in European countries is reported to be around 9% (Hanberger *et al.*, 2001).

Genes encoding β-lactamases found in Gram-positive and negative bacteria and can be located either on plasmids or the bacterial chromosome. ESBLs are often plasmid-mediated (Paterson *et al.*, 2001) and most of those from *Escherichia coli* and *Klebsiella pneumoniae* are mutants of the TEM- or SHV-type β-lactamases (Jarlier *et al.*, 1988; Paterson *et al.*, 2001), such as TEM-1, TEM-2, and SHV-1 (Livermore 1995).

Other possible mechanisms are the hyperproduction of chromosomal AmpC β-lactamases, and strains producing AmpC β-lactamases are emerging in several species of Gram-negative bacteria (Pfaller *et al.*, 1997) and mostly confer resistance to cephamycins (cefoxitin and cefotetan). Further mechanisms include OXA β-lactamases, plasmid cephalosporinase production, or even changes in membrane permeability (Briñas *et al.*, 2002).

1.8.7 Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic that acts by inhibiting the peptidyltransferase step in protein synthesis by binding to the 50S ribosomal subunit. It was extensively used in human and veterinary medicine until concerns over its toxicity emerged (Settepani *et al.*, 1984) and it's use in veterinary medicine is now limited because of concerns about resistance.

1.8.7.1 Chloramphenicol resistance

Resistance to chloramphenicol can be either enzymatic or non-enzymatic. The most frequent resistant mechanism to chloramphenicol is the inactivation of the antibiotic by a chloramphenicol acetyltransferase (CAT), which is responsible for most enzymatic resistance to chloramphenicol (Shaw, 1983). The *catI* gene is the most reported and widespread gene responsible (Alton & Vapnek, 1979). Non-enzymatic resistance to chloramphenicol is through drug efflux, associated with the *cmlA* genes (Williams, 1996). The *cat* genes are commonly found on plasmids. Other mechanisms of resistance involve decreased outer membrane permeability or active efflux, sometimes observed in Gram-negative bacteria (Fluit & Schmitz, 1999).

1.8.8 Florfenicol

Florfenicol (FLO) is a novel broad-spectrum antibiotic not-approved for human use. Florfenicol is a fluorinated chloramphenicol derivative, licensed in Europe in 1995 and in the USA in 1996 for the control of bacterial infections of the respiratory tract of cattle (Doublet *et al.*, 2002). Florfenicol is bacteriostatic, and, like chloramphenical acts by binding to the 50S subunit of the bacterial ribosome disrupting bacterial protein synthesis (Schlünzen *et al.*, 2001). Florfenicol has been shown to be having spectrum activity similar to chloramphenicol, but acts at lower concentrations and is active against chloramphenicol-resistant bacteria (Graham *et al.*, 1998), whether caused by CAT or mediated by the CmlA efflux pump (Bissonnette *et al.*, 1991; Cloeckaert *et al.*, 2001).

1.8.8.1 Florfenicol resistance

Florfenicol resistance is assumed to be associated with efflux of the antibiotics (Du *et al.*, 2004). The *flo* gene was described first in *P. piscicida* from fish in Japan (Kim & Aoki, 1996) and is approximately 50% similar to the efflux gene *cml*. It is now found in a wide range of bacteria, including *S.* Typhimurium DT104 (Briggs & Fratamico, 1999), *E. coli* (Blickwede & Schwarz, 2004) and *Vibrio cholerae* (Hochhut *et al.*, 2001). However, despite this, little resistance has been detected in the major bacteria of cattle (Priebe & Schwarz, 2003; Blickwede & Schwarz 2004): on the other hand florfenicol resistance has emerged in China, even though it is not licensed for use there (Du *et al.*, 2004).

1.8.9 Glycopeptides

Glycopeptides are infrequently used in animals, so until recently, little work had been done on glycopepetide resistance in bacteria from domestic animals. These drugs are used to treat Gram-positive bacterial infections, and act by binding to the D-alanyl–D-alanine side chains of peptidoglycan or its precursors, thereby preventing cross-linking of the peptidoglycan chain (Reynolds *et al.*, 1989). Glycopeptide drugs include vancomycin, teicoplanin and ristocetin

Vancomycin is the most important glycopeptide in clinical use. It is a naturally-occuring antibiotic, produced by *Streptomyces orientalis*. Introduced in 1956, it is mainly used to treat severe infections such as staphylococcal and enterococcal infections (MRSA) in humans. Teicoplanin can be used in a similar way, but resistance to vancomycin usually involves resistance to teicoplanin as well (Fekety *et al.*, 1995).

Glycopeptide resistance is discussed further below in the section on vancocmycin resistant enterococci (VRE).

1.8.10 Macrolides

The macrolides include erythromycin, azithromycin, clarithyromycin, and tylosin, which is available for veterinary use only. They are bacteriostatic and inhibit bacterial synthesis by binding to 23S rRNA within the 50S ribosome, preventing protein synthesis. They have a fairly narrow spectrum of activity and Gram-negative bacilli, with some exceptions that include campylobacters, are often resistant.

1.8.10.1 Macrolide resistance

Macrolide resistance is by modification of the ribosome by methylation, and this leads to cross-resistance to macrolides, lincosamides and streptogramin B, known as the MLS_B phenotype (Weisblum, 1995). Expression of the MLS_B phenotype can be inducible or constitutive (Leclercq, 2002), the inducible phenotype being encoded *erm* (erythromycin ribosome methylase) genes. The most important macrolide resistance genes found in pathogenic bacteria are *ermA, ermB, ermC* and *ermF*, and these are often plasmid encoded (Weisblum, 1995, Roberts *et al.*, 1999). Other, efflux mechanisms have been found in certain Gram-positive populations (Arpin *et al.*, 1999) encoded by *mef, msr*, and *vga* genes.

1.8.11 Vancomycin resistant enterococci (VREs)

Vancomycin resistant enterococci have emerged as important nosocomial pathogens over the last decade (Aarestrup *et al.*, 1998; Butaye *et al.*, 2000; Casewell *et al.*, 2003). In Europe and the USA, VRE were first described in 1987 (Goossens *et al.*, 1997; Leclercq *et al.*, 1988) and in Japan in 1996 (Fujita *et al.*, 1998). Since then, VRE infections, often as outbreaks, have been reported world-wide (Uttley *et al.*, 1988; Devriese *et al.*, 1996; Goossens *et al.*, 1997; Bager *et al.*, 1997).

Enterococcus faecium and *E. faecalis* are responsible for almost all nosocomial enterococcal infections in humans, and vancomycin resistance in these is associated with the *vanA* and *vanB* genes, both of which encode high levels of resistance to vancomycin. The VanC phenotype, encoded by the *vanC-1, vanC-2* and *vanC-3*

genes, is associated with *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* respectively (Leclercq *et al.*, 1992), which rarely cause human disease, and the VanC phenotype is associated with only low-level resistance. VanC resistance appears to be intrinsic, and not transferable like *vanA* and *vanB*. Finally, *vanD* and *vanE* –encoded resistances appear to be associated with E. faecium and E. faecalis, respectively, but again are not transferable.

It remains unclear, and controversial, as to whether animal VRE have been transferred to humans or not (Butaye *et al.*, 2000). The demonstration of genetic differences between animal and human enterococci (Van den Braak *et al.*, 1998), and the similarity of vancomycin resistant genes recovered from animals and humans (Willems *et al.*, 1999) together might suggest that resistant genes may have moved before, rather than the enterococci themselves. The emergence of VRE has been attributed to the use of glycopeptides (avoparcin) in as growth promoters in food animals (Bager *et al.*, 1997), and as a result avoparcin has been banned in the EU since 1997. The ban was followed by a drop in the prevalence of VRE in different European countries. In the USA avoparcin was never used in animals and it is believed that high levels of resistance emerged there from the over-use of vancomycin in human medicine. It is interesting that 5-10% of healthy people are colonized with vancomycin resistant enterococci in the European Union where avoparcin was used (Endtz *et al.*, 1997), but in the USA where this drug was never used, VRE are absent from healthy people (Coque *et al.*, 1996). Streptogramins are now the drugs of choice for the treatment of VREs (Smith *et al.*, 2003). The streptogramin virginiamycin has also been banned as a growth promoter, along with the related compounds dalfopristin and quinupristin (van den Bogaard *et al.*, 2000).

If VREs are found in horses it seems most likely that this would be due to the transfer of these pathogens from other animals as horses are rarely treated with glycopeptides, and no growth promoters are used in the horse (Devriese *et al.*, 1996).

Chapter 2
General Materials and Methods

Details of the materials not mentioned in this chapter, and their sources, are listed in appendix 1.

2.1 Collection of faeces from horses.

Fresh faecal samples were collected and placed into a universal bottle. Samples were then returned to the laboratory for processing, either immediately or by first class post.

2.2 Preparation of faecal sample mixture

On arrival at the laboratory, 5g of faeces was mixed with 5ml of brain-heart infusion broth containing 5% glycerol in a universal bottle. The remainder of faecal suspension were then stored at -80°C.

2.3 Isolation of bacteria

2.3.1 Escherichia coli

One ml of the faecal suspension (2.2 above) was added to 3ml of brilliant green broth and incubated aerobically for 24 hours at 37°C. It was then streaked onto eosin methylene blue agar (EMBA) and again incubated for 24 hours at 37°C. Three metallic colonies typical of *E. coli* were randomly selected and plated on nutrient agar, and these plates were again incubated for 24 hours under the same conditions for further confirmation by the following biochemical and bacteriological tests.

Positive control of known *E. coli* was used initially, but because *E. coli* was easy to culture, these were discarded later on.

Gram stain: A drop of sterile water was placed on a clean slide and a part of a colony added, mixed and allowed to air dry at ambient temperature, and then fixed by passing through a Bunsen flame. The Gram stain was performed as follows:

1. The slide was flooded with crystal violet for 1 minute
2. Lugols iodine was added for a further 1 minute.
3. The slide was than washed with acetone, followed by water.
4. Finally, safranin was added for 2 minutes.

Under oil immersion x100 magnification, *E. coli* appeared as Gram negative (i.e. pink) rods.

Oxidase test: Using a sterile loop, a few colonies were picked and placed onto an oxidase strip. A change of colour to purple after 30 seconds indicated a positive reaction and oxidase activity. *E. coli* should be negative in this test and not change the colour of the strip.

Catalase test: With a sterile loop, a few colonies were picked and placed onto a glass slide, and mixed with a few drops of 3% hydrogen peroxide. Catalase breaks down hydrogen peroxide into water and oxygen, so the production of bubbles indicated positive reaction. *E. coli* should be catalase positive in this test.

Indole test: Colonies were grown on tryptone soy agar for 24 hours, then a strip of filter paper soaked in Kovac's reagent was placed across the colonies. *E. coli* caused a change in colour to pink as a result of indole production.

Lactose fermentation: Suspected colonies were plated onto MacConkey agar and incubated for 24 hours at 37°C. *E. coli* ferments the lactose and produces pink colonies.

Citrate utilization test: Colonies were inoculated onto Simmon's citrate agar and incubated at 37°C for 48 hours. *E. coli* can not use citrate as a carbon source, and therefore grow poorly on this media.

2.3.2 *Salmonella*

One ml of the faecal suspension was added to 10ml of buffered peptone water and incubated aerobically at 37°C for 24 hours. Following incubation, 1ml was transferred to Rappaport-Vassiliadis broth (10ml) and incubated in aerobic conditions at 42°C for 24 hours. A loopful of broth was then inoculated onto xylose lysine decarboxylase (XLD) agar and incubated for further 24 hours. Suspected colonies, based on colour, were transferred onto nutrient agar and cultivated in the same way for further confirmation by biochemical and laboratory tests.

The positive control was a confirmed isolate of *Salmonella enterica* Typhimurium.

Lactose fermentation test: As for *E. coli*, but salmonellas do not ferment lactose, and so produce yellow colonies.

Agglutination tests: An emulsion was prepared on glass slide of the suspected *Salmonella* colonies with few drop of sterile water. For each isolate, two emulsions were made on each slide. A drop of polyvalent O and H antisera was added to each drop and the slide was rotated gently from side to side. *Salmonella* spp. should show agglutination with both O and H antisera.

API tests: Api 20E test strips were used according to the manufacturer's instructions to biochemically confirm isolates as *Salmonella* spp..

2.3.3 *Campylobacter*

Approx. 0.2ml of the faecal mixture was added to 3ml of Campylobacter enrichment broth (containing 5% lysed horse blood and cefoperazone 20mg/L, vancomycin 20mg/L, trimethoprim 20mg/L, cycloheximide 50mg/L). Broths were incubated under microaerophilic conditions (N_2 74%, O_2 11%, H_2 3%, CO_2 12%) at 37°C for 24 hours and then plated on to modified Campylobacter selective agar (mCCDA) (containing Cefoperazone 32mg/L and Amphotericin 10mg/L) and incubated at 37°C for 2-3 days. Suspect colonies were transferred to Columbia agar (with 5% defibrinated horse

blood) for incubation under the conditions listed above. Colonies were then further investigated after 48 hours incubation.

A *C. jejuni* strain (NCTC) was used as a positive control.

Gram stain: This was done as described previously. *Campylobacter* spp., are Gram negative small, curved rods.

Catalase and oxidase tests: These tests were as described above. *Campylobacter* spp. are mostly positive for both tests, although some species can be catalase negative.

Growth in oxygen: *Campylobacter* spp., are microaerophilic organisms and do not grow in aerobic conditions.

Molecular identification: *Campylobacter* spp., were confirmed by a genus specific PCR and assigned to species by different PCR assays. Details of the PCR method are given later, in section 2.6.

2.3.4 Isolation of vancomycin resistant enterococci (VRE)

Approx 0.2ml of the faecal mixture was added to kanamycin aesculin azide broth (containing 0.5mg/l of bile salts), and was incubated under aerobic conditions at 37°C for 24 hours. A loopful was then streaked out onto kanamycin aesculin azide agar with a 5µg vancomycin disc placed in the middle of each plate, and incubated at 37°C for 48 hours. Black colonies which grew close to the 5µg vancomycin disc were picked and cultured on Columbia agar containing 5% horse blood and incubated as above for 24 hours for further identification as described below.

Gram stain: as described above - enterococci appear as Gram-positive (purple) cocci.

Molecular identification: A multiple PCR was used to identify species and antibiotic resistance genes as detailed later in 2.6.1.
Positive controls were kindly provided by Dave Mallon of Medical Microbiology, University of Liverpool and were further used in PCR reactions later.

2.4 Antibiotic susceptibility

2.4.1 Antibiotic susceptibility tests of E. coli

Antibiotic susceptibility testing was performed according to the BSAC guidelines. The methods and the guidelines were according to the BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing Version 2.1.4 May 2003, with updates taken from www.bsac.org.uk. This version provides the protocols for the following methods as well as the breakpoint diameters and MICs. Further details are given in Appendix 1C.

2.4.1.1 Disc diffusion test

E. coli isolates were grown overnight on nutrient agar and suspended in 3ml of sterile water consistent to a 0.5 McFarlands standard. A swab from the suspension was then streaked onto ISO-sensitest agar plate using a rotary plater. Antibiotic discs were then placed on the surface of ISO plates using a sterile needle. The following antibiotic discs were used:

- Ampicillin (10μg)
- Apramycin (30μg)
- Chloramphenicol (30μg)
- Nalidixic acid (30μg)
- Tetracycline (30μg)
- Trimethoprim (2.5μg).

Plates were incubated for 24 hours at 37°C in aerobic conditions. The zones of inhibition around the discs were measured in millimetres and interpreted according to BSAC guidelines as described and updated by BSAC (www.bsac.org.uk).

2.4.1.2 Minimum inhibitory concentrations (MIC) of antibiotics against E. coli

The MICs of the antibiotics listed above were determined using antibiotic-incorporated media. Standard microbiology methods for the determination of the

minimum inhibitory concentration (MIC) of antimicrobial agents were used and are described in detail in Appendix 1-D and materials are menthioned in Appendix 1.

2.4.1.3 Further antibiotic susceptibility tests of resistant E. coli isolates

Isolates of *E. coli* resistant to specific antibiotics were also tested further against the following antibiotics by the disc diffusion test.

Trimethoprim resistant E. coli

E. coli that showed resistance to trimethoprim were tested against a wider range of antibiotics as this kind of resistance is often carried on integrons, which also carry other resistance genes. Briefly, the antibiotics were: spectinomycin, streptomycin, sulphamethoxazole and gentamicin, and these are listed in detail in appendix 1-C.

β–lactam antibiotics

E. coli isolates resistant to ampicillin were tested for the production of β-lactamase using the chromogenic cephalosporin, nitrocefin. A colour change from yellow to pink within 10 minutes indicated a positive reaction and β-lactamase production.

Ampicillin resistant isolates were also tested against a range of β-lactam drugs including cephalosporins using the disc diffusion method described above according to BSAC Guidelines. The drugs used were:

- Aztreonam (30μg)
- Co-amoxyclav (10μg)
- Ceftazidime (30μg)
- Cefoxitin (30μgl)
- Cefuroxime (30μg)
- Piperacillin (85μg)
- Tazobactam (75μg)

Inducible β-lactam resistance

Ampicillin resistant isolates were further tested for the presence of inducible resistance genes by culturing the ampicillin resistant *E. coli* isolates on plates containing an ampicillin disc. After 24 hour incubation, growth around the disc was tested for β-lactamase production.

Fluoroquinolones

Nalidixic acid resistant *E. coli* were tested for resistance to ciprofloxacin (1μg) by disc diffusion. The MIC of ciprofloxacin was determined according to BSAC guidelines.

Florfenicol

E. coli resistant to chloramphenicol were tested for resistance to florfenicol (30μg) using the disc diffusion test according to BSAC guidelines.

2.4.2 Antibiotic susceptibility testing of Salmonella spp.

This was done according to BSAC guidelines as set out for *E. coli* above. The antibiotics used for testing were:

- Ampicillin (10μg)
- Apramycin (30μg)
- Chloramphenicol (30μg)
- Tetracycline (30μg
- Nalidixic acid (30μg)
- Trimethoprim (2.5μg).
- Sulphamethoxazole(100μg)
- Streptomycin(10μg)
- Gentamicin(10μg)
- Ciprofloxacin (1μg) for nalidixic acid resistant isolates

2.4.3 Antibiotic susceptibility testing of Campylobacter spp.

This was done according to BSAC guidelines and method is described in section 2.4.1, except for the replacement of iso-sensitest agar with Mueller Hinton agar supplemented with 10% lysed horse blood. The antibiotics used for testing were:

- Co-amoxyclav (30μg)
- Ampicillin (10μg)
- Erythromycin (5μg)
- Nalidixic acid (30μg)
- Trimethoprim (2.5μg)
- If isolates were resistant to nalidixic acid they were also tested against ciprofloxacin (1μg).

2.4.4 Antibiotic susceptibility testing of vancomycin resistant enterococci (VREs)

Confirmed VRE isolates were tested for antibiotic resistance using the disc diffusion method and drug concentrations according to BSAC. The method used was that set out previously in 2.4.1, except that Iso-sensistest agar supplemented with 5% defibrinated horse blood was used. Briefly, the antibiotics used were:

- Ampicillin (10μg)
- Azithromycin (15μg)
- Gentamicin (200μg)
- Imipenem (10μg)
- Linezolid (10μg)
- Meropenem, (10μg)
- Synercid (15μg)
- Teicoplanin (30μg)

2.5 Conjugation experiments

Mating experiments were done using nalidixic acid resistant *E. coli* K12 as recipient (and were therefore only undertaken with isolates susceptible to nalidixic acid). The *E. coli* K12 recipient was inoculated into 20 ml nutrient broth and the donors (resistant isolates) were inoculated into 2ml nutrient broth and incubated at 37°C. After overnight incubation, 4 ml of the recipient (K12) culture was transferred to the donor (resistant isolate) culture and incubated for one hour at 37°C. Iso-sensitest agar plates were prepared containing nalidixic acid (30μg/ml) plus the antibiotic drug to which the donor isolate was resistant to and the concentration needed was obtained by using the same preparation protocol that have been used for the MICs determination as described in appendix 1-D. The concentration of the antibiotics were as follows (ampicillin 8mg/L, chloramphenicol 8mg/L, tetracycline 4mg/L, trimethoprim 4mg/L). A loopful of mating broth was streaked out and incubated for 24 hours on each antibiotic incorporated agar plate.

Resistant colonies were observed and picked for further characterization.

2.6 Molecular methods

2.6.1 Polymerase chain reaction (PCR)

E. coli and Campylobacter DNA extraction.

A few colonies from a fresh culture were suspended in 500μl sterile water in an Eppendorf tube and boiled for 20 minutes at 100°C, cell lysates were kept at 4°C for up to two weeks.

Alkaline extraction of vancomycin resistant enterococci)

The alkaline digest method (Bown *et al.*, 2003) was used to extract DNA from a few colonies of bacteria which were resuspended in 500 μl of 1:20 dilution of 1.25% ammonia (NH_3) and than heated at 100°C for 15-20 minutes (until the total volume was roughly halved, by which time most of the ammonia had also evaporated).

PCR identification of Campylobacter

Method 1: (Linton *et al.*, 1996). This method was used to identify isolates to the *Campylobacter* genus, and is specific for the 16S rRNA region. The total reaction volume was done in 25µl containing: 20mM Tris HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.2mM DNTP's, 0.4µM of each primer, 0.625U *taq* DNA polymerase and 1µl DNA template. The reaction was run as: 25 cycles of 94°C for 1 minute then 55°C for 1 minute and 72°C for 1 minute.

Method 2: (Houng *et al.*, 2001). This PCR enables the identification of *C. jejuni* and *C. coli.* Again, the total reaction volume was 25µl, this time containing 25µM each primer, 0.1mM of each DNTP, 1.5mM $MgCl_2$, 1x PCR buffer, 1.25U *taq* DNA polymerase and 1µl DNA templates. The reaction details were; 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 1 minute.

The primers used were:

	Primers sequences 5' to 3'	Ampicon size(bp)
Method 1	GGATGACACTTTTCGGAG CATTGTAGCACGTGTGTC	816
Method 2		
C. jejuni:	CTGCTACGGTGAAAGTTTTGC GATCTTTTTGTTTTGTGC	645
C. coli :	GATTTTATTATTTGTAGCAGCG TCCATGCCCTAAGACTTAACG	783

PCR identification of enterococci and VREs

The method used was described by Dutka-Malen *et al.*, 1995. The reaction mix (25µl) contained 3.5mM $MgCl_2$, 1.25mM DNTP's, 0.75U *taq* polymerase, 10pMol/µl of each primer, and 1µl DNA template and were run at 94°C for 2minutes, 30 cycles of 94°C per 1 minute, 54°C for 1 minute, 72°C for 1 minute and a finally 72°C for 10 minutes.

The primers used were:

Primers sequences		Amplicon size(bp)
vanA	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732
vanB	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635
E. gallinarium	GGTATCAAGTGAAACCTC CTTCCGCCATCATAGCT	822
E. casseliflavus	CTCCTACGATTCTCTTG GCAGCAAGACCTTTAAG	439
vanD	TTAGGCGCTTGCATATACCG TGCAGCCAAGTATCCGGTAA	461
E. faecalis	ATCAAGTACAGTTAGTCTT ACGATTCAAAGCTAACTG	941
E. faecium	GCAAGGCTTCTTAGAGA CATCGTGTAAGCTAACTTC	550

PCR identification of antibiotic resistance genes

Trimethoprim

PCR was used in order to identify the *dfr1, 9, 12* and *7-17* genes.

dfr1and dfr9 genes

The method was described by Gibreel & Sköld (1998). PCRs were performed in 25µl volumes containing 50pMol/µl each primer, 23µl 1.1xReddyMix, PCR MasterMix1.5mM $MgCl_2$ [(which is composed of the following: 1.25units *taq* DNA polymerase, 75mM Tris-Hcl (pH 8.8 at 25°C, 20mM (NH4), 1.5mM $MgCl_2$, Tween 20 (0.01%(v/v))], 0.2mM each dATP, dCTP, dGTP, and dTTP), and 1µl DNA template. PCR conditions were 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes. Positive controls were isolates that were consistently positive.

dfr 12, dfr 7-17 genes

The method used was that described by Lee *et al.* (2001) in order to identify each gene. PCR reactions for all types was carried out in 25µl volumes containing 1µl of each DNA template, 50pMol/µl of each primer, 1.1xReddyMix, PCR

MasterMix1.5mM $MgCl_2$ (as previous) and 1µl DNA template. Each gene reaction was run separately. PCR products of *dfrA7*and *dfrA17*, *dfrA12* were identified by enzymatic cleavage with 20U *pst1*, and *EcoRV* respectively. Positive controls were isolates that were consistently positive.

The primers used were:

Primers sequences 5' to 3'		Ampicon size(bp)
Dfr1	ACGGATCCTGGCTGTTGGTTGGACGC CGGAATTCACCTTCCGGCTCGATGTC	254
Dfr9	ATGAATTCCCGTGGCATGAACCAGAAGAT ATGGATCCTTCAGTAATGGTCGGGACCTC	399
dfrA7, dfrA17	GTCGCCCTAAAACAAAGTTA CGCCCATAGAGTCAAATGT	195
dfr12	CCGTGGGTCGATGTTTGATG GCATTGGGAAGAAGGCGTTCAC	485

Tetracycline

The method used was that described by Ng *et al.* (2001). This PCR detects the presence of six genes responsible for tetracycline resistance: *tetA, tetB, tetC, tetD, tet E* and *tetG*. PCRs were run as two groups:

Group 1 (*tet A, G, E*): 25µl reaction contains 0.3 mM DNTP's, 4mM $MgCl_2$, 0.25µM each primer, 2.5U *taq* polymerase and 1µl DNA template.

Group 2 (*tet B, C, D*): as described for group 1 but with 3mM $MgCl_2$ and 1µl each primer. The reactions cycle for both groups were: one initial cycle at 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, then 55°C for 1 minute, and 72°C for 1.30 minutes.

The primers used were:

	Primers sequences 5' to 3'	Ampicon size(bp)
*tet*B	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659
*tet*C	CTTGAGAGCCTTCAACCCAG ATGGTCCTCATCTACCTGCC	418
*tet*D	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC	787
*tet*A	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	210
*tet*E	AAACCACATCCTCCATACGC AAATAGGCCACAACCGTCAG	278
tetG	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	468

Positive controls were respectively *tetA*, *tetB* positive isolates that were consistently positive.

<u>***Chloramphenicol***</u>

Two protocols were applied in order to identify *catI, catII, catIII* and *cml* genes.

The method used was that described by Vassort-Bruneau *et al.* (1996) identifies three different genes responsible for chloramphenicol resistance, *catI, catII* and *catIII.* The reaction was 25μl total, containing 25pMol/μl primers, 23 μl of 1.1xReddyMix, PCR MasterMix1.5mM $MgCl_2$ (as previous) and 1μl DNA template. The reaction program was 30°C for 5 minutes, than 30 cycles at 94°C for 30 seconds, followed by 50°C for 30 seconds and finally 72°C for 1.30 minutes.

The method used for identification of *cmlA* gene was as described by Keyes *et al.*, (2000). Reactions were carried out in 25μl volumes containing 50pMol/μl of each primer, 23μl of 1.1xReddyMix, PCR MasterMix 1.5mM $MgCl_2$ (as previous) and 1μl

DNA template. The reaction was run in the following program; 30 cycles of 94°C for 1 minute, 40°C for 1minute and 72°C for 15 seconds.

Primers used were:

	Primers sequences 5' to 3'	Ampicon size(bp)
CatI	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	585
CatII	ACACTTTGCCCTTTATCGTC TGAAAGCCATCACATACTGC	495
CatIII	TTCGCCGTGAGCATTTTG TCGGATGAGTATGGGCAAC	508
cmlA	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	698

Positive controls were *CatI* positive isolates that were consistently positive.

Ampicillin

The methods for this PCR were published by Pitout *et al.* (1998). This PCR was used to detect both the presence of the *tem* and *shv* genes and the protocol was as follows: every reaction of 25μl contained 23μl 1.1xReddyMix, PCR MasterMix 1.5mM $MgCl_2$ (as above), 0.2mM primers and 1μl of DNA template. The PCR programme was denaturation at 96°C for 15 second, then 24 cycles of 96°C for 15 seconds followed by 50°C for 15 seconds and 72°C for 2 minutes.

Primers used were:

	Primers sequences 5' to 3'	Amplicon size (bp)
Shv	CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGC	885 bp
Tem	TCGGGGAAATGTGCGCG TGCTTAATCGTGAGGCACC	971 bp

Positive controls for *Tem* and *Shv* were provided by the department.

Gel electrophoresis and visualization

Amplicons were visualised by electrophoresis of 20µl of reaction product with 6.2µl ethidium bromide through 1.5% agarose gels in 1 x Tris Acetate EDTA (TAE) buffer. Electrophoresis conditions were 120V for 75-80 minutes, except that VRE PCR products were run for 95 minutes at 110V. A molecular weight marker (100bp ladder) was used to estimate the molecular weight of the products under UV light using the BioRad gel documentation system.

2.6.2 Rapid pulse field method (PFGE) for E. coli and salmonellae

The protocol is displayed on http://www.cdc.gov/pulsenet/protocols.htm by the Center for Disease and Control Prevention (CDC): "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)."

The protocol can be summarized as follows:

- A loopful of a 24 hour culture of *E. coli* was inoculated into 2ml of cell suspension buffer (CSB) [100Mm tris, 100Mm EDTA, pH 8.0] in a sterile plastic bijou.
- The suspension was then diluted into 1:10 and measured at optical density of OD_{610}.
- 200µl of the suspension was adjusted to OD_{610}= 1.35 and 10 µl 20mg/ml proteinase K solution (stored frozen) was added.
- An agarose mixture was freshly made from 1% Bio-Rad PFGE-grade agarose, 1% SDS in 1xTE buffer, and this was added and mixed, then quickly transferred into duplicate plug molds and left at 4°C. Two plugs were made for each isolate.
- The plugs were then placed into a bijoux containing 3ml of cell lysis buffer (CLB) [50 Mm tris, 50mM EDTA, pH 8.0, +1% sarcosyl] and 15 µl proteinase K, and incubated with shaking (175-200rpm) at 54°C for 2hrs.

- The buffer was removed and plugs were washed by adding 3ml pre-heated sterile water, and incubated at 54°C for 15 minutes. This step was performed twice.
- The wash was than removed and 3 ml pre-heated 1xTE buffer was added and incubated at 54°C for 15 minutes. This step was performed four times in total.
- The buffer was then removed and one plug was transferred into a 1.5ml tube containing 200 µl 1x restriction buffer (specific for *XbaI*) and incubated at 37°C for 15 minutes (the remaining plug was kept 1ml of 1xTE and stored at 4°C).
- The buffer was again removed and 200 µl 1x restriction buffer containing 50U *XbaI* was added and incubated at 37 °C for 2 hours.
- 2L of 0.5xTBE buffer was prepared and added to CHEF-DRIII system tank.
- A 1% agarose gel in 0.5x TBE was prepared and half of the digested plug was loaded onto the gel and the other half was returned into the tube containing 200 µl 1x restriction buffer.
- A Lambda genomic molecular weight marker was loaded onto the first, middle and last lane.
- All plugs including the Lambda marker were covered with 1% agarose and placed into the tank.
- The gel was run at 14°C for 20 hrs, initial switch 2.2s, and final switch 54.2s at 6V/cm^2.
- The gel was stained in ethidium bromide solution for 20-30 minutes and visualized under UV light using the BioRad gel doc system.

Chapter 3
The prevalence of zoonotic enteric bacteria and antibiotic resistance in the horse

3.1 Introductions

Little is known about companion animals, such as the horse, as a source of zoonotic enteric bacteria or antibiotic resistant bacteria, most previous research having been done on food animals. This chapter describes a survey of faeces from horses for *E. coli*, *Salmonella*, *Campylobacter*, and vancomycin resistant enterococci (VRE), and also antibiotic resistance in commensal *E. coli*.

E. coli is known to colonize the gastrointestinal tract of many mammals, including the horse, and become part of the normal intestinal flora (Bettelheim *et al.*, 1996). *E. coli* can also be found in the environment, in soil and in water. Although most *E. coli* are not considered pathogenic, some strains express genes associated with pathogencity in humans and/or other species (Gaastra *et al.*, 1996; Carvalho *et al.*, 2003). These *E. coli*, while being pathogenic to humans, may cause no disease in their non-human hosts (Bettelheim *et al.*, 1996): for example O157:H7 are not normally pathogenic in cattle (DeanNystrom *et al.*, 1997; Wilson, 2005). Indeed, studies of a range of virulence factors and their associations with disease in dogs (Beutin *et al.*, 1999), horses (van Duijkeren *et al.*, 2000), calves (Aidar *et al.*, 2000), and also primates (Carvalho *et al.*, 2003) have found very different degrees of association with diarrhoea in different host species.

In a study conducted between 1987 and 1989 in the UK and Ireland, the prevalence of *E. coli* in horses was found to be similar in normal and in diarrhoeic foals (Browning *et al.*, 1991), and in a study of adult horses, the number of *E. coli* genotypes, and the prevalence of virulence factors (e.g. F17) did not differ significantly between horses with and without diarrhoea (van Duijkeren *et al.*, 2000). However, another study by Holland *et al.*, 1996 did find a higher proportion of virulence genes (*STb*, *STaP*, *LT*, *slt1*, *slt2* and *eae*) in diarrhoeic than in healthy horses.

The main cause of infectious gastroenteritis in humans is *Campylobacter* of which most cases are due to infection with *C. jejuni*. *Salmonella* is also a common cause of enteric disease in man, with both *Salmonella* and *Campylobacter* believed to be mainly food-borne pathogens (Desenclos *et al.*, 2002). However, infection by other routes is also possible and horses are known hosts of both these bacteria, albeit usually with a low prevalence (Browning *et al.*, 1991).

Campylobacter spp infection in horses has not been widely investigated, and animals such as cattle, sheep and pigs are believed to constitute a larger potential source of *Campylobacter* infection for man (Manser & Dalziel, 1985). Among domestic livestock, high faecal carriage rates of *C. jejuni* and *C. coli* have been reported for poultry, pigs, cattle, and sheep (Prescott & Munroe, 1982). *C. jejuni* has been reported as the cause of enteritis, and *C. coli* associated with gastrointestinal lesions in horses (Al-Mashat & Taylor, 1986). Furthermore, in a case of an aborted 7-month-old equine foetus, *C. fetus* subsp fetus was detected in the fetal stomach contents as well as other organs (Hong & Donahue, 1989). In another study the same organism was cultured from jugular venous blood, but not from any other organs from a case of granulomatous enteritis (Johnson & Goetz, 1993). In a survey of several species of domestic animals *Campylobacter* spp were not isolated from any horse (Rosef *et al.*, 1983), nor from a survey of horses with and without diarrhoea (Prescott & Bruinmosch, 1981).

Salmonella has also been recognized as an important food-borne pathogen for humans (Gorman & Adley, 2004), with many cases attributed to contact with or consumption of contaminated products such as poultry meat. *Salmonella* infections in horses has been well studied and discussed as a nosocomial infection within equine hospitals, and most reports of equine salmonellosis describe isolates collected in hospitals or as a result of hospital outbreaks. *Salmonella* spp., shedding in horses is often associated with colic cases as reported by Kim *et al*, (2001), from which shedding was found to be higher when diarrhoea was evident during hospitalization. In another report, horses with colic were 4.2 times as likely to have *Salmonella* isolated as those admitted to a hospital for other reasons (Hird *et al.*, 1986). Other risk factors for shedding *Salmonella* include antibiotic administration, the route of antibiotic administration, and stress (Hird *et al.*, 1984; Hird *et al.*, 1986; Kim *et al.*, 2001). A study by Schott *et*

al. (2001), reported a foal with multi-drug resistant *S.* Typhimurium infection, and found that antibiotic sensitivity testing and pulsed-field gel electrophoresis were useful techniques for tracing the source of the case and showing it to be nosocomial. Moreover *Salmonella* was reported to be isolated from faeces and also variety of environmental surfaces in an equine animal hospital (Ewart *et al.*, 2001).

The isolation of *Salmonella* spp. from different animals resistant to one or more antibiotics has risen in the United Kingdom as shown by Low *et al.* (1997), and between 1990 and 2000, there has been a dramatic increase in the prevalence of multi-drug resistance (MDR - resistance to four or more antibiotics) in England and Wales (Threlfall *et al.*, 2004). *S.* Typhimurium is well documented as the most likely serotype to be MDR, and definitive phage type 104 (DT104) is considered a major public health problem, as it is resistant to five antimicrobial agents: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (referred to as ACSSuT). *S.* Typhimurium DT104 has become a major cause of enteric infection in Britain, USA, and Canada (Brenda *et al.*, 2002) and has been documented worldwide (Wall *et al.*, 1994). It was first identified in the UK in 1984 (Threlfall *et al.*, 1996) and emerged in the early 1990s as the dominant type of *Salmonella* in the USA (Sanchez *et al.*, 2002). However, *S.* Typhimurium DT104, which has emerged as a common cause of salmonellosis in humans and cattle, has rarely been reported in the horse.

Vancomycin-resistant enterococci (VRE) are important nosocomial pathogens in humans (Boyle *et al.*, 1993). The recent emergence of VRE in humans worldwide has led to a number of studies investigating its prevalence in domestic and wild animals and other sources (Sundsfjord *et al.*, 2001). Although it now seems most likely that VREs are not zoonotic (or at least, zoonotic spread is not important in the overall epidemiology of infection), animals as a source of infection remain a possibility, and no previous extensive study appears to have been done on VRE in horses.

VRE have been isolated from various farm animals and their products (Bates *et al.*, 1997; Bager *et al.*, 1997) and more recently from horses also (Devriese *et al.*, 1996). Mostly, the VanA phenotype has been recorded, although VanC phenotype has also been reported (Rice *et al.*, 2003).

In addition to being a source of agents directly pathogenic to human beings, animals, including horses, or rather their normal enteric 'flora', might be a source of antibiotic resistance to clinical isolates. Research on antibiotic resistance is scarce in horses, and the potential for transmission from horse to man (or in reverse) has been largely ignored, despite horses playing having close and common contact with humans.

The aims of the work described in this chapter therefore were to investigate the prevalence of *E. coli*, *Salmonella*, *Campylobacter* and VRE in horse faeces, and the prevalence of antibiotic resistant *E .coli*.

3.2 Materials and methods

3.2.1 Sample collection

Horse faecal samples were collected from an equine veterinary hospital as well as two different riding establishments, all on the Wirral Peninsula in North West England. Samples were processed and tested for *E. coli*, *Salmonella* spp., *Campylobacter* spp. and VRE as described Chapter 2. Samples were processed with a few hours of collection, and subsamples stored at -80°C.

3.2.2 Sources of Samples

Hospital samples: - 109 faecal samples from 64 horses were collected at the Philip Leverhulme Equine Hospital (PLEH) of Liverpool University (samples are listed in Appendix 2A).

Riding school ('premises A'): - 112 horse faecal samples were collected from 37 horses at a riding school in Wirral (samples are listed in appendix 2B).

Livery yard ('premises B'): - 43 faecal samples were collected from 35 horses at a livery yard in Wirral (samples are listed in appendix 2C).

A questionnaire (Appendix 1.F.1) was designed and completed for the horses.

3.2.3 Isolation of bacteria and identification

3.2.3.1 *E. coli*

From each faecal sample, 3 colonies suspected of being *E. coli* were picked and subcultured for more detailed bacteriological and biochemical identification as described in Chapter 2.3.1.

3.2.3.2 *Salmonella and Campylobacter*

Suspect colonies were collected and further analyzed by bacteriological and biochemical tests for both of the bacteria except for *Campylobacter* which were

further confirmed by molecular means as described in as described in Chapter 2.3.2 and 2.3.3 respectively.

3.2.4 Antibiotic susceptibility tests

3.2.4.1 E. coli

Presumptive *E. coli* isolates were tested for antibiotic resistance using the disc diffusion method as described in Chapter 2. The antibiotics tested were ampicillin (if ampicillin resistant then isolates were also tested against other β-lactams, as listed in chapter 2.4.1), apramycin, chloramphenicol (and to florfenicol when isolates were resistant to chloramphenicol), nalidixic acid (and to ciprofloxacin if nalidixic acid resistant), tetracycline and trimethoprim, chosen to represent widely used antibiotics in human and veterinary medicine. The minimum inhibitory concentrations (MICs) for these drugs were also tested to confirm resistance, as mentioned in Chapter 2.4.1 and according to the method in appendix 1-D.

Antibiotic sensitivity tests and MIC determination were done according to the BSAC guidelines, using concentration breakpoints as listed in Appendix 1-C.

3.2.4.2 *Salmonella* and *Campylobacter*

The methods used for susceptibility testing are described in Chapter 2.4.2 and 2.4.3 and antibiotics used are listed in the same sections.
Briefly the drugs tested were:

- *Salmonella*: Ampicillin, Apramycin, Chloramphenicol, Tetracycline, Nalidixic acid, Trimethoprim, Sulphamethoxazole, Streptomycin, Gentamicin, and Ciprofloxacin
- *Campylobacter* : Co-amoxyclavulanate, Ampicillin, Erythromycin, Trimethoprim, Nalidixic acid and Ciprofloxacin

Drugs are also listed with their breakpoints and MICs value in appendix 1-C.

3.2.5 Vancomycin-resistant enterococci (VREs)

The isolation methods and the bacteriological and molecular methods for identification of suspect isolates are all described in Chapter 2.3.4, and the molecular methods for confirmation are described in 2.6.1 (Dutka-Malen *et al.*, 1995). The antibiotic susceptibility tests were as described in chapter 2.4.4, and the drugs tested were: Ampicillin, Azithromycin, Gentamicin, Imipenem, Linezolid, Meropenem, Synercid and Teicoplanin.

Drugs used are further listed in Appendix 1-C with their breakpoints and MICs values.

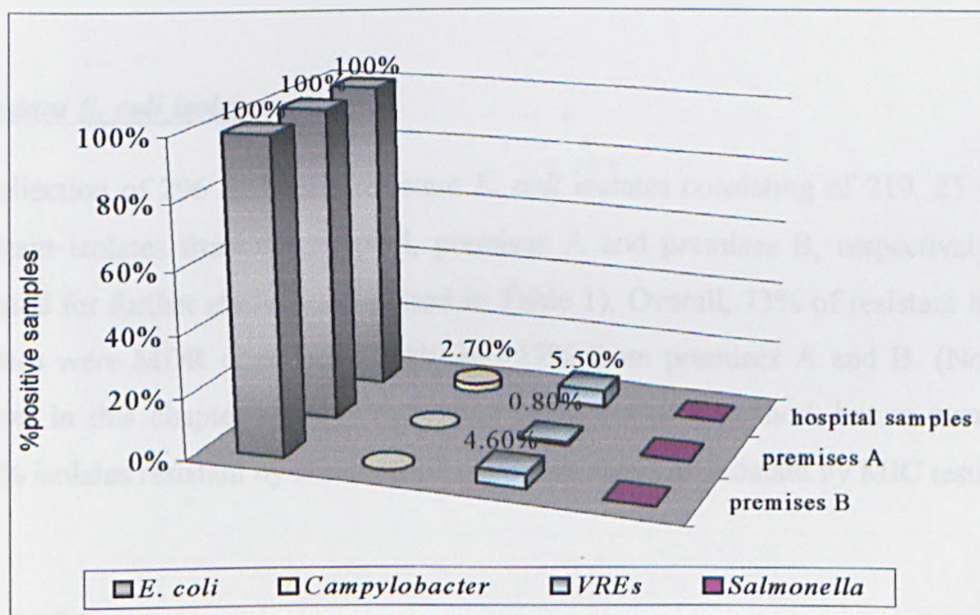
3.3 Results

Prevalence of bacteria and antibiotic resistance

General bacterial prevalence

As expected, *E. coli* was the most prevalent organism isolated from all equine faecal samples, but, perhaps more surprisingly, *Salmonella* was not isolated from any of the samples tested. *E. coli* was isolated from 100% of faecal samples, and VRE, although not common, were the second most frequently isolated bacteria in all the three horse populations, ahead of *Campylobacter* which was isolated only in samples from the hospital.

Figure1. The prevalence of *E. coli*, *Campylobacter*, VRE and *Salmonella* in horse faecal samples

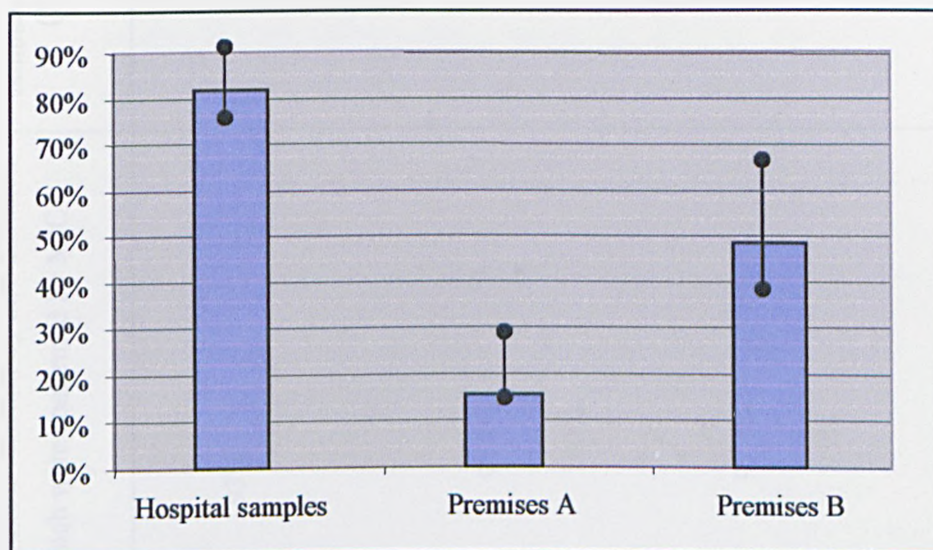


3.3.1 Prevalence *E. coli*

E. coli was isolated from all horse faecal samples, regardless of the horse population. Antibiotic resistant *E. coli* was isolated from all sources, but with a noticeable difference in the prevalence of resistant isolates at the different premises (Figure 2). Among samples from the hospital, 89/109 contained at least one antibiotic resistant *E.*

coli, whereas premises A and B had 17/112 and 21/43 resistant samples, respectively (Figure 2).

Figure2. The prevalence of resistant *E. coli* isolates: the proportion of samples containing antibiotic resistant *E. coli* with 95% binomial confidence intervals (CI)



Resistant E. coli isolates

A collection of 296 antibiotic resistant *E. coli* isolates consisting of 219, 25 and 52 resistant isolates from the hospital, premises A and premises B, respectively were selected for further study (summarised in Table 1). Overall, 73% of resistant hospital isolates were MDR compared to 4% and 17% from premises A and B. (Note: the figures in this chapter refer to resistance using the disc method, but in every case >90% isolates resistant by this method were confirmed as resistant by MIC testing.)

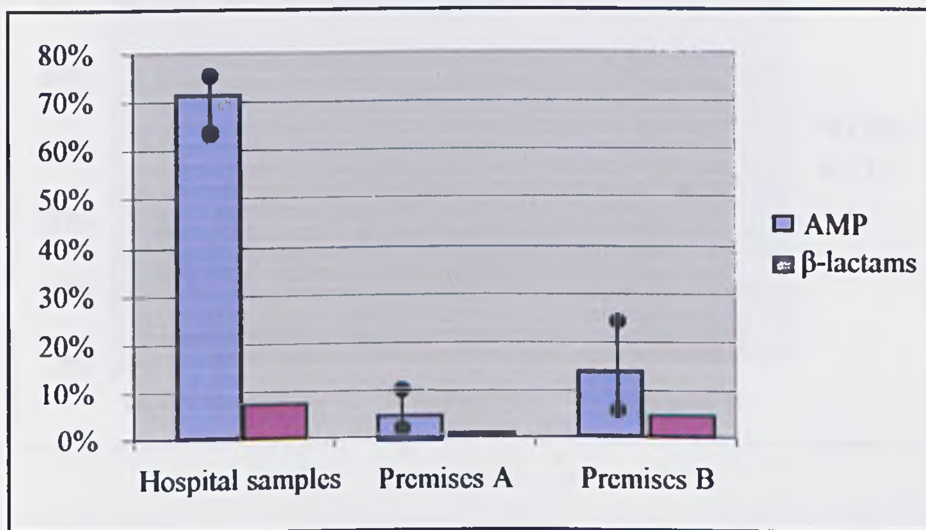
Table1. Summary of results for resistance to antibiotics of *E. coli* from all premises combined (264 faecal samples in total).

Antibiotic	Number of faecal samples with resistance *E. coli* by disc diffusion method (%)	Total number of resistant *E. coli* isolates	Proportion of isolates resistant by disc diffusion method, which were confirmed by MIC	Number of isolates that are MDR (% by faecal samples)
AMP	89 (33%)	191	93%	57 (21.5%)
APR	1 (0.3%)	1	-	1 (0.3%)
NAL	36 (13.6%	72	-	35 (13.2%)
CIP	28 (10.6%)	59	93.8%	28(%)
CHL	49 (18.5%)	102	100%	47 (17.8%)
FLO	9 (3.4%)	14	-	9
TET	92 (34.8%)	198	93.4%	57 (21.5%)
TRI	135 (51%)	279	95%	57 (21.5%)

3.3.1.1 Ampicillin and beta-lactam drug resistance

Ampicillin resistant *E. coli* were the most prevalent resistance phenotype in hospital samples: 78/109 from the PLEH contained ampicillin resistant isolates compared to just 5/112 and 6/43 from premises A and B (Figure 3). Further testing of the ampicillin resistant *E. coli* isolates for β-lactamase activity was done using the chromogenic cephalosporin nitrocefin, and were found positive.

Figure3. The prevalence of ampicillin resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)



Ampicillin resistant *E. coli* isolates were also often resistant to other antibiotics. Defining multi-drug resistance (MDR) as resistance to 4 different antibiotic classes, 93% of ampicillin-resistant *E. coli* from horses in the PLEH were MDR, none from premises A, and 82% on premises B.

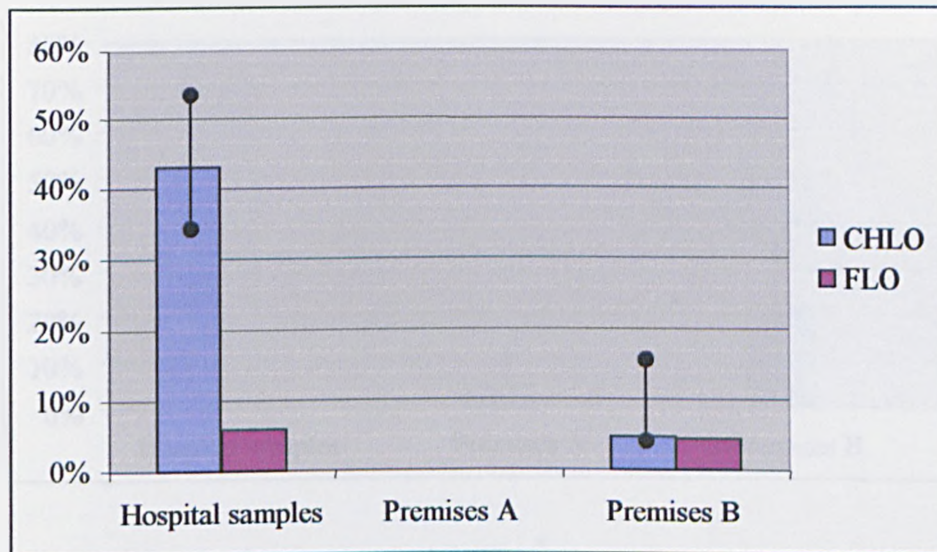
Further ß-lactam resistance

Ampillin isolates were tested for susceptibility/resistance to further β-lactam drugs and cephalosporins (as described in chapter 2). Resistance to most of the β-lactam drugs was demonstrated in 17 isolates in total: 12 from the PLEH, and 1 and 4 from premises A and premises B respectively. All these isolates, except for one isolate from premises A were MDR. The MICc of ampicillin for these isolates were all ≥ 256 ug/ml apart from one isolate for which the MIC of ampicillin was 128ug/ml.

3.3.1.2 Chloramphenicol and florfenicol resistance.

Chloramphenicol resistant *E. coli* were detected in 47/109 samples from the hospital group, 2/43 from premises B and 0/112 from premises A. Of these, 9 samples from the hospital and 2 from premises B had also contained *E. coli* resistant to florfenicol.

Figure4. The prevalence of chloramphenicol and florfenicol resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)



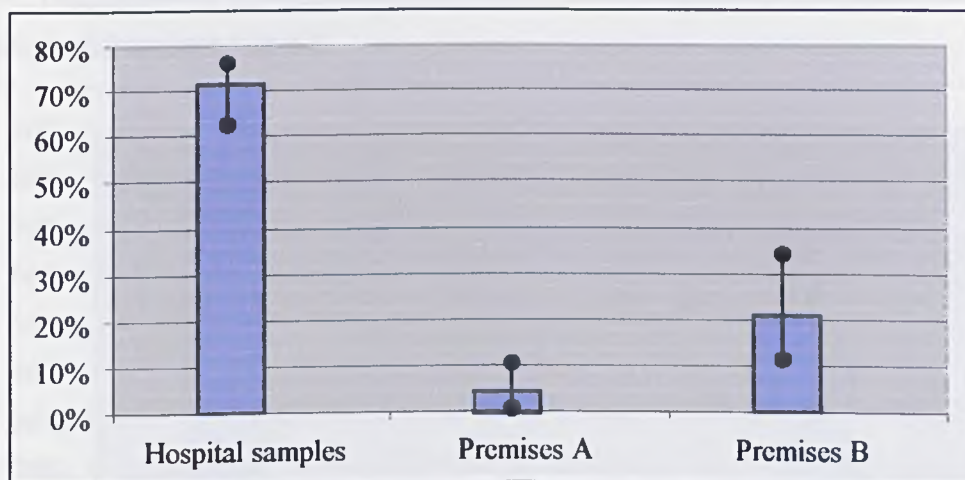
Overall, 102 chloramphenicol resistant isolates were studied further: 97 from the PLEH and 5 from premises B. MIC determination confirmed that all the chloramphenicol resistant isolates by disc diffusion were resistant. Almost all (98%) of the chloramphenicol resistant isolates were MDR, except for 4 isolates from the hospital, which each showed a similar resistance pattern (CHL,TET,TRI) (see appendix 3).

Also further testing of the chloramphenicol resistant isolates (n=102) showed that 10 isolates from hospital and 4 from premise B were also resistant to florfenicol by the disc diffusion method, and also all of them were MDR.

3.3.1.3 Tetracycline resistance

Resistance to tetracycline had the second highest prevalence, and was found in 78/109 hospital samples, and 5/112 and 9/43 samples from premises A and B respectively.

Figure5. The prevalence of tetracycline resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)

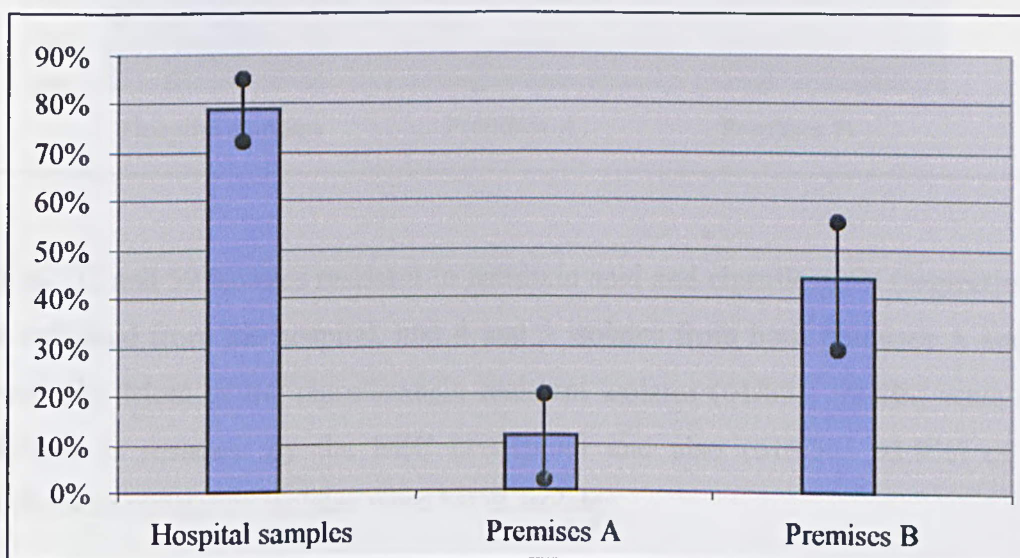


The total number of isolates with resistance to tetracycline was 198 distributed as follows: hospital (n=177), premises A (n=5) premises B (n=16). Of these, 93.4% were confirmed as resistant by the MIC determination method. Of tetracycline isolates, 60% had the MDR phenotype from the hospital and 56% from premises B.

3.3.1.4 Trimethoprim resistance

Resistance to trimethoprim had the highest prevalence of all the antibiotics tested for in samples from all sites: 86/109 PLEH samples contained trimethoprim resistant *E. coli*, and 13/112 and 19/43 samples from premises A and B respectively as shown in Figure 6.

Figure6. The prevalence of trimethoprim resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)

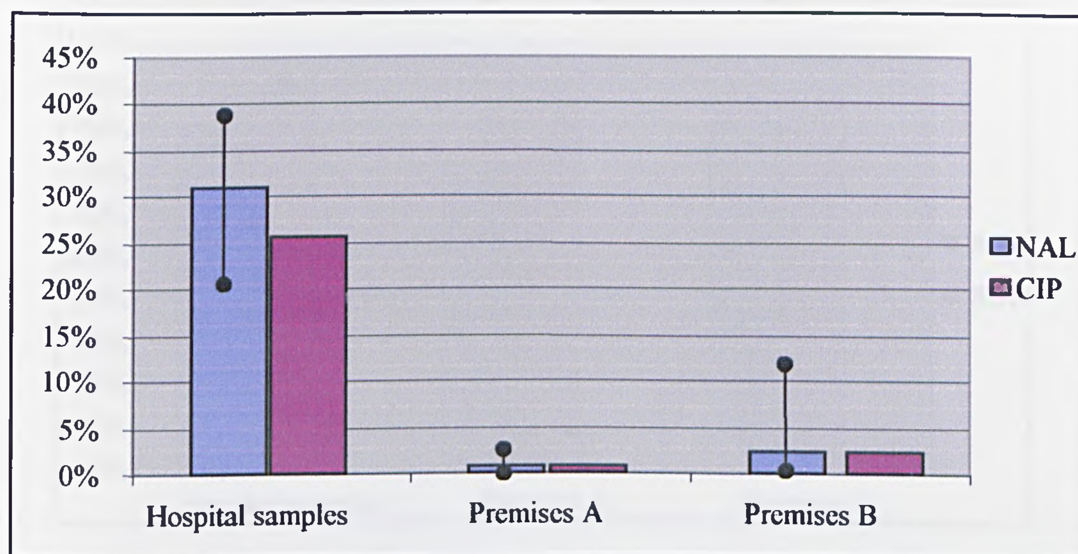


Overall, 279 trimethoprim resistant isolates and were collected: 209 from the hospital, 20 from premises A and 50 from premises B. MDR was a feature of 50.7% of trimethorprim-resistant hospital isolates, none from A and 18% of premises B.

3.3.1.5 Fluoroquinolone resistance

Resistance to nalidixic acid was found in 34/109, and to ciprofloxacin in 28/109 hospital faecal samples, and one sample contained *E. coli* resistant to both drugs on each of premises A and B (figure 7).

Figure7. The prevalence of fluoroquinolone resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)

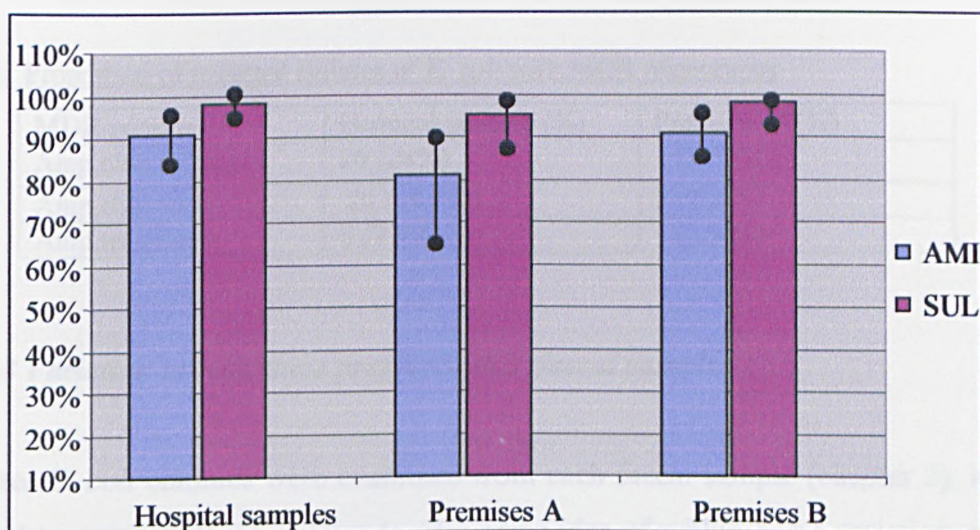


Over all, 72 and 59 isolates resistant to nalidixic acid and ciprofloxacin (respectively) were collected from the hospital, and 4 and 3 isolates from both premises A and B, respectively. Most of the ciprofloxacin resistant isolates (61/65 - 93.8%) were also confirmed as resistant by the MIC breakpoint and also (61/65 - 93.8%) of the ciprofloxacin resistance isolates were MDR isolates.

3.3.1.6 Aminoglycosides and sulphonamides.

Further testing of all 296 of the isolates resistant to any antibiotic among hospital samples found that 100 and 107 samples were also resistant to aminoglycosides and sulphamexazole respectively.

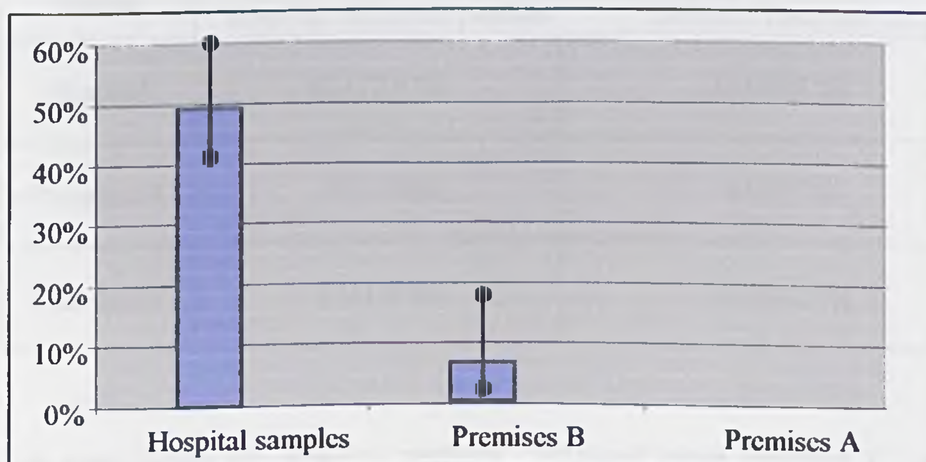
Figure8. The prevalence of aminoglycosides, and sulphonamides, resistant *E. coli* (proportion of samples) with 95% binomial confidence intervals (CI)



3.3.1.7 Multi-drug resistant (MDR) E. coli

MDR was defined as resistance to four or more classes of antibiotics. The prevalence of faecal samples containing MDR isolates was 54/109 samples (49.5%) from the hospital, 3/43 samples (7%) from premises B, and no samples from premises A. Overall, 106 MDR isolates were obtained from the hospital and 9 isolates from premises B (figure 8).

Figure9. The prevalence MDR *E. coli* isolates: the proportion of samples containing MDR *E.coli* with 95% binomial confidence intervals (CI)



The resistance profiles of the MDR isolates mostly fell into three distinct groups of resistance (Table 2).

Table2. Proportion of resistant isolates of *E. coli* with MDR phenotypes

MDR pattern	Hospital isolates (%)	Premises B (%)
Amp,chlo,tet,tri,nal	26 (24%)	1 (2.3 %)
Amp,chlo,tet,tri	20 (18.3%)	1 (2.3 %)
Amp,tet,tri,nal	8 (7.3%)	1 (2.3 %)

3.3.1.8 Variation in resistance profiles within faecal samples

As three *E. coli* colonies were examined from each faecal sample (chapter 2), it was decided to compare these isolates to gain some idea of within horse variation, which might indicate the strength of selection for particular resistant strains. Table 3 summarizes these comparisons.

Table3 show the proportion of samples faecal samples that contained *E. coli* with different or identical resistance profiles. Identical resistance profiles indicate at least two (of the three) isolates were identical, and different resistance profiles indicate all three isolates had different resistance profiles.

Table3. Proportion of samples faecal samples that contained *E. coli* with different or identical resistance profiles

Source	Number and proportions of samples containing isolates with	
	Identical resistance profiles	Different resistance profile
Hospital	41 (37.6 %)	55 (50.4 %)
Premises A	2 (1.7 %)	4 (3.5 %)
Premises B	5 (11.6 %)	13 (30.2 %)

As can be seen, while the hospital samples contained more resistant *E. coli* than samples from other sites, the ratio of identical to different profiles in the hospital (0.75) isolates was approximately twice that at the two stables (0.48 and 0.38).

3.3.2 Prevalence of *Salmonella*

No *Salmonella* spp. were isolated from any of the faecal samples in this study.

3.3.3 Prevalence of *Campylobacter*

Campylobacter was isolated from only three samples collected at the hospital, and PCR confirmed these as *C. jejuni*. No antibiotic resistance was demonstrated in these isolates apart from the intrinsic resistance to trimethoprim, and no further work was done on these isolates owing to the low prevalence found.

3.3.4 Prevalence of vancomycin resistant *enterococci* (VRE).

The prevalence of VRE in faecal samples was low: six isolates were obtained from the PLEH samples, one from premises A and two from premises B (Tables 4 and 5). The majority of isolates were characterized as *E. gallinarum*, although the two isolates from premises B were untypable by PCR. Resistance in the *E. gallinarum* isolates appeared to be due to *vanC-1*, characteristic of these species. *VanA* and *vanD* genes were detected in the untypable isolates.

Table4. Proportion of samples containing VRE with 95% binomial confidence intervals

Origin	Proportion	95% Confidence interval
Hospital samples	0.0550	0.0205-0.1160
Premises A	0.0098	0.0002-0.0487
Premises B	0.0465	0.0057-0.1581

Table5. Characteristics of VRE from horse faeces

Source	Isolate number (see appendix 2A)	Genes	Species	Antibiotic resistance profile
PLEH	43	*vanC-1*	*E. gallinarum*	Van,Teic,Qu,Azit,,Mer,
	4	*vanC-1*	*E. gallinarum*	Van, Teic,Qu,Azit,,Mer
	42	*vanC-1*	*E. gallinarum*	Van,Gent,Amp,Teic,Qu,azitr,Imp,Mer
	89	*vanC-1*	*E. gallinarum*	Van, Teic,Qu,Azit,,Mer
	40	*vanC-1*	*E. gallinarum*	Van,Gent,Amp,Teic,Qu,azitr,Imp,Mer
	86			Van, Teic,Qu,Azit,,Mer
Premises A	164	*vanC-1*	*E. gallinarum*	Van, teic,Qu,azitr, Mer
Premises B	317	*vanA*	-	Van,Teic,Qu,Azit,,Mer
	333	*vanD*	-	Van,Teic,Qu,Azit,,Mer

Antibiotic abbreviations: Vancomycin (Van), Ampicillin (Amp), Gentamicin(Gent), Teicoplanin(Teic), Quinupristin/dalfopristin (Qu), Azithromycin (Azit), Imipenem (Imp), Meropenem (Mer)

3.4 Discussion

This study showed, as expected, that *E. coli* is the most frequently isolated bacterium of those tested in horse faeces. That no *Salmonella* was isolated was slightly unexpected, as salmonellosis is a well-documented nosocomial problem in hospitalised horses (Dargatz *et al.*, 2004), and has previously been reported to be associated with risk factors including hospitalization and subsequent antimicrobial therapy. The very low prevalence of *Campylobacter* in horses contrasts with its high prevalence in the normal faeces of other large, intensively farmed herbivores (Prescott& Munroe, 1982). The significance of the low prevalence of VRE found in this study is difficult to assess, and is discussed more lately.

Salmonellosis is often regarded as an emerging problem in equine hospitals, with significant potential for both nosocomial and zoonotic spread and is largely documented as outbreaks (Ward *et al.*, 2005a). Two sources of infection have been suggested: an environmental source (Ewart *et al.*, 2001) and transmission amongst horses after arrival at the hospital; or asymptomatic carriage (Owen *et al.*, 1983) among horses, and re-introduction into hospitals through increased shedding subsequent to the stress of transport or illness. That no *Salmonella* was found in this study agrees with previous studies in which no salmonellas were isolated from horses with and without diarrhoea (Van Duijkeren *et al.*, 2000), but is perhaps slightly surprising given that the PLEH specializes in colic, and salmonellosis was found to have a higher prevalence in colic cases (Kim *et al.*, 2001). However, having had cases of salmonellosis in the past, the PLEH has defined infection control protocols in place, and these may have contributed to the lack of *Salmonella* isolated in this study from this equine hospital. However this low isolation rate may also indicate the need for better isolation methods for *Salmonella* as previous reports indicated that 17% of faecal specimens from healthy horses were positive for *Salmonella* spp. by PCR, but bacterial culture failed to recover the bacterium (Cohen *et al.*, 1996).

Recent studies have found *Campylobacter* infections to be common, regardless of disease, in a variety of domestic and wild animals, although other studies have suggested a low rate of infection in horses (Manser & Dalzie, 1985). In this study, protocols were used that have been optimized for isolating a wide range of

Campylobacter spp., from faeces (Aspinall *et al.*, 1993), but only three samples were positive, and all were of *C. jejuni* and from hospitalised cases. This might indicate that hospitalization might play a role in *Campylobacter* shedding, either through providing a source of infection or through stressing horses and causing increased susceptibility to infection and excretion. Either way, a prevalence of 2.7 % of this bacterium does not pose a great zoonotic risk.

Perhaps the most interesting finding of this study is the prevalence of antibiotic resistance in *E. coli*. It is generally agreed that the use of antibiotics selects for resistance (Hughes & Datta, 1938; Russell, 1998; Barber *et al.*, 2002). Antibiotic usage in both human and animal hospitals provides both selection for resistance and the opportunity for nosocomial transmission (Corpet *et al.*, 1989, Moken *et al.*, 1997, Levy *et al.*, 1998b, Russell, 2000). The much higher prevalence of antibiotic resistance, and of MDR, found in hospitalized horses in this study fits well with these observations. However, horses generally spend less time in hospital than humans, and all the horses at the PLEH are referrals from private veterinary practices, so many of the horses will have undergone treatment, perhaps including antibiotics, prior to arrival. Thus the higher prevalence of resistant *E. coli* in hospitalized horses might reflect prior selection rather than the immediate hospital environment. Transportation and other stress factors have also been shown to increase the shedding of antibiotic resistant enteric bacteria (Moro *et al.*, 1998), and this too might have influenced the prevalence of shedding in horses within the PLEH.

The differences in the prevalence between Premises A, a riding stable, and Premises B, a livery stable, however, were also marked. This could be a product of season as the premises B samples were collected between spring and summer when horses were going through changes in the routine of how they were kept. The type of work, and therefore nutrition and stresses placed on the horses, also differed between the two sites, and may have influenced the shedding rates. Questionnaires were completed for all the individual horses in this study, and also at the premises level for sites A and B, and the data collected from these will be analyzed further.

Although resistance was found at all sites, the prevalence of resistance to individual antibiotics, while largely following the general pattern, did vary between sites.

Resistance to trimethoprim, tetracycline and ampicillin were those most frequently encountered at all sites, yet no MDR was found on Premises A, for example.

Others have suggests that resistance to fluorquinolones is most likely to emerge in through the usage of fluoroquinolone drugs (Wiuff *et al.*, 2000), and that may also explain the higher rates of resistance seen in hospital isolates and MDR isolates in this study. Ciprofloxacin and β-lactam antibiotics constitute 50-60% of medications subscribed by doctors in the UK, so resistance to these in companion animals such as horses might be considered of importance to public health. Further work is needed to characterise the resistance found here: PCR of the resistant isolates produced ampicons of the gyrase gene, and these amplicons should be sequenced.

Chloramphenicol is never prescribed for horses in the UK, yet resistance to choloramphenicol was found in almost half of hospital samples. That almost all the chloramphenicol resistant isolates were MDR, suggests that co-selection may occur through the use of other antibiotics, especially if resistance to several antibiotics is encoded on single plasmid or other mobile genetic element (Carattoli *et al.*, 2001). Certainly the lack of direct selection for chloramphenicol resistance does not appear to have led to loss of chloramphenicol resistance from *E. coli* in horses (Phillips, 1998).

It was interesting to find vancomycin resistant enterococci (VREs) in the hospitalised horses, although PCR characterisation showed that the horse isolates were not like those associated with human infection, which usual carry *vanA* and are *E. faecium*. Human *E. faecium* isolates have been reported in the UK with resistance to quinupristin/dalfopristin as well as vancomycin, and this resistance has been shown to be transferable (Woodford *et al.*, 1997). Thus, the isolates in this study were also tested for resistance to a range of drugs including quinupristin/dalfopristin, azithromycin, imipenem, meropenem and also to teicoplanin. Previously, the VanC resistance phenotype was described in *E. casseliflavus* and *E. gallinarum*, which normally have intrinsic, low-level resistance to vancomycin and are susceptible to teicoplanin (Arthur & Courvalin, 1993, Yesim *et al.*, 2000). However the isolates in this study with *vanC*-encoded resistance were strongly resistant to vancomycin and

also to teicoplanin and other streptogramins drugs by the disc diffusion tests. Further characterization of these isolates is therefore needed.

The multi-drug resistant isolates were not characterized further due to time and economic constraints. Further work is required to identify the multidrug resistant mechanisms, which may be responsible for the high level of prevalence of these profiles (i.e. Amp,chlo,tet,tri,nal, Amp,chlo,tet,tri, Amp,tet,tri,nal respectively). That, 84% and 41% of resistant isolates were also resistant to the aminoglycosides streptomycin and spectinomycin, respectively, and 95% and 20% for sulphonamides and gentamicin, respectively, indicates that the resistance genes may present on a mobile genetic elements such as integrons. Integrons containing cassettes of genes encoding resistance to trimethoprim, chloramphenicol, β-lactam drugs, sulphonamides, streptomycin, spectinomycin, and amynoglycosides have been reported previously (Hall & Collis, 1998), and integrons conferring resistance to streptomycin, trimethoprim and β-lactams have been documented in multiresistant *E. coli* isolates from the normal intestinal flora of healthy fattening pigs (Sunde *et al.*, 1986).

3.5 Conclusions

This survey indicates that horses should not be considered a reservoir for *Campylobacter* infections, and also suggests that *Campylobacter* is not associated with disease in the horse. The absence of *Salmonella,* even in hospitalized horses, suggests that salmonellosis in horses is largely associated with occasional individuals, who may introduce it to larger horse populations, rather than endemic. Thus more work is needed to understand the sources of infection to horses, in order to prevent and control nosocomial infections.

This is one of the first reports of VRE in horses. However, the species and types of antibiotic resistance found suggest that horses are unlikely to be sources of zoonotic VRE infection

On the other hand, antibiotic resistance, including MDR and resistance to antibiotics important in human medicine, is common in *E. coli* from horses, and especially those hospitalized. Thus, the next objectives should be (1) to evaluate the impact of management and environmental conditions on the prevalence of resistance in *E. coli* in horses and (2) to define the type and location of genes that code for resistance in *E. coli*. These issues will be discussed later.

Chapter 4
Molecular characterization of antibiotic resistance genes of equine faecal *E. coli* isolates

4.1 Introduction

This chapter describes the genetic analysis of the antibiotic resistant isolates described in Chapter 3.

Resistance to antimicrobials in bacteria is a global problem, and understanding the molecular basis of how resistance genes are acquired and transmitted may contribute to the development of new antimicrobial strategies (Swartz, 1997). Antibiotic resistance in *E. coli* has been described in numerous studies (e.g. Piddock *et al.*, 1999), but these have mostly focused on isolates from man and food animals. Studies of antibiotic resistance and the mechanisms of resistance among isolates from horses are rare. Horses have the potential to be reservoirs of infection for humans, and, in addition, antibiotic resistance may inhibit veterinary treatment of economically valuable animals.

It is generally acknowledged that the use of antibiotics in both human and animal health has selected for the evolution of antibiotic-resistant bacteria (Neu, 1992). Furthermore, the use of antimicrobial agents to treat pathogens may select for resistance genes in non-pathogenic bacteria, which later may transfer the acquired resistance to pathogenic bacterial species (Phillips *et al.*, 2004). The resistance genes can be transferred and exchanged between bacterial species as well as genera, which further poses the risk of the transfer of resistance between the natural microflora and pathogenic organisms (Shoemaker *et al.*, 2001). Commensal bacteria therefore represent a reservoir of antibiotic resistance genes that has the potential to be transferred to human and animal pathogens (Perreten *et al.*, 2005).

The aim of this chapter was to analyze the genetic basis of the antibiotic resistance described in chapter 3, and to compare the findings in the horse with those observed for other species and to begin to understand the sources and transmission of resistance.

4.2 Materials and methods

4.2.1 Source of E. coli resistant isolates

Antibiotic resistant *E. coli* isolates were obtained from equine faeces collected at the University of Liverpool's Philip Leverhulme Equine Hospital (PLEH) (219 isolates), from a riding school referred to as premises A (25 isolates) and from a livery stable referred to as premises B (52 isolates). All 296 isolates are listed in detail in Appendix 3. The methods used to identify them as *E. coli* and to determine their resistance profiles (by disc diffusion and through MIC assays) are described in Chapters 2 and 3.

4.2.2 Determination of antibiotic resistance genes of E. coli resistant isolates

Genes known to be responsible for resistance in isolates from humans or domestic animals were tested for by a panel of primers and PCR assays. The PCR primers for genes commonly associated with resistance to ampicillin, chloramphenicol, tetracycline and trimethoprim, and the relevant PCR protocols are fully described in Chapter 2.6. Also a selection of resistant isolates positive to different PCR protocols is shown in appendix 3).

4.2.3 Conjugation assays

Mating experiments to determine if resistance could be transferred by conjugation, were done using a nalidixic acid resistant *E. coli* K12 as recipient as described in detail in chapter 2.5.

4.3 Results

<u>*Table1. The numbers of E. coli isolates from equine faeces in which antibiotic resistance genes were detected*</u>

Antibiotic (No. of resistant isolates investigated)	Source and number (N) of isolates	Identified by PCR	Not identified by PCR	Genes targeted by PCR with positive PCR in **bold**
Ampicillin (191)	Hospital N =177	169	8	***tem***
	Premises A N =3	1	2	***shv***
	Premises B N =11	4	7	
Chloramphenicol (102)	Hospital N = 97	75	22	***catI***
	Premises A N = 0	0	0	*catII*
	Premises B N = 5	0	5	*catIII*
				cmlA
				tetA
Tetracycline (198)	Hospital N = 177	154	23	***tetB***
	Premises A N = 5	3	2	*tetC*
	Premises B N = 16	15	1	*tetD*
				tetE
				tetG
Trimethoprim (279)	Hospital N = 209	195	13	***dfr1***
	Premises A N = 20	18	2	***dfr9***
	Premises B N = 50	47	3	***dfr12***
				dfr17-7

4.3.1 Ampicillin resistance

PCR assays for the *tem* and *shv* genes were performed on all 191 ampicillin resistant isolates and produced amplicons for 174 (91%) isolates. In the vast majority of cases, the *tem* gene, which encodes a β-lactamase was identified, and only one isolate was positive for the *shv* β-lactamase (Table 2).

Table2. Proportion of identified genes responsible for ampicillin resistance

R-gene	Hospital /MICs ug/ml		Premises A/MICs ug/ml	PremisesB/MICs ug/ml
tem	168	(65%) ≥ 256 (26%)128 (8.9%) 64	1 ≥256	4 ≥256
shv	1	≥256	0	0
Undet-rmined	8		2	7

The ampicillin resistant isolates were further investigated to look for any inducible ampicillin resistant genes as described in chapter 2.4.1.3, however no indication was found among all isolates of any inducible ampicillin resistant genes apart from increase in the resistance in most isolates.

Resistance to further β-lactams and cephalosporins

Seventeen ampicillin resistant isolates showed resistance to other β-lactamase drugs and cephalosporins and were all *tem* positive and had MICs ≥256ug/ml except in the case of two isolates from the PLEH and premises A that had MICs of 128ug/ml.

4.3.2 Chloramphenicol and florfenicol resistance

Amplicons were observed for 73.5% (75/102) of chloramphenicol resistant isolates, all of which were hospital isolates and *catI* genes.
Only one of the chloramphenicol isolates was also resistant to florfenicol, and this isolate was also positive for the *catI* gene.

4.3.3 Tetracycline resistance.

In all, 198 tetracycline resistant isolates were tested by PCR for 6 resistance genes and the results, with the MIC values, are listed in table 4.

Overall 86.8% (172/198) resistant isolates were positive by PCR. The *tetB* was the most prevalent gene, accounting for 71% of identifiable genes, followed by *tetA* at 18% and *tet(A+B)* at 11%.

Table3. Proportion of identified genes responsible for tetracycline resistance

R-genes	Hospital /MICs ug/ml		Premises A/ MICs ug/ml		Premises B/MICs ug/ml	
tetA	29	31% (32) 27% (64) 17% (256) 13% (128)	1	100% (64)	1	100% (64)
tetB	109	36% (128) 29% (64) 14% (32)	2		11	54% (64) 18% (32) 18% (256)
tet(A+B)	16	37% (64) 25% (128) 18% (32)	0		3	(64)
undeter-mined	23		2		1	

4.3.4 Trimethoprim resistance

In total, 279 isolates were tested for four resistance genes, and the results are shown in table5. The PCR assays identified the resistance genes in 93% (260/279) of the isolates. The genes detected were, in order of prevalence, *dfr1*, *dfr (17-7)*, *dfr12* and *dfr9* both overall and from each site.

Table4. Proportion of identified genes responsible for trimethoprim resistance

R-genes	Hospital / MICs ug/ml		Premise A / MICs ug/ml		Premise B/ MICs ug/ml	
dfr1	105	46.6 % (256) 38 % (>256) 10.4 % (128) 4.7% (64)	18	50% (256) 22.2%(>256) 16.6%(64) 11.11%(128)	41	68% %(256) 12.1%%(>256) 12.1%%(128) 7.3%(64)
dfr12	45	75.5%(>256) 22.2%(256) 2.2%(128)	0		0	
dfr17-7	73	91.7%(>256) 8.2%(256)	0		4	100%(>256)
dfr9	1	100% (>256)	0		0	
undetermined	13		2		3	

4.3.5 Conjugation experiments

Mating experiments were performed on all isolates except those which exhibited nalidixic acid resistance. Ten transconjugants isolates were obtained and they were all from mating with hospital isolates. Table6 lists the properties of the donor isolates. The resistance profiles of the transconjugants were identical to those of the donors in every case. PCR of resistance genes was not done on the transconjugants.

Table5. Resistant isolates that transferred resistance via conjugation, listed according to their resistance phenotypes

Resistant isolates profile	Culture collection number (see appendix3)	Donor genes
AMP,CHLO,TET,TRI (n= 4)	222	*dfr17,tetA,catI,tem*
	221	*dfr17,catI,tem*
	126	*tetA, dfr1, dfr(7-17), tem,*
	106	*dfr(7-17), dfr12,tetB,tem*
	101	*dfr12, ,tetA ,tetB, tem, catI*
	81	*dfr1, dfr(7-17), tetB, tem, catI*
AMP,TET,TRI (n=2)	89	*dfr(7-17), dfr12, tetB, tem*
	58	*dfr1, tetB ,tem,*
CHLO,TET,TRI (n=1)	99	*dfr12, tetB , catI*
TET,TRI (n=1)	112	*tetB, dfr1*

4.4 Discussion

The results suggest that most of the antibiotic resistance observed in *E. coli* from horses is the result of well-known and well-characterized genes, which are common to *E. coli* from man and domestic animals. The normal flora may play a role as an acceptor and donor of transmissible antimicrobial resistance mechanisms (Yolanda *et al.*, 2004).

Ampicillin resistance was almost entirely associated with *tem* genes, with only one isolate positive for a *shv* β-lactamase gene: previous studies have also found *tem* genes to be the most prevalent in ampicillin resistant *E. coli* (Petrosino *et al.*, 1999). Resistance to ampicillin is mainly due to β-lactamase production, with these enzymes divided into four functional classes A, B, C and D (Bush *et al.*, 1995). Class A β-lactamases encoded by *tem* and *shv* genes are most common in *E. coli* and *Klebsiella pneumoniae* (Heritage *et al.*, 1999).

In the 1970s and 1980s, novel β-lactam antibiotics, such as the cephalosporins, were developed to counter the growing problem of β-lactamase-mediated resistance in gram-negative bacilli. The prevalence of extended spectrum beta-lactamase (ESBL) resistance in European countries of *E. coli* human isolates is reported to be around 9% (Hanberger *et al.*, 2001) with variations from country to another. Reports of resistance against these extended-spectrum β-lactams drugs (ESBLs) (Kim *et al.*, 1998) and against β-lactamase inhibitors have become an increasing problem, and result from mutation of *tem* and *shv* β-lactamase genes (Livermore *et al.*, 1995, Paterson, 2001). In this study, 17 isolates were identified as being resistant to cephalosporins, all of which were *tem* positive and had MICs of ampicillin ≥256ug/ml (except for two isolates against, which the MICs =128ug/ml). TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria, being responsible for up to 90% of ampicillin resistance in *E. coli* (Livermore *et al.*, 1995). Therefore our results showed that resistance genes *E. coli* from horses are similar to those found in other animals and humans however these need further investigation, especially the *tem* genes responsible for β-lactam and cephalosporin resistance found in this study should be further investigated by sequencing the *tem* PCR products.

Amongst tetracycline resistance genes, *tetB* was the most prevalent among all isolates at all sites, followed by *tetA*, and no other tetracycline genes were identified. The *tet B* gene has the widest host range among gram-negative pathogens (Chopra *et al.,* 2001). The *tetA* and *tetB* genes encode tetracycline efflux pumps which are energy-dependent, membrane-associated proteins, which export tetracycline out of the cell (George & levy, 1983). These genes confer resistance to both tetracycline and doxycycline: however *tetB* specifically confers resistant to minocycline as well, though not to glycylcyclines.

The *tetA* gene in these isolates appears to confer resistance requiring a higher MIC of tetracycline than *tetB,* and the presence of both *tetA* and *tetB* together did not affect the MIC value among the isolates containing both of these genes. The presence of these genes together does not mean higher levels of resistance and may be a result of both of the genes encoding energy-dependent efflux systems.

CatI was the only acetyltransferase (CAT) enzyme, which prevents the binding of chloramphenicol to the 50S ribosomal subunit, detected in the isolates in this study. The presence of florfenicol resistance might suggest the presence of the *flo* gene, which is reported to be highly similar to *cmlA* gene among *salmonella* (Bolton *et al.,* 1999), which encodes a putative efflux pump that confers resistance to both chloramphenicol and florfenicol (Bolton *et al.,* 1999). However, the fact that *cmlA* gene was identified by PCR, amongst the isolates might suggest that an unknown *flo* gene might be present, or that the florfenicol resistance might result from mutation in *catI* genes: one of isolates had resistance to both chloramphenicol and florfenicol and was positive for the *catI* gene. However the lack of a positive control for the *cmlA* gene PCR made it difficult to determine if the PCR was working correctly and therefore further investigation is needed to characterize the genes responsible.

Around 90% of the trimethoprim resistant isolates were positive for one or other of the *dfr* genes tested for. These are commonly encoded on mobile genetic elements such as integrons and transposons. The most common gene detected was, as expected, *dfrA1*. *Dfr1* spread rapidly during the 1970s and 1980s on the transposon Tn7 (Jansson *et al.,* 1992; Yu *et al.,* 2004) and became the most

prevalent gene responsible for trimethoprim resistance in the UK (Towner *et al.*, 1994), followed by *dfr12* and *dfr17*, as found in this study.

The *dfr9* was not found in this study except in one isolate, consistent with other studies that found that *dfr9* is most likely to be associated with bacteria from swine (Jansson *et al.*, 1992).

Both *dfr7-17* (30%) and *dfr12* (17%) are also enocoded in class 1 integrons enabling horizontal transmission among Gram-negative bacteria (Rowe-Magnus *et al.*, 2002a). The integrons that are associated with trimethoprim resistance also encode resistance to other antibiotics, including sulfamethoxazole, aminoglycosides, quinolones, chloramphenicol, tetracycline and some ß-lactams (Martinez *et al.*, 1998).

The conjugation experiments demonstrated that resistance could be transferred for a small number of isolates. It may be that mating experiments would have been more successful if they had just been done on isolates that conferred resistance to 3 or more different classes of drugs, especially those positive for trimethoprim resistance *dfr17* and resistance to drugs such as chloramphenicol, ampicillin, and aminoglycosides. In studies on *E. coli* isolates of human origin, the wide dissemination of *dfrA17* in urinary *E. coli* isolates has been found to be mainly due to the horizontal transfer of class 1 integrons, via conjugative plasmids (Yu *et al.*, 2004). It was clear that resistant genes have transferred and the recipient (*E. coli* K12) had acquired resistance from the donor (resistant isolates) to all drugs that the donor was resistant to, which clearly suggests that transfer has happened on genetic elements that confer resistance to a number of antibiotics.

4.5 Conclusions

The genes responsible for antibiotic resistance in equine *E. coli* are largely those commonly found in other domestic animals and humans. Moreover, the conjugation experiments and some of the genes detected suggest that MDR might be associated with horizontally transmitted genetic elements like integrons or plasmids. All of this suggests that the antibiotic resistance found in horses probably originates from, and has been selected by, the same sources and mechanisms as in other species. The finding of resistance genes which are normally associated with other animals, e.g. the *dfr9* gene, suggests transmission of resistance between different animal populations. Thus horses may be both recipients of, and sources of, the zoonotic transmission of antibiotic resistance.

Chapter 5

Antibiotic resistance and molecular analysis of faecal *Salmonella* spp. of equine origin

5.1 Introduction

Salmonellosis is an important nosocomial infection of hospitalised horses (Powell *et al.*,1989; Murray *et al.*, 1996; Dargatz *et al.*, 2004; Ward *et al.*, 2005a), but is rarely detected in horses in cross-sectional studies (Pichner *et al.*, 2005; Chapter 3). Hence molecular and antibiotic resistance studies of *Salmonella* from horses are few when compared to the considerable information available on salmonellosis in humans and food animals.

Clinical signs of salmonellosis in the horse are mostly associated with gastrointestinal problems, however horses can also be infected with *Salmonella* asymptomatically and still shed *Salmonella* in their faeces without developing any clinical signs (Owen *et al.*,1983). Horses admitted to hospitals may be more susceptible to *Salmonella* infection due to stress, including surgery (Rhoads *et al.*, 1999). In the USA, antibiotic administration has been identified as a risk factor for *Salmonella* shedding in horses at hospitals (Hird *et al.*, 1986). Foals and adult horses treated with antibiotics prior to hospitalization and horses that have had abdominal surgery were all more likely to shed *Salmonella* (Ernst *et al.*, 2004). Antibiotics have been shown to select for resistant salmonellae, for example in chickens (Smith and Tucker, 1975). The use of some antimicrobials is likely to add to selection pressure for multi-drug resistant (MDR) *Salmonella* to colonize equine patients (Dargatz *et al.*, 2004).

Multiresistant (to four or more antimicrobial agents) *Salmonella enterica* serovar Typhimurium definitive phage type 104 (MR DT104) is a zoonotic pathogen isolated frequently from food animals (Lawson *et al.*, 2004). It was associated with cattle, but is now also isolated commonly from pigs, sheep and poultry (Threlfall, 2000).
It has been suggested that the emergence of the MDR strain, *Salmonella* Typhimurium DT104, might have been due to the licensing of enrofloxacin as a veterinary medicine (Threlfall, *et al.*, 1998). Studies have shown genomic similarities

between quinolone-resistant *Salmonella* strains isolated from both humans and animals (Heurtin-Le Corre *et al.*, 1999). Fluoroquinolone resistance, however, has been reported among several different members of the *Enterobacteriaceae* (Cometta *et al.*, 1994), and is generally attributed to the widespread use of this class of agents (Hooper *et al.*, 2001). Multi-drug resistance in bacteria is commonly attributed to the acquisition of plasmids or transposons (Jacoby & Archer, 1991; Gold & Moellering, 1996) or through clonal selection of strains, as is the case with *S.* Typhimurium DT104 (Ridley & Threlfall, 1998).

Although no salmonellae were isolated from horses in the study in Chapter 3, isolates had been archived from previous cases in horses at the PLEH. Given the animal health and zoonotic risks associated with equine salmonellosis, and the high prevalence of antibiotic resistant *E. coli* from horses in this study, the archived *Salmonella* were investigated for antibiotic resistance and subjected to macrorestriction of genomic DNA and pulsed-field gel electrophoresis (PFGE) to provide further information on the sources of infection through molecular analysis.

5.2 Materials and methods

5.2.1 Source of isolates

Thirty-one confirmed *Salmonella* ssp isolates from equine faeces were obtained from the veterinary pathology archive, which were collected between 1995 to 2002 from horses at Philip Leverhulme Equine Hospital (PLEH). These had already been phage-typed and serotyped (personal communication from Mr A. Wattret). All the isolates were serotyped by the Veterinary Laboratory Agency (VLA) and results are shown in table1.

5.2.2 Serotyping and phage-typing

Of the 31 isolates, the majority were *S.* Typhimurium (of which 5 isolates were *S.* Typhimurium DT104), 2 were *S.* Enteritidis, one each of *S.* Newport, *S.* Ohio, and the rest (n=4) were untypable. All are listed in table 1.

5.2.3 Antibiotic suscebtibility tests

The disc diffusion method was used as described in Chapter 2, section 2.4.2 according to BSAC guidelines to determine the antibiotic susceptibility of the isolates. The antibiotics used and their concentrations are listed in detail in Appendix 1-C. Briefly, the drugs used were ampicillin, apramycin, chloramphenicol, tetracycline, trimethoprim, sulphamexazole, gentamicin, nalidixic acid and ciprofloxacin. The breakpoints of these drugs are listed in Appendix 1-C.

5.2.4 Macrorestriction PFGE analysis

Pulse field gel electrophoresis (PFGE) was used to compare 27 of the *Salmonella* isolates as described in Chapter 2, section 2.6.2, using the restriction enzyme *Xba*I, (Schott *et al.,* 2001). *Salmonella* isolates included in the PFGE analysis are listed in bold table 5.1, and numbered from 1-27 as stated in the table. The MDR isolates were all included for this analysis.

Table1. Archived *Salmonella* isolates and antibiotic resistance phenotypes from horses at the PLEH (*Salmonella* in bold were the included in the PFGE Analysis

Isolate no.	Year	P/T	Serotype	Resistance phenotype	Isolate no.	Year	P/T	Serotype	Resistance phenotype
1	1995	104	***S.* Typhimurium**	A,C,T,Tr,Su,NAL,FLO	17	1997	-	***S.* Typhimurium**	A,C,T,Su,FLO
2	1995	4	***S.* Enteritidis**	T,Su	18	1997	-	***S.* Typhimurium**	A,C,T,Su,FLO
3	1995 -		***S.* Typhimurium**	A,T,Su	19	1997	-	***S.* Typhimurium**	A,C,T,Su,FLO
4	1995	-	**untypable**	T,Tr,Su,NAL	20	1997	-	***S.* Typhimurium**	A,C,T,Su,NAL,FO
5	1995	193	***S.* Typhimurium**	T,Tr,Su,NAL	21	1997	-	***S.* Typhimurium**	A,C,T,Su,FLO
6	1995	-	***S.* Typhimurium**	T,Su	22	1997	-	***S.* Typhimurium**	T
7	1996		***S.* Typhimurium**	T,Su	23	1998	104	***S.* Typhimurium**	A,C,T,Su,FLO
8	1996	-	***S.* Typhimurium**	A,C,T,Tr,Su,FLO	24	1998	195	***S.* Typhimurium**	T
9	1996	6	***S.* Typhimurium**	A,C,T,Tr,Su,FLO	25	1999	-	***S.* Enteritidis**	T
10	1996	104	***S.* Typhimurium**	A,C,T,Tr,Su,FLO	26	2000	-	untypable	T,Su
11	1996	-	***S.* Typhimurium**	A,C,T,Tr,Su,FLO	27	2000	-	***S.* Typhimurium**	T
12	1996	-	***S.* Ohio**	T,Tr,Su	28	2000	104	***S.* Typhimurium**	A,C,T,Tr,Su,FLO
13	1996	-	**untypable**	T,Tr,Su	29	2001	-	***S.*** Newport	T,NAL
14	1997	-	**untypable**	T	30	2002	-	***S.*** Typhimurium	C,T,FLO
15	1997	104	***S.* Typhimurium**	A,C,T,Su,FLO	31	2002	-	***S.*** Typhimurium	C,T,Su,FLO
16	1997	-	***S.* Typhimurium**	A,C,T,Su,FLO					

*abbreviations : (-) non, (A) Ampicillin, (C) chloramphenicol, (T) tetracycline, (Su) sulfamethoxazole, (Tr) trimethoprim, (NAL) nalidixic acid, (FLO) florfenicol

5.3 Results

5.3.1 Antibiotic resistance results

All the isolates were resistant to at least one antibiotic and 26/31 isolates were resistant to more than one antibiotic: these results are shown in Table 1. Over half (17/31) of the isolates were MDR, i.e. were resistant to four or more antibiotics, and of these MDR strains, 16/17 were typed as *S.* Typhimurium (one that was untypable): 14/17 of the MDR isolates had ACSUT phenotype, including the five confirmed isolates of DT104. Of the 17 MDR isolates, fourteen (including the five confirmed as DT104) shared the phenotype ACTSuFLO, two isolates had the similar phenotype TTrSuNAL and just one had the phenotype CTSuFLO

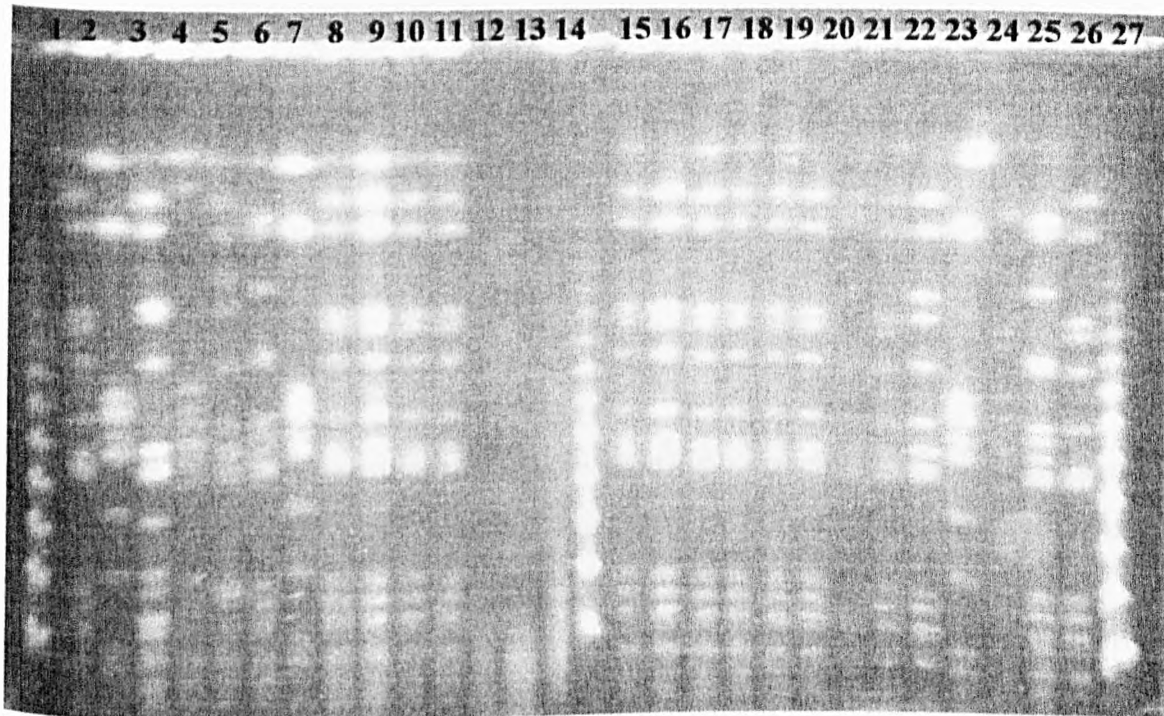
Sensitivity tests showed all the isolates to be resistant to tetracycline and also the majority of them resistant to sulphonamides. All the isolates were susceptible to ciprofloxacin, although resistance was found to the quinolones, nalidixic acid, and they were also sensitive to gentamicin. All the *Salmonella* isolates resistant to chloramphenicol, apart from one, were MDR, and all were also resistant to florfenicol. Isolates resistant to ampicillin were mostly MDR

To sum up, two distinctive phenotypes were noticed among the MDR isolates: ACTTrSuFLO and ACTSuFLO. These were found in 6 and 7 of the 31 isolates, respectively.

5.3.2 Molecular results (pulse field gel elestrophoresis (PFGE)

Twenty-seven isolates were compared by PFGE. Most gave clear banding patterns (Figure 1.)

Figure1. Macro-restriction of 27 archived *Salmonella* isolates using *Xba*I and PFGE. The lanes refer to the isolates as in Table 1, and unlabelled lanes contain the λ-concatemer molecular weight marker.



Gels were analysed by visual interpretation of the banding patterns according to Tenover *et al.*, 1995. Isolates were considered to be genetically indistinguishable if the RFLP patterns were identical, or closely related if there were 2-3 band differences, possibly related if there were 4 - 6 band differences and unrelated if there were more than 7 band differences. Different gels were compared using the λ-molecular weight marker that was run on each gel.

Profiles in lane 8-11 and 15-20 and 22 all had identical PFGE patterns and were all *S*. Typhimurium with the same resistance profile, even though some of these isolates were different phage types. Isolate 22, was resistant to tetracycline only, and isolate 23 had 2 band differences, but the same resistance profile as 15-20, suggesting it closely related or a sub-type.

5.4 Discussion

No salmonella were isolated from horses in the cross sectional study described in Chapter 3, and several different serotypes of *Salmonella* had been collected from horses intermittently over seven years to produce the archive used in this study. This, suggests that salmonellosis probably is not endemic at the hospital, or a nosocomial infection, contracted during hospitalisation, but rather an infection periodically introduced by individual horses. On the other hand, the similarity of the MDR phenotypes and PFGE patterns in the collection suggests that there may have been an 'epidemic' among equine salmonellas, probably the result of spillover of salmonellosis from other species.

Most of the isolates in this study were *Salmonella enterica* serotype Typhimurium, the serovar most commonly reported in other studies in horses (Van Duijkeren *et al.*, 2002). This strain is considered endemic in developing countries and epidemic in developed countries. Many studies have shown the increasing prevalence of antibiotic resistance in *S* Typhimurium (Threlfall & Ward, 2001) in both farm animals and humans, and all the salmonella in this study were resistant to at least one antibiotic, and many were multidrug resistant. Two distinctive resistance phenotype were noticed among the multidrug resistant isolates in this study: ACTTrSuFLO and ACTSuFLO. These were found in 5 and 7 of the 31 isolates, respectively, and are similar to the resistance profiles of *Salmonella* Typhimurium from two human outbreaks in England in 2000 (Horby *et al.*, 2003), which were attributed to the consumption of contaminated food and from horses in Netherlands (Van Duijkeren *et al.*, 2002).

On the other hand, no ciprofloxacin resistance was found in this collection, despite the use of fluoroquinolones in horses (enrofloxacin "Baytril"), in contrast to studies done in cattle and chickens (Piddock *et al.*, 1999).

Trimethoprim resistance in salmonellas has been attributed to the use of this agent to combat the emergence of DT104 in cattle in the UK (Threlfall *et al.*, 1996; Threlfall *et al.*, 1999), so finding trimethoprim resistance among equine salmonellae may suggest that these isolates originated in food animals, or might have acquired this resistance type through genetic elements originating from food animal bacteria. Similarly,

florfenicol is not licensed for use in the horse, although it is licensed for cattle with respiratory infections. *S.* Typhimurium DT104 with *floSt* have been isolated from many different animals including horses (Bolton *et al.*, 1999) and it has been suggested that this resistance is indicative of DT104 (Khan *et al.*, 2000; Yang *et al.*, 2001). In this study, florfenicol resistance was found in MDR salmonellas, suggesting that this resistance is encoded within a MDR gene cassette, which probably, again, originated in food animals. It is well documented that MDR is most likely to be related to the carriage of integrons, as in the case of DT104, and horizontal transfer of these genes might have occurred. The possibility of transfer of plasmids amongst salmonellae in the intestinal tract of humans has been reported (Balis *et al.*, 1996), as has transfer of resistance between different organisms such as *Salmonella* and *E. coli* (Hunter *et al.*, 1993; Winokur *et al.*, 2001). DT104 contains chromosomal integrons (Recchia & Hall, 1997; Carlson, 1999). It has been suggested that this may allow resistance genes to persist even in the absence of antibiotics (Threlfall *et al.*, 1994a).

Overall, this study shows that the resistance phenotype of *salmonella* can be a useful epidemiological tool, especially when combined with PFGE. The MDR isolates in this study appeared to be closely related, and probably represent spillover into horses of an epidemic of MDR salmonellas, including DT104, in other domestic animals and humans. Although the multidrug resistant *Salmonella* types especially DT104 have been associated with consumption of meat and contact with infected animals (especially cattle) (Wall *et al.*, 1994; Wall *et al.*, 1995), these findings may also suggest horse faeces as a potential source of human infection with MDR salmonellas.

The existence of the MDR phenotype complicates the use and selection of antibiotic drugs for treatment of clinical *Salmonella* infections. Furthermore, infected horses may be a source of human infection (and vice versa), particularly in a hospital setting.

Chapter 6
Antibiotic resistance in faecal *E. coli*: a cohort-control study of hospitalised horses

6.1 Introduction

In the cross sectional study described in Chapter 3, a higher prevalence of antibiotic resistance was found in *E. coli* isolated from faeces of hospitalised horses than those at local riding and livery stables. This chapter, therefore, describes a preliminary study undertaken of the dynamics of antibiotic resistance in faecal *E. coli* during hospitalisation in the PLEH. The two main questions to be answered by this study were:

1) Does the prevalence of antibiotic resistant *E. coli* increase during hospitalisation?
2) Does the prevalence of antibiotic resistance decrease after the horses leave the hospital environment, or is it maintained (making hospitalisation a community risk)?

Some studies have shown that hospitalization of humans is associated with an increase in antibiotic resistance in pathogenic bacteria, while others have found that it did not have a significant affect on the prevalence of resistance in *E. coli* (Koterba *et al.,*1986; Gaynes & Monnet, 1997; Bruinsma *et al.*, 2003). Resistance might be selected for by antimicrobial administration, while the shedding of resistant bacteria might produce a reservoir of resistant bacteria in the environment (Levy *et al.*, 1998b). If the resistance is encoded for by genes on mobile elements that also encode resistance to other antibiotics, then exposure to one antimicrobial agent could lead to selection for resistance against another (Braoudaki & Hilton, 2004). Furthermore, stopping treatment, and the removal of selective pressure, does not mean necessarily lead to the loss of resistance (Phillips *et al.*, 1998).

In this chapter, the faeces of horses entering the PLEH were collected and examined for antibiotic resistant *E. coli* on arrival, during hospitalisation, and after discharge, in order to assess the dynamics of the prevalence of antibiotic resistance.

6.2 Materials and methods

Horses admitted to the PLEH for more than 7 days were sampled as follows:

- On arrival, and before treatment began; (one sample, referred to as 1st)
- One day and 2-3 days after treatment had started; (two samples, referred to as 2nd, 3rd)
- Immediately before discharge (one sample, referred to as 4th)

Further faecal samples were collected by the horse's owners, 4-8 weeks after discharge (5th), and also 6 months after discharge (6th), and sent to the laboratory for analysis.

The protocols for dealing with the samples, isolation and identification of *E. coli*, and analysis of antibiotic susceptibility and resistance, were as described previously in Chapters 2 and 3.

Two Questionnaires (Appendix 1.F.2 & 1.F.3) were designed and completed by the clinician responsible for the case. For the purposes of analysis, horses were divided into three groups as follows:

1) Horses with gastrointestinal conditions and under antibiotic therapy (abbreviated to GI+).
2) Horses with non-gastrointestinal conditions and under antibiotic therapy (abbreviated to non-GI+).
3) Horses with non-gastrointestinal conditions and no antibiotic therapy (abbreviated to non-GI-).

6.3 Results

The horses sampled were: GI+ group (n=6 horses), non-GI+ (n=4 horses) and non-GI- (n=5 horses), from each of which six samples were collected (n=90 total horse faecal samples) (faecal samples are listed in appendix 4).

Table1. The number of horses, faecal samples collected and faecal samples positive for at least one antibiotic resistant (AR) *E. coli* isolate

Cohort group	*No of horses*	*No of samples collected*	*No of samples positive for AR E. coli*	Distribution of resistance among samples					
				1st	2nd	3rd	4th	5th	6th
GI +	6	36	21	2	4	5	5	3	2
NON GI +	4	24	18	3	4	4	4	3	0
NON GI –	5	30	16	1	3	4	4	4	0

Table2. Distribution of the positive samples* according to sampling time (cohort groups combined)

Sampling time	Distributing of positive resistant samples to individual antibiotic (groups combined) & VREs										
	AMP	ß-LAC	APR	CHL	FLO	NAL	CIP	TET	TRI	MDR	VRE
1st	4	0	0	1	0	0	0	0	4	0	2
2nd	10	5	1	6	2	8	6	9	10	8	2
3rd	11	4	0	6	2	8	7	10	11	9	3
4th	9	5	0	7	2	6	6	10	13	8	3
5th	6	1	0	2	1	4	3	7	9	4	2
6th	1	0	0	0	0	0	0	1	3	0	1

* The number of samples (out of 15) containing *E. coli* with resistance to the listed antibiotic or VRE

Table3. Summary of horses, faecal samples, faecal samples containing resistant *E. coli*, and the number of faecal samples with *E. coli* resistant to each individual antibiotic

Source of samples	*Samples collected*	*Positive samples*	*Distributing of E. coli isolates resistant to different antibiotics*									*No of samples with mult-drug resistant E. coli*
			AMP	ß-LACTAMS	APR	CHL	FLO	NAL	CIP	TET	TRI	
GI +	36	21	15	1	1	5	5	8	6	15	19	8
NON GI +	*24*	18	12	10	0	10	0	11	11	11	18	11
NON GI –	*30*	16	14	4	0	7	2	7	5	11	13	*10*

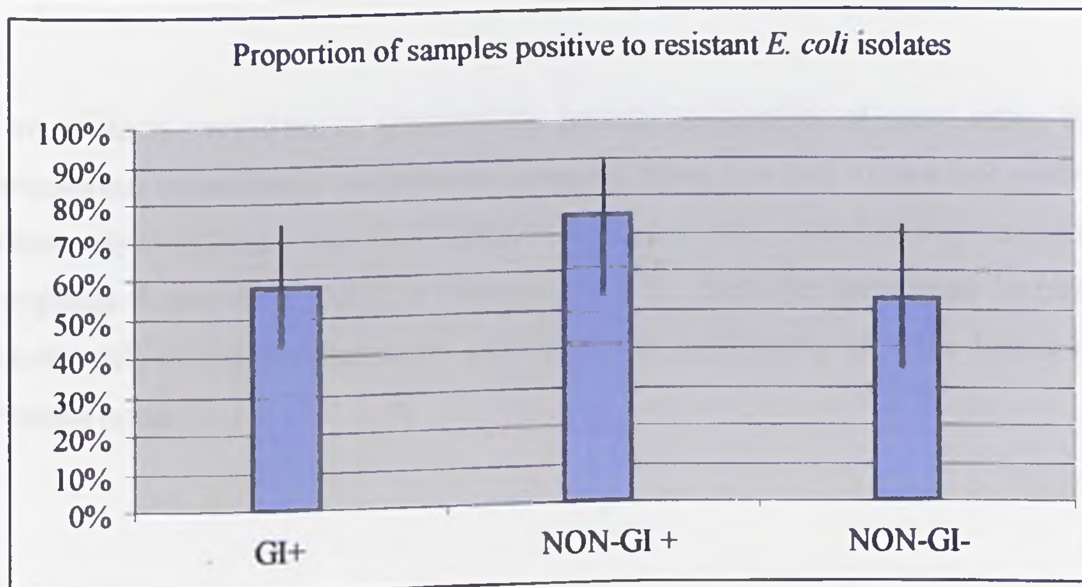
Abbreviations: Ampicillin (AMP), Beta lactam drugs (ß-LACTAM)*, Apramycin (APR), Chloramphenicol (CHL), Florfenicol (FLO), Nalidixic acid (NAL), Ciprofloxacin (CIP), Teracycline (TET), trimethoprim (TRI).

*The ß-lactam drugs represent 6 different cephalosporins and beta-lactam drugs which are listed in chapter 2

In total, 138 *E. coli* isolates that were resistant to at least one antibiotic were collected (See appendix 4). Tables 2 and 3 summarise the isolates resistant to individual antibiotics by treatment group and sampling time. Overall, the relative prevalences of resistance to individual antibiotics were similar to those found in the earlier cross-sectional study (Chapter 3).

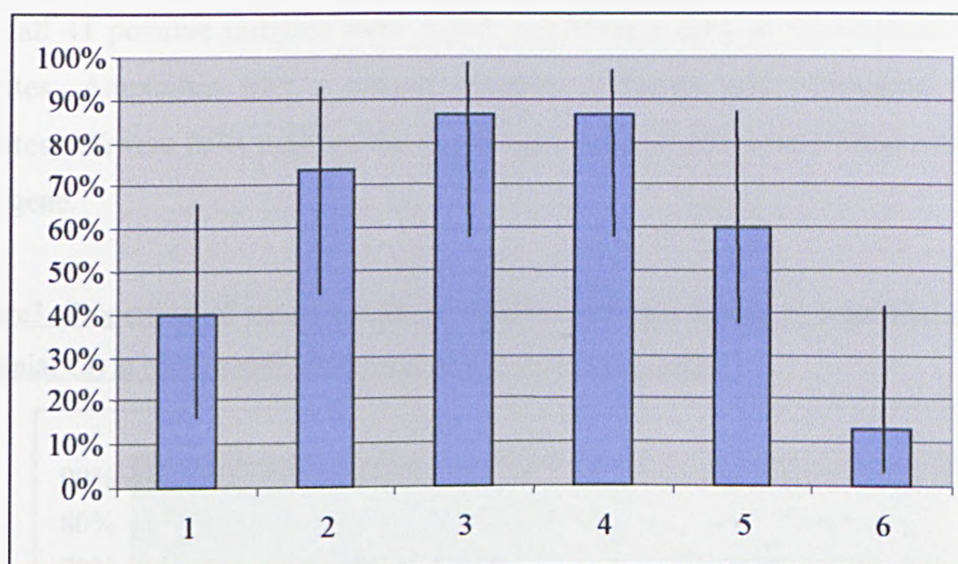
The proportion of samples that had an *E. coli* isolate resistant to at least one antibiotic did not vary significantly between treatment groups (over 50% prevalence in each group – Figure 1)

Figure1. Proportion of samples with at least one resistant isolate to at least one antibiotic (with 95% binomial CI)



Therefore, for the analysis over time the treatment groups were combined (Figure 2). A definite trend for the prevalence of resistance to increase during hospitalisation, and decrease after release (table 1 and figure 2) was observed.

Figure2. Proportion of samples with at least one resistant isolate to at least one antibiotic (with 95% binomial CI) at each sample time (treatment groups combined) (table1).

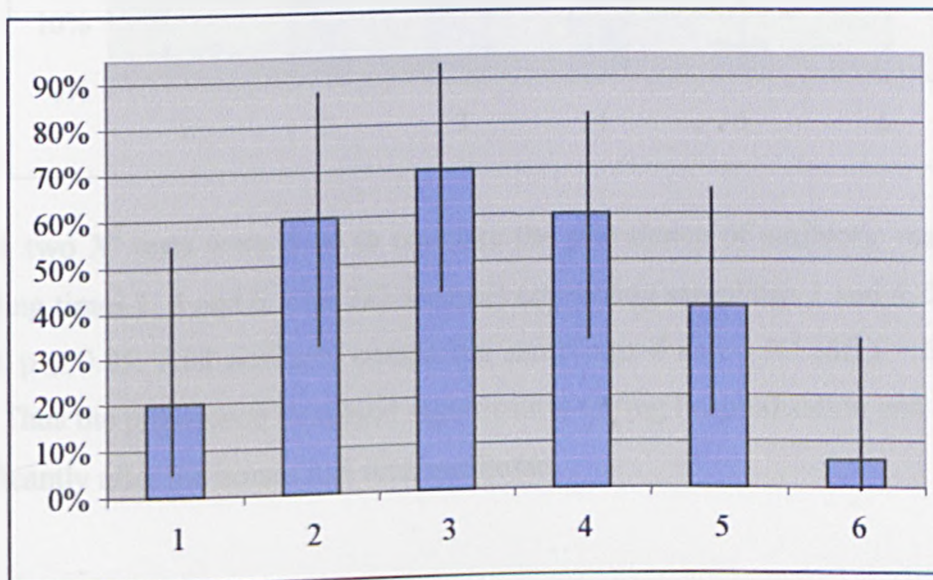


Two X^2 tests were done to compare the prevalence (number of cases rather than proportions) of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 7.03, $p \leq 0.01$. And similarly comparing samplings 4 and 6 X^2 (df1) = 13.4, $p \leq 0.001$. Thus the prevalence increased significantly during hospitalisation and decreased significantly after the horses had returned home.

6.3.1 *Ampicillin resistance*

Overall 41 positive samples were found, providing a total of 95 ampicillin resistant isolates. Ampicillin MIC's ranged between 128ug/ml and >256ug/ml. Of these isolates, 2%were PCR positive for the *shv* gene and 60.8% were PCR positive for the *tem* gene.

Figure3. Proportion of samples with at least one resistant isolate to ampicillin (with 95% binomial CI) at each sample time (treatment groups combined)

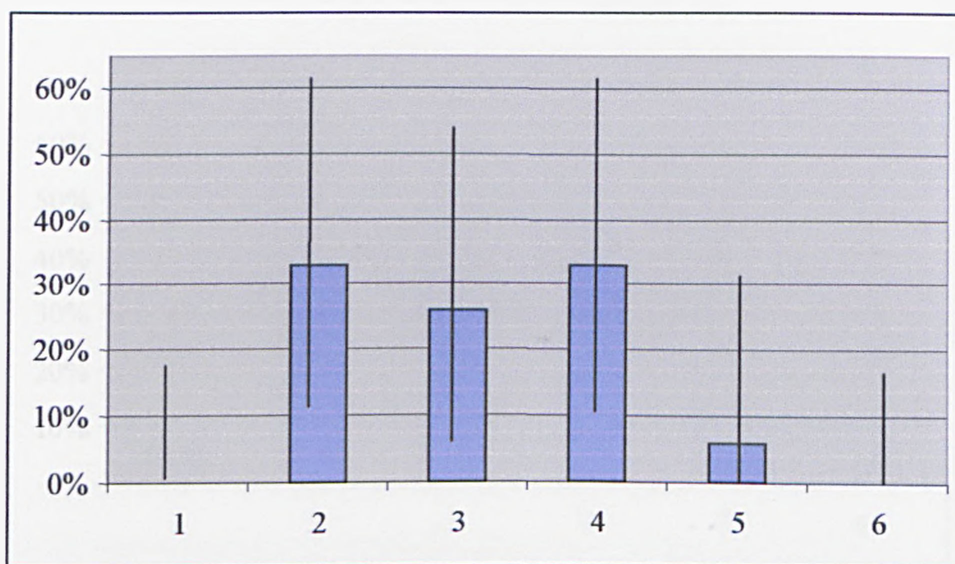


Two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 3.394, $p \geq 0.05$. And similarly comparing samplings 4 and 6 X^2 (df1) = 9.600, $p \leq 0.01$. Thus there was a non-significant trend for prevalance to increase during hospitalisation and the prevalence decreased significantly after the horses had returned home.

6.3.2 Expanded β-lactam resistance

The ampicillin resistant isolates collected were tested against further β-lactams and cephalosporins (see chapters 2 and 3). A total of 34 resistant isolates were collected from 15 samples and no further characterization was done of these isolates.

Figure4. Proportion of samples with at least one resistant isolate to β-lactam drugs (with 95% binomial CI) at each sample time (treatment groups combined)

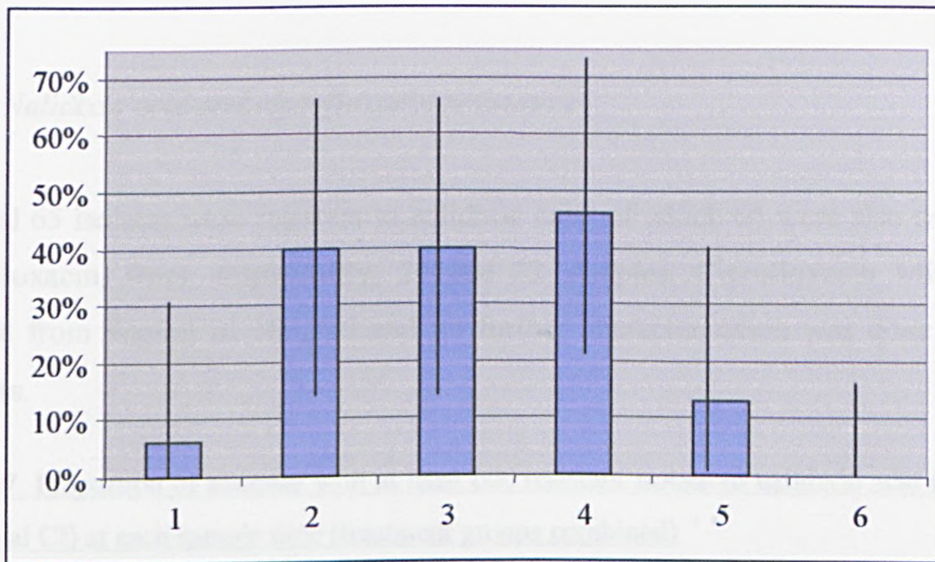


Again, two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 6.000, $p \leq 0.05$. And similarly comparing samplings 4 and 6 X^2 (df1) = 6.000, $p \leq 0.05$. Thus the prevalence increased significantly during hospitalisation and decreased significantly after the horses had returned home.

6.3.3 *Chloramphenicol and florfenicol resistance*

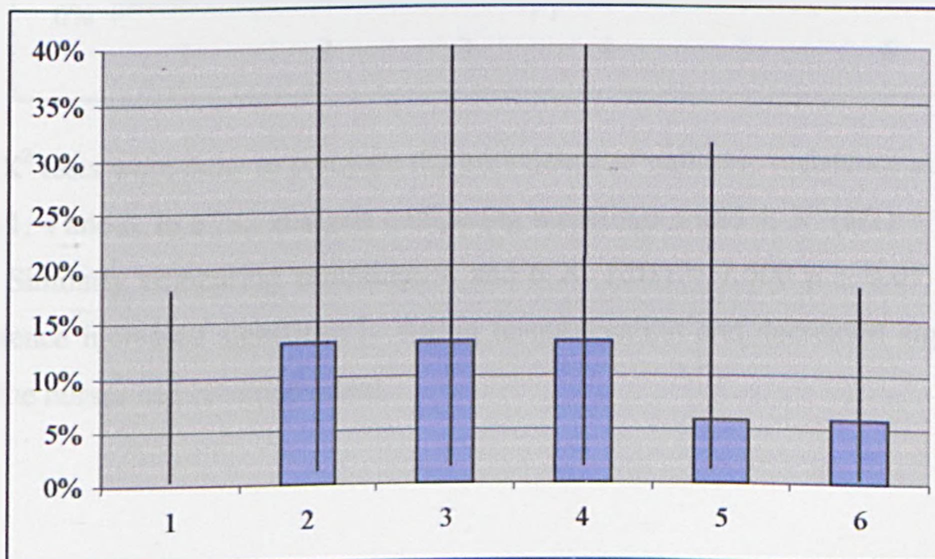
In total 22 isolates were resistant to chloramphenicol, of which 7 were also resistant to florfenicol, and a total of 51 and 7 resistant isolates respectively to chloramphenical and florfenicol were collected. The MICs further confirmed these isolates' resistance, and mostly ranged between 256ug/ml and ≥256ug/ml. In total, 78% of all chloramphenicol resistant isolates were positive by PCR, and only *CatI* genes were identified.

Figure5. Proportion of samples with at least one resistant isolate to chloramphenicol (with 95% binomial CI) at each sample time (treatment groups combined)



Again, two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (dfl) = 6.136, $p \leq 0.05$. And similarly comparing samplings 4 and 6 X^2 (dfl) = 9.130, $p \leq 0.01$. Thus the prevalence increased significantly during hospitalisation and decreased significantly after the horses had returned home.

Figure6. Proportion of samples with at least one resistant isolate to florfenicol (with 95% binomial CI) at each sample time (treatment groups combined)



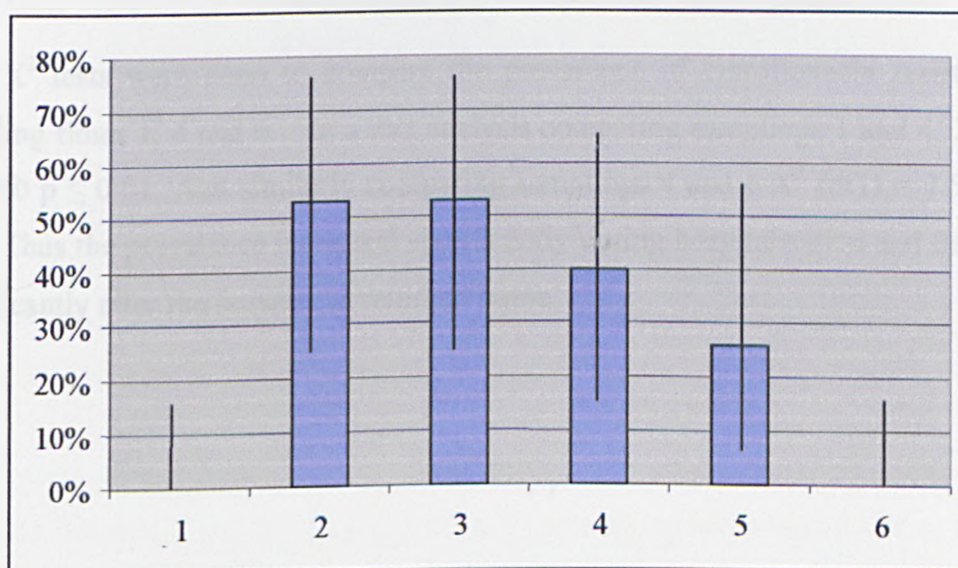
Similarly, two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (dfl) = 2.143, $p \geq 0.05$. And similarly comparing samplings 4 and 6 X^2 (dfl) = 2.143, $p \geq$

0.05. Thus there was no support for the hypothesis of a trend for prevalence to increase during hospitalisation or after the horses had returned home.

6.3.4 *Nalidixic acid and ciprofloxacin resistance*

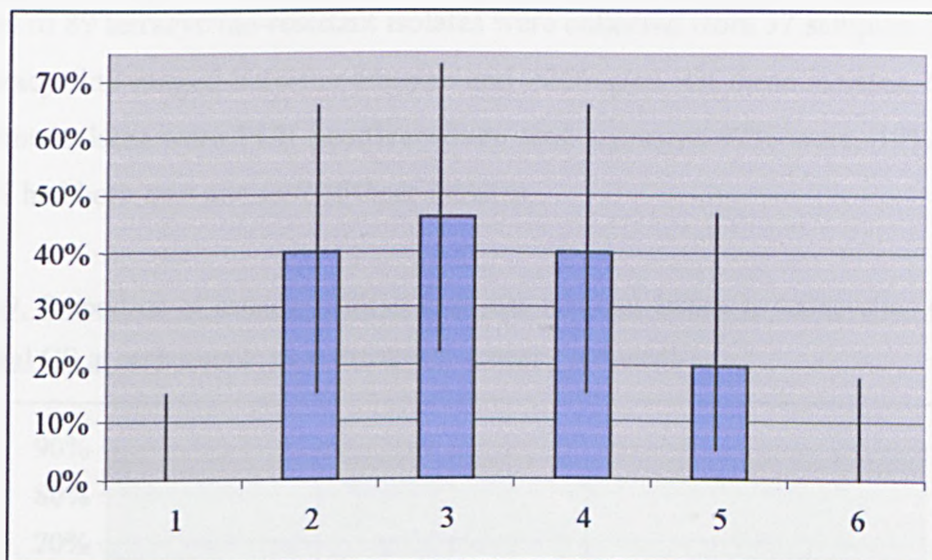
In total 65 isolates were resistant to nalidixic acid, of which 64 were also resistant to ciprofloxacin, from, respectively, 26 and 22 samples. Ciprofloxacin MIC values ranged from 4ug/ml to 16ug/ml and no further characterization was done on these isolates.

Figure7. Proportion of samples with at least one resistant isolate to nalidixic acid (with 95% binomial CI) at each sample time (treatment groups combined)



Two X^2 tests were done to compare the prevalence of nalidixic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 7.500, $p \leq 0.01$. Similarly comparing samplings 4 and 6 X^2 (df1) = 7.500 $p \leq 0.01$. Thus the prevalence increased significantly during hospitalisation and decreased significantly after the horses had returned home.

Figure8. Proportion of samples with at least one resistant isolate to ciprofloxacin (with 95% binomial CI) at each sample time (treatment groups combined)

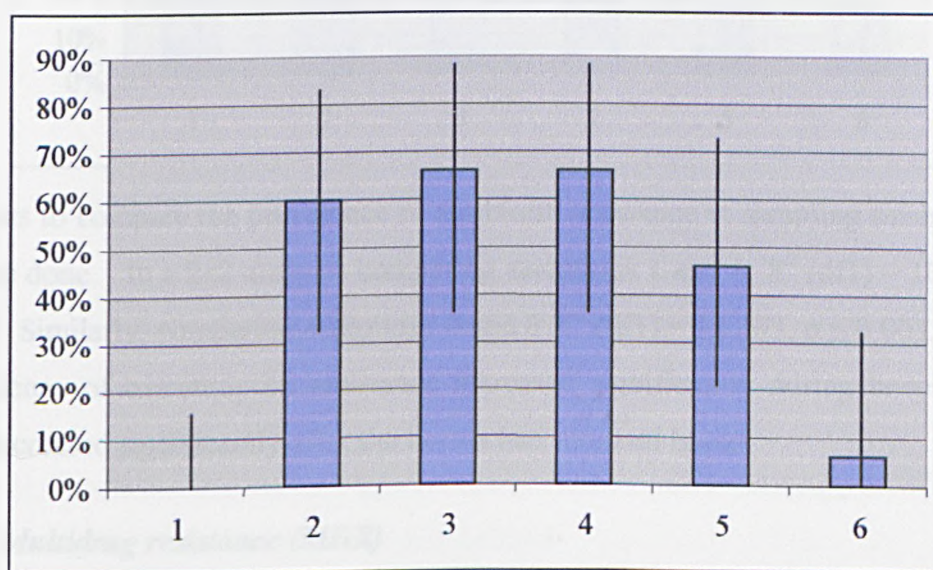


Two X^2 tests were done to compare the prevalence of ciprofloxacin resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 7.500 $p \leq 0.01$. And similarly comparing samplings 4 and 6 X^2 (df1) = 7.500, $p \leq 0.01$. Thus the prevalance increased significantly during hospitalisation and decreased significantly after the horses had returned home.

6.3.5 *Tetracycline resistance*

A total of 89 tetracycline-resistant isolates were collected from 37 samples. The MICs of tetracycline ranged between 64ug/ml and ≥256ug/ml. Of these isolates, 60.8 % of resistant isolates were PCR positive where *tetA* represent 92% were, 19% *tetB* and 11.9% had both *tetA* and *tetB* of these isolates.

Figure9. Proportion of samples with at least one resistant isolate to tetracycline (with 95% binomial CI) at each sample time (treatment groups combined)

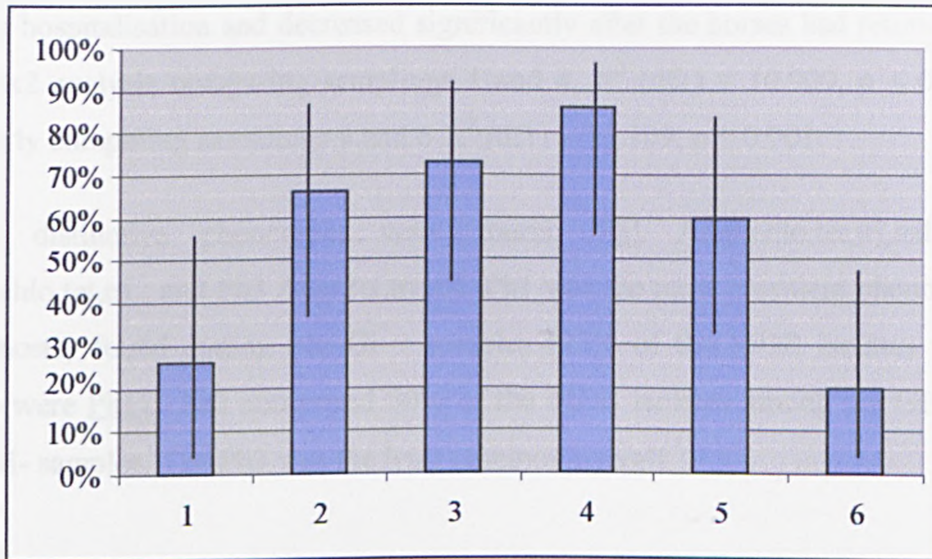


Again, two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 15.000, $p \leq 0.001$. And similarly comparing samplings 4 and 6 X^2 (df1) = 11.627, $p \leq 0.001$. Thus the prevalance increased significantly during hospitalisation and decreased significantly after the horses had returned home.

6.3.6 *Trimethoprim resistance*

In total, 127 isolates resistant to trimethoprim were collected from 50 samples. The MICs were all >256ug/ml. Of these isolates 79% were positive by PCR, comprising *dfr1* 38.5%, *dfr*(7-17) 50.4% and 20% was positive for *dfr*12.

Figure10. Proportion of samples with at least one resistant isolate to trimethoprim (with 95% binomial CI) at each sample time (treatment groups combined)

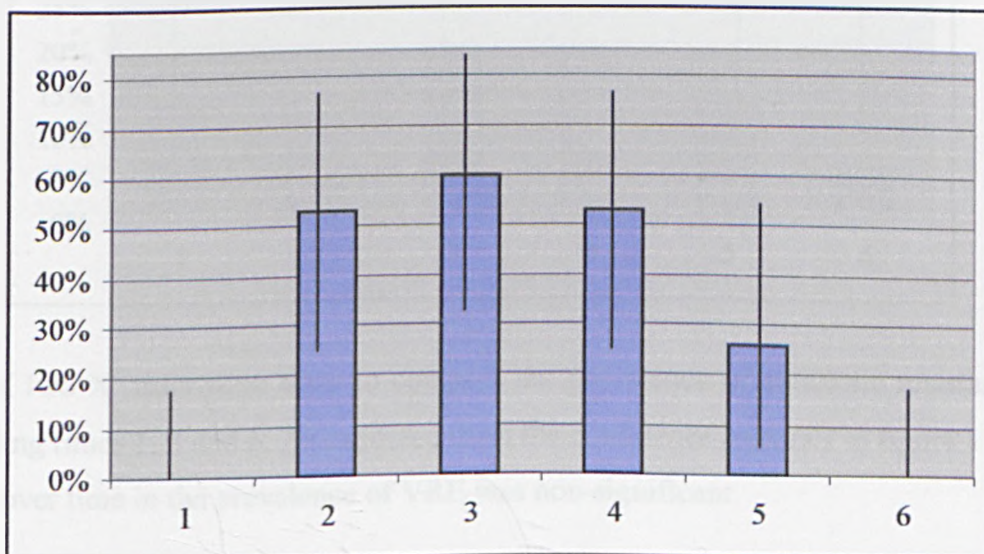


X^2 tests to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6 were done. In a 2x2 analysis comparing samplings 1 and 4, X^2 (df1) = 10.995, $p \leq 0.001$. Similarly, comparing samplings 4 and 6 X^2 (df1) = 13.393, $p \leq 0.001$. Thus the prevalence of trimethoprim resistance increased significantly during hospitalisation and decreased significantly after the horses had returned home.

6.3.7 *Multidrug resistance (MDR)*

The total number of isolates with a MDR profile was 71 from all groups, which represents 51.4% of the isolates and 29 samples.

Figure11. Proportion of samples with at least one MDR isolate (with 95% binomial CI) at each sample time (treatment groups combined)



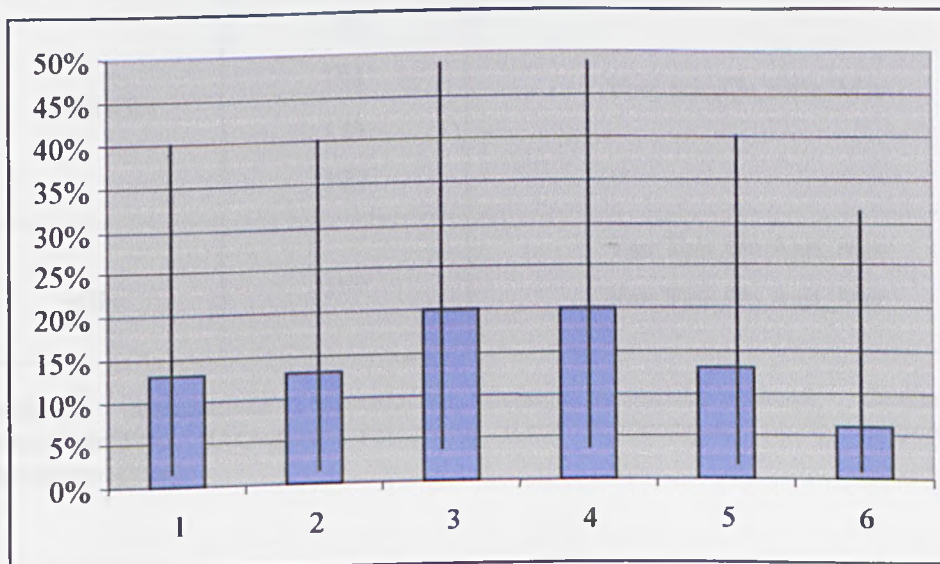
Again, two X^2 tests to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6 were done, and the prevalence found to have increased significantly during hospitalisation and decreased significantly after the horses had returned home. In a 2x2 analysis comparing samplings 1 and 4, X^2 (dfl) = 10.909, $p \leq 0.001$, and similarly comparing samplings 4 and 6 X^2 (dfl) = 10.109, $p \leq 0.001$.

Three distinctive phenotypes were found: Ph1 Amp,chlo,tet,tri,nal ; Ph2 Amp,chlo,tet,tri ; and Ph3 Amp,tet,tri,nal. Ph1 was the most prevalent phenotype, and was mostly found among non-GI + samples (93% of the MDR isolates from that group were Ph1). Ph2 comprised 50% of the MDR isolates among the GI+ and the non-GI- samples. The Ph3 was the least common overall.

6.3.8 *Vancomycin resistant enterococci (VREs)*

Overall, 13 (1, 4 and 8 samples from GI+, NON-GI+ and NON-GI- groups) samples contained VRE.

Figure12. Proportion of samples with at least one VRE resistant isolate (with 95% binomial CI) at each sample time (treatment groups combined)



Again, two X^2 tests were done to compare the prevalence of antibiotic resistance at sampling times 1, 4 and 6. As expected from the confidence intervals in figure 11, any trend over time in the prevalence of VRE was non-significant.

Table4 shows the resistance profiles of the VREs, all of which were typed by PCR and resistance profile as *E. gallinarum*.

<u>Table4. Characteristics of VRE from horse faeces</u>

Source	Number of isolates (App-5)	Summary of the Antibiotic resistance profiles
NON-GI-	141 142 153 251 101 102 107 108 119 120 121 122 123	Van,Teic,Qu,Azit,,Mer, Van, Teic,Qu,Azit,,Mer Van,Gent,Amp,Teic,Qu,azitr,Imp,Mer Van, Teic,Qu,Azit,,Mer Van,Gent,Amp,Teic,Qu,azitr,Imp,Mer Van, Teic,Qu,Azit,,Mer
NON-GI+	138 161 175 172	Van, teic,Qu,azitr, Mer
GI+	197	Van,Teic,Qu,Azit,,Mer Van,Teic,Qu,Azit,,Mer

Antibiotic abbreviations: Vancomycin (Van), Ampicillin (Amp), Gentamicin(Gent), Teicoplanin(Teic), Quinupristin/dalfopristin (Qu), Azithromycin (Azit), Imipenem (Imp), Meropenem (Mer)

6.3.9 Further sensitivity testing and experiments

6.3.9.1 *Aminoglycosides and sulphonamides resistance*

All the resistant isolates (n=138) were tested for susceptibility to further antibiotics. Overall, 38.5% were resistant to gentamicin, 71% to spectinomycin, 96% to streptomycin and 90% to sulphamexazole.

6.3.9.2 *Conjugation experiments*

In total 16 isolates (11%) were able to transfer resistance by conjugation, and these were distributed amongst all cohort groups. Details are shown in appendix 5. Mating experiments were performed on all isolates (n=138) except those which exhibited nalidixic acid resistance. Table5 lists the properties of the donor isolates, the resistance profiles of the transconjugants, which were identical to those of the donors in every case, and the results of PCR for resistance genes in the donor isolates (not repeated for the transconjugants).

Table5. Resistant isolates that transferred resistance via conjugation, listed according to their resistance phenotypes

Resistant isolates number (culture collection see app-5)	Origin	Resistance phenotype	Donors genes
54	NON-GI-	AMP	*tem*
55	NON-GI-	AMP	*tem*
204	NON-GI-	MDR	*dfl,dfl2,tetA,tem,catI,dfl*
205	NON-GI-	MDR	*dfl,dfl2,tetA,tem*
215	NON-GI-	MDR	*dfl,dfl2,tetA,tem,catI*
235	NON-GI-	MDR	*tem*
236	NON-GI-	MDR	*dfl,dfl2,tetA,tem*
28	GI+	AMP	*tem*
98	GI+	AMP,TRI	*dfr(7-17)*
136	GI+	AMP,TRI	-
147	GI+	AMP	*tem*
148	GI+	AMP	*tem*
149	GI+	AMP	*tem*
172	GI+	AMP,TRI	*dfl*
294	GI+	AMP,TRI	*tem*
189	NON-GI +	AMP	*tem*

6.4 Discussion

Previous studies have given rise to conflicting conclusions as to whether or not hospitalisation is associated with an increase in antibiotic resistance in bacteria (Koterba *et al.*,1986; Gaynes & Monnet, 1997; Bruinsma *et al.*, 2003). Resistant organisms might be excreted in the faeces of animals, following administration of antimicrobials, which contributes to the reservoir of resistant bacteria in the environment (Levy *et al.*, 1998). Therefore resistant bacteria acquired in the hospital environment may be disseminated via horses discharged from that environment.

In this study, no obvious association was seen between antibiotic treatment, or clinical condition, and resistance profiles in faecal *E. coli*. This may be because numbers were relatively small, or because horses entering the PLEH are largely referral cases and likely to have received antibiotic therapy prior to admission.

However, overall, resistance to most individual antibiotics, and MDR all increased during hospitalisation and then decreased after release from hospital. In an earlier study in a university equine hospital that investigated antibiotic resistance in *E. coli* and *Klebsiella*, similarly found that the rate of resistance amongst bacteria were higher day 7 of hospitalization compared to day of admission (Koterba *et al.*, 1986). This may be due to selection during hospitalisation through antibiotic therapy, and also the ready availability of resistant isolates in the hospital environment. It would be interesting to undertake PFGE analysis of the *E. coli* over time and see if the resistance is due to infection with resistant strains or horizontal transmission of resistance to the existing gut flora.

That the prevalence of resistance dropped markedly after discharge from the hospital might suggest that both the increase and decrease in resistance might be due to the turn over of *E. coli* between the gut and the environment.

The determination of the MICs and genetic analysis of resistance isolates, suggested that, as in Chapter 4, resistance was due to the same genes that commonly cause resistance in *E. coli* in other species. It was interesting, though, that while some MDR transferred in the conjugation studies; many transconjugants were resistant to

ampicillin or ampicillin and trimethoprim (table 5). Again, this suggests that both resistance profiles are encoded on mobile genetic elements. Horses in the GI+ and non-GI+ groups were the donors for most of the Amp and Amp/Trim transconjugants, and all the horses in both groups had had therapy with β-lactam drugs. It may, therefore be that these isolates represent either an endemic strain in the hospital, or an endemic plasmid moving rapidly between horses.

On the other hand, the PCR results for the determination of the resistance genes were not entirely similar to the results of first study. The trimethoprim resistant genes *dfr(7-17)* were the most prevalent genes identified among the positive PCR isolates and *dfr1* was the second most prevalent. The tetracycline resistant gene *tetA* was the most prevalent gene identified in this collection in contrast with previous results. This might mean that the *dfr*(7-17) and *tetA* resistant genes are more involved in the MDR mechanisms. The *tem* gene was again the most prevalent gene among the isolates. The *catI* genes which were mostly found in MDR isolates. As in chapter 4, the florfenicol resistance isolates were positive by PCR (5 out 7 were positive to *catI* gene) and the mechanisms of this resistance require further research.

The VREs were also isolated from all groups and all of them were found to be of the same species (*E. gallinarum*). Although numbers were small, there was no obvious association between antibiotic use, hospitalisation or clinical condition, suggesting that low prevalences of VRE are present in a variety of environments in horses.

6.5 Conclusions

No association between therapy and resistance profile was seen in this study. However, the prevalence of resistance, and MDR, did increase during hospitalisation and then decreased after release from the hospital. Thus therapy and the general environment of the hospital do appear to select for resistance, which may be of clinical and public health concern. Resistance isolates may also be disseminated once horses have been discharged.

Chapter 7

Risk factors associated with Equine Gastrointestinal Antimicrobial Resistant *E. coli*

7.1 Introductions

Currently there is a paucity of information concerning antimicrobial resistant bacteria in horses. However, there would be some expected common risk factors as found with other species. For example, hospitalization has been shown to be a risk factor for antimicrobial resistant bacteria shedding in some animals. Furthermore, hospitalization, in some cases combined with prior antibiotic therapy, significantly increases the chance of sick animals acquiring infections from other animals shedding infectious agents (Hird_ *et al.*, 1986; Ernst *et al*,. 2004).

Among humans, therapy with certain antibiotics has been shown to be a risk factor for shedding resistance and particularly to the same class of antibiotic as that used in therapy, for example fluoroquinolones (Ena J *et al.*, 1998; Garau *et al.*, 1999, Cheong *et al.*, 2001; McDonald *et al.*, 2001; Kahlmeter, 2003). Studies have shown that risk factors associated with antibiotic resistance among *E. coli* isolates of human origin were prior quinolone therapy, urinary tract abnormalities, and prior therapy with other antimicrobial agents, while other variables such as age, sex, long-term care, use of urinary or other catheters, were not indicated as a risk factors (Huotari *et al.*, 2003). Furthermore, resistance to fluoroquinolones is almost always reported associated with multi drug resistance and has never been found to occur independently of resistance to at least one other agent (Sahm *et al.*, 2001). Others have similar risk factors associated with *E. coli* resistance to other antibiotic drugs, such as ampicillin (Sotto *et al.*, 2001; Killgore *et al.*, 2004; Bolon *et al.*, 2004; Lautenbach *et al.*, 2000; Rao, 1998) and mutlidrug resistance (Glynn *et al.*, 2004).

7.2 Materials and methods

The protocols used for the determination of antimicrobial resistance, and the sources of samples have been described fully in Chapters 2 and 3. For each horse entering the cross sectional study, a questionnaire was completed concerning it's environment and clinical history. In addition questionnaires were completed for each of the premises. Examples of these questionnaires are given in Appendix 1.

7.2.1 Statistical Analysis

The following statistical analysis was conducted on the chapter 3 samples in order to identify any risk factors associated with antibiotic resistance. In this work I was helped enormously by Dr. Keith Baptiste (University of Copenhagen).

Initially all data was assessed by descriptive statistics and univariable analysis using SAS-version 9.1 computer software. The frequency of *E. coli* colonies resistant to antimicrobials (and multi-drug resistant) was compared between different groups (e.g. origin of horse, sampling time) using a McNemar's test.

The association between antimicrobial resistant gastrointestinal bacteria and risk factors related to the horse and environment were explored with logistic regression analysis. Separate models were explored for the dependent outcomes – antimicrobial resistant *E. coli*, and multi-resistant *E. coli*, respectively. For each dependent variable, two overall analyses were done involving: 1) hospitalised horses; 2) horses residing at two different outside premises. Each dependent variable was represented as a bacterial-level proportion index (No. positive colonies / No. colonies examined). Furthermore, multi-level logistic regression models were explored for each dependent outcome, where either the number of faecal samples taken from a horse or the premises of origin were included in models as random effects using SAS-Glimmix macro.

Independent variables included horse-related variables (e.g. age, sex, breed, origin), treatment-related variables (e.g. disease, diagnosis, drug type, drug amount, days

hospitalised, route of drug administration, treatment days), and bacteria-related characteristics (e.g. antimicrobial resistance pattern, number of isolates).

Initially, all continuous independent variables were screened in a univariable analysis, as both a linear and quadratic terms, against all outcome variables for possible inclusion in models. From the univariable analysis, independent variables were chosen for inclusion in preliminary models based on a p-value ≤ 0.30, as well as the size of the parameter estimate $\pm$ standard error. For example, parameter estimates with unusually large values were excluded from the analysis. A Pearson's scale was used to correct for overdispersion.

Forward and backward selection procedures were used to identify variables with a p-value ≤ 0.05 for inclusion in final models. All continuous independent variables were centred against the mean-value before inclusion in final models to reduce muticollinearity. Final models were selected based on parameters estimates, p-values and examination of model residuals.

7.3 Results

In total, 66 horses were sampled at the equine hospital (Leahurst) but just 64 were analyzed, as well as 35 and 37 horses from two other horse boarding premises, respectively. From the equine hospital, 32 horses were sampled twice, 8 horses sampled on three separate occasions, as well as four horses and one horse sample four and five times, respectively. From the two other premises, 42 horses were sampled twice, and 27 and 14 horses sampled three and four times, respectively. The frequency of horses positive for antimicrobial resistant *E. coli* and multi-drug resistant *E. coli* is given previously in chapter 3 and demonstrates a proportionally higher number of positive horses in the hospital environment compared to the other two horse premises. From the equine hospital, there were a significantly greater proportion of positive horses from the first sampling compared to other sampling times.

A summary of the univariate analysis to explore horse-related associations of antimicrobial resistant *E. coli* and multi-resistant *E. coli* is given in Table 1a, 1b. From this univariate analysis, multivariable logistic regression models were explored. No useful models could be developed for multi-resistant *E. coli*, both with hospital data and the other premises. However, models could be developed for antimicrobial-resistant *E. coli* both in the hospital setting and other premises (Table 2). In the hospital, logistic regression models showed that horses were more likely to shed antimicrobial-resistant *E. coli* if presented for a GI problem, received a number of antimicrobial treatments in hospital and if the horse received oral antimicrobial treatments. For example, horses presented to the hospital for GI problems were 2.81 times more likely to be positive for antimicrobial resistant *E. coli* than horses not presented for GI problems. Also, for each antimicrobial treatment in hospital, horses were 1.18 times more likely to be positive for antimicrobial resistant *E. coli*. However, the model indicated that the greatest odds (3.52) of shedding antimicrobial resistant *E. coli* were from horses treated with oral antimicrobial drugs.

For horses from premises outside the hospital, two risk factors were identified as associated with antimicrobial-resistant *E. coli*. Horses were 1.17 times more likely to positive for antimicrobial resistant *E. coli* for each year increase in age. Also, the number of years the horse had spent on the premises was found to be a protective

factor for not acquiring antimicrobial resistant *E. coli*. In other words, horses were 0.76 odds less likely to be positive for antimicrobial resistant *E. coli*.

Table1a: Summary of the univariate analysis of variables used for the logistic regression analysis. Results expressed as either mean+Std err., or frequency of positive horses for each type of *E. coli.*

	Hospital Resistant *E. coli*			**Hospital Multi-resistant *E. coli***		
	Positive	Negative	p-value	Positive	Negative	p-value
Age (yrs)	10.1±0.6	7.5± 1.2	0.118	10.8±0.8	8.2±0.7	0.09
Days before sampling AB given	6.9±0.9	3.1±0.7	0.078	6.1±1.1	6.1±1.0	0.672
No. Tx in Hospital	6.9±0.9	2.9±0.7	0.058	6.2±1.1	5.8±1.0	0.662
Days in hospital	9.8±1.9	29.2±23.0	0.282	10.0±2.7	18.0±10.0	0.442
No. AB before sampling	0.9±0.1	0.9±0.3	0.258	1.1±0.1	0.70±0.2	0.662
ABs before sampling	20	5	0.057	15	10	0.980
Non-AB TX before sampling	32	8	0.258	22	18	0.518
NSAID	19	5	0.621	11	13	0.265
B-lactam drugs	11	3	0.447	7	7	0.453
Aminoglycosides	3	1	0.824	2	2	0.518
Quinilones	2	0	0.985	2	0	0.452
GI problem	17	3	0.003	14	6	0.287
ABs Route IV	16	7	0.878	12	11	0.868
ABs Route IM	4	1	0.045	3	2	0.366
ABs Route PO	9	1	0.030	9	1	0.219
Stallion	23	5	0.086	14	14	0.750
Gelding	27	8	0.090	19	16	0.874

Table1b: Summary of the univariate analysis of variables used for the logistic regression analysis. Results expressed as either mean+Std err., or frequency of positive horses for each type of *E. coli*.

	Premise Resistant *E. coli*			**Premise Multi-resistant *E. coli***		
	Positive	Negative	p-value	Positive	Negative	p-value
Age (yrs)	14.8±1.1	13.3±0.7	0.018	14.4±1.9	13.7±0.6	0.062
Yrs on Premise	5.8±0.6	6.6±0.5	0.004	5.0±0.7	6.6±0.42	0.664
Stallion	8	15	0.247	5	18	0.012
Gelding	11	37	0.247	3	45	0.001
On medication	1	0	0.900	0	1	0.99
Medication in last 6 months	3	1	0.020	1	3	0.108
Abs in last 6 months	1	1	0.100	0	2	0.344
Cephalosporins	1	2	0.020	1	2	0.022

Table 2: Summary of logistic regression models developed to explore the association of antimicrobial resistant E. coli in horses either at the equine hospital or other premises.

Logistic Regression Models	Variables	Parameter Estimate	Std. err.	Odds Ratio	Odds Ratio 95%CI	p-value
Hospital – Resistant *E. coli*	Intercept	-0.541	0.386			
	GI problem	1.035	0.410	2.81	1.26-6.289	0.0117
	No. Tx in Hospital	0.166	0.060	1.18	1.05-1.33	0.006
	AB Route PO	1.257	0.587	3.52	1.11-11.10	0.032
Premises – Resistant *E. coli*	Intercept	-1.760	0.420			
	Age	0.156	0.034	1.17	1.09-1.25	<.0001
	Yrs on Premise	-0.280	0.058	0.76	0.68-0.85	<.0001

7.4 Discussion

There were significant differences in the proportion of horses that tested positive for antimicrobial resistant *E. coli* with respect to the different sampling times. This suggests there is a fair degree of variation in shedding patterns of *E. coli* from horses. Future studies should take this into consideration and sample horses more than once to investigate antimicrobial resistant bacteria.

Horses presenting to hospital were more likely to be shedding antimicrobial resistant *E. coli*. This may be due to the fact that they are diseased and more likely to harbour these type of bacteria. Or possibly they have picked up resistance in the hospital. From the data collected in this study, the environment that horses live in appears to have an influence on the presence of antimicrobial resistant bacteria independent of treatment with antibiotics.

The logistic regression models indicate that horses presented for gastrointestinal diseases were more likely to shed antimicrobial resistant *E. coli*. This may have implications for hospital management in that maybe different isolation procedures may be in order to control antimicrobial resistant bacteria in the hospital.

Surprisingly, there was not a strong association between types (e.g. Quinolones) and doses of antimicrobial drugs given to horses and the presence of antimicrobial resistant *E. coli*. However, the relatively small scale of this study in terms of the number of horses sampled may be the reason for the lack of association. A larger scale study would be needed to further investigate associations of equine gastrointestinal antimicrobial resistant bacteria and drug usage.

Chapter 8
General discussion

The main aims of the work described in this thesis were to investigate the prevalence of zoonotic, enteric bacteria in horses, and also antibiotic resistance, itself a form of zoonosis. The investigation concentrated on *E. coli*, *Salmonella enterica*, *Campylobacter* spp and vancomycin resistant enterococci (VREs), and on antibiotic resistance in *E. coli*.

Initially, a relatively simple cross sectional study was carried out, in which faecal samples were collected from healthy horses at two stables, and from horses hospitalised at the Philip Leverhulme Equine Hospital, the referral hospital of the University of Liverpool Veterinary School.

The prevalence of *Campylobacter* was found to be low in horses – only three isolates were found, all *C. jejuni*, and all from horses within the PLEH. This fits with previous studies in which campylobacteriosis has been found to be uncommon in horses (Browning *et al.*,1991; Guy, 2001) unlike many other herbivores, and particularly cattle. Furthermore, there was no evidence of resistance among these isolates apart from resistance to trimethoprim, which is normal in *C. jejuni* (Gibreel *et al.*, 2000; Moore *et al.*, 2005). It may be that campylobacteriosis is a nosocomial infection, resulting from a high density of horses and high turn over within the hospital, or it may be that 'ill' horses are more susceptible to infection and shedding. However, it is equally likely that these isolates merely reflect the horses in the PLEH coming from so many different populations. Whatever the source of these isolates, campylobacteriosis appears uncommon in horses and therefore *Campylobacter* of equine origin do not pose a zoonotic concern.

Salmonella enterica was not found in any horses in the cross sectional study, although they had been isolated previously from the PLEH, and other studies have found salmonellosis to be mainly a problem in hospitalized horses (Hartmann *et al.*, 1996; Guy, 2001). The combination of the therapy (antibiotics in particular) and other stressors, reportedly can lead to shedding of this bacterium from horses (Cherry *et al.*,

2004). That *Salmonella* was not found in this study, and previously also had a very low prevalence among the hospitalized horses in the hospital indicates that *Salmonella* in horses is mostly likely brought to the hospital by individuals (Ward *et al.*, 2005 a & b), which may lead to small outbreaks of nosocomial infection.

An archive of *Salmonella* isolates from the PLEH was therefore investigated. Antibiotic susceptibility assays proved to be a useful tool in identifying the possible similarities between the *Salmonella* isolates, especially when combined with restriction length polymorphism and pulse field electrophoresis (PFGE). *Salmonella enterica* Typhimurium was the most frequently isolated serovar, as has been found in previous studies in horses (Van Duijkeren *et al.*, 2002). Furthermore, several isolates were typed as multidrug resistant DT104. The sensitivity tests along with the PFGE revealed the similar profiles for several clusters of isolates, despite their being isolated from different horses at different times. This suggests that horses are probably spill-over hosts, becoming infected with whatever strains are prevalent at the time, although some nosocomial transmission may occur once an infected horse enters the hospital. Overall this suggests that horses can be infected with zoonotic salmonellas, but that the infection rate is low, and the public health risk therefore also low.

Not surprisingly, *E. coli* was the bacterium most frequently isolated from horse faeces, the isolation rate reaching almost 100%, whatever the background of the horse. This study did not concern itself with pathogenicity and the zoonotic potential of *E. coli*. This would make an interesting further study, as the archived isolates could be screened for genes known to encode human pathogenicity factors. Rather, this study focussed on *E. coli* as a potential source of antibiotic resistance, an issue important in both equine and human medicine.

Further analysis of the cross sectional survey in Chapter 3 showed that antibiotic resistance, and multi-drug resistance, was more common in *E. coli* isolated from horses in the hospital than from the two local stables. Interestingly, there was also a significant difference between the prevalences of antibiotic resistant *E. coli* in the two stables, and further study is needed to determine why this might be. Using logistic

regression models, horses were found to be more likely to shed antimicrobial-resistant *E. coli* if they had a GI problem, received antimicrobial treatment in hospital and if the horse received oral antimicrobial treatments. While not unexpected, these results may be useful to those trying to control resistance in the hospital population. It was also interesting that among non-hospitalized horses factors like age and time spent on the premises were found to be protective, ie associated with not acquiring antimicrobial resistant *E. coli*

That more resistance was seen in hospitalised horses is perhaps not surprising, as these are animals that have been treated with antibiotics and in which selection for resistance might be expected. A longitudinal study of horses (chapter 6) and the rates of resistance in their *E. coli* clearly demonstrated a rise in the prevalence of resistance and multi-drug resistance during hospitalisation, and a decline after discharge. No difference was detected in that study between horses being treated with antibiotics and those not, however. This may be due to sample sizes and lack of statistical power or it may be that antibiotic resistance is endemic in the hospital *E. coli*, and the horses become infected with resistant strains without any need for selection. It also may be that hospitalised horses had been treated prior to arrival with antibiotics.

It is interesting that the prevalence of resistant *E. coli* fell after discharge from hospital. It has been argued that the use of antibiotics selects for resistance, but that resistance, in the absence of antibiotics, makes bacteria less 'fit' than the wild-type strain (Lenski, 1997). However, it has also been shown that compensatory mutations soon occur leading to the evolution of resistant strains no less fit than their wild type ancestors (Lenski, 1997), and empirical evidence, such as finding relatively high prevalences of resistance in wildlife faeces (Gilliver *et al.*, 1999), also suggests that antibiotic selection is not necessary for the maintenance of resistance in bacterial populations. It may be, therefore, that this loss of resistance is due to lack of selection, or it may be simply that horses have a high rate of turnover of gut flora in exchange with their environment, and so the resistant strains acquired during hospitalisation are simply diluted back in the horse's normal environment. If this turnover argument is true, then it might also explain why there was no difference between antibiotic–

treated and non-treated horses in the hospital: the rise in resistance may have simply been due to all the horses ingesting resistant strains of *E. coli* from the environment.

That so much resistance is found amongst *E. coli* from hospitalised horses is worrying from both an equine medicine and a public health perspective. Particularly worrying are the MDR strains, with resistance to important drugs such as ciprofloxacin and beta-lactams, which are still widely prescribed in human medicine. Further molecular typing, perhaps using PFGE might be useful in order to study who infects whom, and to follow the transmission of strains or genes amongst horses and also in the environment.

The existence of MDR strains, at least sometimes containing transferable MDR (although a lot more work needs to be done on the isolates from this study in order to determine the transferability and genetic basis of resistance) is particularly worrying clinically as any selection for resistance to one antibiotic will lead to selection for others encoded on the same genetic element (Ambler *et al.*, 1993; Garau *et al.*, 1999; Braoudaki & Hilton, 2004). Chloramphenicol, for example, has rarely if ever been prescribed for any horses within the hospital, and generally ceased to be used in animals in Britain in the early 1990s, yet resistance to chloramphenicol was common in MDR isolates.

The PCR studies of resistant isolates overall revealed that the genes responsible were largely those reported from other domestic animals and from humans. Very few isolates contained resistance genes not detectable with PCR primers targeting these common resistance genes. The detection of genes like *tem*, *tetA*, and *dfr* genes shows that horses are probably just part of the world-wide phenomenon of resistance dissemination and development.

The findings of high proportion if MDR phenotypes among the *Salmonella* isolates in chapter 5 may also be linked to the MDR found in *E. coli.* While it is probably most likely that the salmonellae isolated from horses were already MDR before that case, it remains possible that the MDR originated in *E. coli* and was transferred to the salmonellae in the horse intestine, perhaps under selection pressure from the use of

antibiotics. Similar transfer has been reported in other species (e.g. Hunter *et al.*, 1993).

Vancomycin resistant enterococci were found in samples from the hospital but also from non-hospital sources, and there was little evidence of a change in prevalence of VRE during and after hospitalisation. Furthermore, the VREs were nearly all identified as *E. gallinarum*, containing the *vanC* gene usually associated with intrinsic resistance. Although it was interesting to find VRE in horses, the prevalence was low, and this species and resistance phenotype are not usually associated with human disease. Thus, VRE in horses is unlikely to pose a significant zoonotic threat.

Future work

Several further studies have been discussed already, and given more time and resources, should be done.

It would be interesting to undertake a larger study of the dynamics of antibiotic resistance in *E. coli* and salmonellae in the hospital: does antibiotic therapy select for resistance, or is the rise in resistance seen during hospitalisation a function of the gut flora simply including more and more resistant environmental strains? Is there evidence for transmission of resistance from *E. coli* to salmonellae? Or vice versa? This would require more frequent sampling of horses, the examination of more isolates from each sample, and PFGE or other molecular typing of isolates and resistance-encoding genetic elements (integrons, plasmids etc) in order to follow *E. coli*, *Salmonella* and genes through the horse population.

It would also be valuable to test the archive of *E. coli* for potential pathogenicity genes, and determine whether or not there was any association between such genes and resistance.

It would also be interesting to undertake a longitudinal study of zoonotic enteric bacteria and antibiotic resistance in horses and their owners or carers: this would provide the most direct evidence for or against zoonotic transmission.

Several of the resistance mechanisms have not so far been investigated in the isolates collected in this study, and this work should be done on the *E coli* archive. For example, what mutations in *gyr* or other mechanisms might be involved in ciprofloxacin resistance? And what are the genetic mechanisms underlying MDR.

It would be sensible to undertake a more detailed analysis of the risk factors for antibiotic resistance in horses sampled in the cross sectional study. Questionnaires about the individual horses and about the establishments they came from are available for each sample, including data on disease, antibiotic treatment and general management. The laboratory work described in this thesis left little time for analysis of this data, but that analysis is now underway in collaboration with Dr Keith Baptiste, and a preliminary analysis is described in chapter 7.

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Abbreviations & appendixes

I-Abbreviations

II-Appendix 1:

1. Bacteriological materials

1. A.1 Media

1. A.2 Chemical Reagents

1. A.3 Antibiotic related materials

1. B Molecular materials

1. C Tables show Breakpoints and MICs values for involved bacteria

1. D MIC (minimum inhibitory concentration) protocol

1. F Questionnaires copies

III-Appendix 2:

- 2. A Hospital samples: - 109 faecal samples
- 2. B Riding school ('premises A'): - 112 horse faecal samples
- 2. C Livery yard ('premises B'): - 43 faecal samples

IV-Appendix 3:

- Culture collection (296 resistant *E. coli* isolates)
- Examples of different PCR positive isolates to different resistant genes

V-Appendix 4:

- 4. A (GI+).
- 4. B (NON-GI+)
- 4. C (NON-GI-)

VI-Appendix 5:

- Culture collection (138 resistant *E. coli* isolates)

I-Abbreviations

Media abbreviation:

NA	nutrient agar
EMBA	Eosin Methylene Blue agar
BA	Blood agar
CAB	Campylobacter broth
XLD	Xylose Lysine Decarboxylase
Iso	Iso-sensitest agar

Antibiotics abbreviations:

ABs	Antibiotics
AMP	Ampicillin
APR	Apramycin
CHL	Choloramphenicol
FLO	Florfenicol
TET	Tetracycline
TRI	Trimethoprim
NAL	Nalidixic acid
CIP	Ciprofloxacin
GEN	Gentamycin

Resistance abbreviation:

PLEH	Philip Leverhulme Equine Hospital
BSAC	British association of antimicrobials and chemotherapy
MDR	Multidrug resistance
VRE	Vancomycin resistant enterococci
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant staphylococcus aureus
VRSA	Vancomycin resistant staphylococcus aureus
BETA	β
R	Resistance

Molecular abbreviation:

PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
DNA	Deoxyribo-nucleic acid
RNA	Ribo-nucleic acid

II-Appendix 1

1. Bacteriological materials

1. A.1 Media

All media described are prepared in accordance with their manufacturers instructions and unless stated otherwise were obtained from LabM (IDG)

Brain heart infusion broth (LAB51)
Eosin Methylene Blue agar (LAB61)
Nutrient agar NA (LAB8)
Tryptone Soy agar (LAB11)
MacConkey agar (LAB30)
Simmon's Citrate agar (LAB69)
Campylobacter Enrichment Broth CB (LAB135)
Campylobacter Selective agar CSA (LAB21)
Columbia agar CA (LAB1)
Rappaport-Vassiliadis Broth RVB (LAB86)
Xylose Lysine Decarboxylase (XLD) agar (LAB032-A)
Kanamycin Aesculin Azide broth KAB (LAB107)
Kanamycin Aesculin Azide agar KAA (LAB106)
Iso-sensitest agar Iso (LAB 170)

1. A.2 Chemical Reagents

Hydrogen peroxide: (Sigma)
Lugol's iodine: (Pro-lab Diagnostics)
Kovac's reagent (bioMerieux)
lysed horse blood and defribinated horse blood :(Southern Laboratories Group)
Polyvalent O and H antisera: (Pro-lab Diagnostics)
Oxidase strip: (Mast)
Api 20E: (bioMerieux).
Gram stains materials were obtained from pro-lab and were: Crystal violet, Lugols iodine, Acetone, Safranin.

1. A.3 Antibiotic related materials

Antibiotic discs (Mast Diagnostics)
Nitrocefin (Oxoid-UK)
Antibiotic supplement (x131): for Campylobacter broth [concentration in media: Cefoperazone 20mg/L, Vancomycin 20mg/L, Trimethoprim 20mg/L, Cycloheximide 50mg/L].
Cefoperazone Amphotericin (LabM, X112): for Campylobacter agar [concentration in media: Cefoperazone 32mg/L and Amphotericin 10mg/L].
Antibiotic drugs for MICs (Sigma) and were:
Tetracycline (T-3383), trimethoprim (T-7883), chloramphenicol(C-0378), ampicillin (A-9393), except ciprofloxacin from ICN-Biomedicals (199020).

1. B Molecular materials

Primers listed in chapter 2.were purchased from MWG: (Ebersberg).
PCR reagents were all purchased from Abgene: (Epsom).
1.1xReddyMix PCR MasterMix: (AB-0575/LD/A)
1.5 $MgCl_2$ PCR MasterMix: (AB-0575/DC/A),
Gel loading solution: (Biogene)
Ethium bromide: (Sigma)
Molecular weight marker: Low 100bp ladder for PCR.
Hi-Pure-Low EEO Agarose: (Biogene)
TAE Buffer (Tris-Acetate- EDTA Buffer) [T-9650] composed as following: 0.4 Tris acetate –pH approx. 8.3, 0.01 M EDTA.
TBE Buffer (Tris-Borate- EDTA Buffer) [T-4415] composed as following: 0.89M Tris BORATE –pH approx. 8.3, 0.02 M EDTA.
Molecular weight marker: (Abgene) A Lamda genomic molecular weight marker: (Biolabs) for PFGE supplied in (1% LMP agarose, 10mM Tris-Hcl (pH8.0), 1mM EDTA and 50% glycerol in a Gel Syrenge dispenser).

Other non mentioned materials in this section were supplied by and through the department.

1.C Tables show Breakpoints and MICs values for involved bacteria according to BSAC guidelines and are available and updated on www.bsac.co.uk

Table shows the break point and the MICs for *E. coli* and *Salmonella*

Antibiotics	Disc (µg)	Resistance interpretation			
		Zone in (mm)		MICs (mg/L)	
		R ≤	S ≥	R ≥	S ≤
Ampicillin	10	13	14	32	16
Amoxycillin	10	13	14	32	16
Cefuroxime	30	19	20	16	8
Ceftazidime	30	21	22	4	2
Ciprofloxacin	1	17	18	2	1
Gentamicin	10	19	20	2	1
Aztreonam	30	23	24	2	1
Cefotaxime	30	29	30	2	1
Cefoxitin	30	19	20	16	8
Cefpodoxime	5	33	34	2	1
Chloramphenicol	30	20	21	16	8
Co-amoxyclav	10/20	13	14	32	16
Piperacillin /Tazobactam	85	21	22	32	16
Streptomycin	10	12	13	16	8
Sulphamethoxazole	100	13	14	64	32
Tetracycline	30	33	34	2	1
Trimethoprim	2.5	14	20	4	0.5

Table shows the Breakpoints and MICs for *enterococci*

Antibiotics	Resistance interpretation				
	Disc(μg)	breakpointS zone in (mm)		MICs (mg/L)	
		R ≤	S ≥	R ≥	S ≤
Gentamicin	200	9	10	1024	512
Ampicillin	10	19	20	16	8
Vancomycin	5	12	13	8	4
Teicoplanin	30	19	20	8	4
Quinupristin/ Dalfopristine	15	19	20	4	2
Azithromycin	15	29	30	2	1
Imipenem	10	19	20	8	4
Meropenem	10	19	20	8	4
Linezolid	10	19	20	8	4

1. D MIC (minimum inhibitory concentration) protocol

Standard microbiology method for the determination of the minimum inhibitory concentration (MIC) of antimicrobial agents using agar incorporation tests

Reference for this method is refereed to the NCCLS guidelines and two methods were used which are documented as M7-A3 and M11-A3 and are (Methods for Dilution Antimicrobial Susceptibility Tests for Bactera that grow Aerobiclly) (Methods for Antimicrobial Susceptibility Testing for Anaerobic Bactera) respectively.

The protocol:

1- Preparation of the inoculum

Isolates were grown 24 hours prior to preparing the inoculum, nutrient agar incubated at 37°C for 24 hours as recommended.

2- Media preparation.

Iso-Sensitest agar was used and prepared in 20ml volumes, autoclaved and then kept in a water bath at 50 °C.

3- Antimicrobial agents stock preparation

From each agents a stock solution was prepared and subsequent dilutions prepared as stated below (in the appropriate diluent) labelled A-D, of which different volumes were added to the 20ml volumes of agar to produce plates incorporating antibiotics at concentration between 256µg/ml and 0.016µg/ml as shown below.

A: 32.0 mg test substance in 6.0 ml dilution: 5330µg/ml
B: 1.0 ml of A +7.0 ml diluent ml dilution: 666µg/ml
C: 1.0 ml of B +7.0 ml diluent ml dilution: 83µg/ml
D: 1.0 ml of C +7.0 ml diluent ml dilution: 10.4µg/ml
E: 1.0 ml of D +7.0 ml diluent ml dilution: 1.3µg/ml

Diluents used for the antimicrobials were as following:

-Ampicillin: phosphate buffer (up to 7 ml)

-Chloramphenicol: ethanol (up to 7ml with water)

-Tetracycline: water (up to 7 ml with water)

-Trimethoprim: 0.05 m/l of HCl (up to 7ml with water)

-Ciprofloxacin: water (up to 7ml with water)

4- Preparation of agar plates:

The previous solutions concentration were added to each 20 ml of agars from stock "A":

-1ml into 20 ml agar = 256 µg/ml

-0.5 ml into 20 ml agar = 128 µg/ml

-0.25 ml into 20 ml agar = 64 µg/ml

The same step was than applied on the other stocks B, C, D, E in the same order to give concentrations in µg/ml of (32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, and 0.016).

Control plates were also prepared where no antibiotic was added; just water and plates were than left to solidify at room temperature.

5- Preparation of the inoculum:

The prepared agars were inoculated by using a multipoint inocular rods of 36 rod which represent the number of isolates that can be inoculated on a single plate and for each agent. The multipoint inocular estimated to pick around 5µ of inoculum and the inoculation should start from the most to the least concentration to avoid any miss leading by picking over concentration and plates than were inocubated for 24-48 hours at 37 °C.

A result for each plate was recorded by observing the concentration that ceased the growth of bacteria (MICs).

MICs breakpoints for the aimed bacteria are listed previously.

Contact name: -

Address: -

Post code: -

Form no (   )

Environment Questionnaire

1-On average in the last 6 months how many horses and ponies are kept on these premises? ☐

2- What is the purpose of your premises? ☐

3- On average in the last 6 months how much grazing do horses and ponies have access to?

None ☐ up to 5 acres ☐ 6-25acres ☐ >25acres ☐

4-On average in the last 6 months how many horses graze together at any one time? ☐

5-On average in the last 6 months did you share grazing with other people?

Yes ☐ No ☐

If yes, how many other horses belonging to other owners apart from your horses ☐

6-Could you give details about the surrounding area around your premises?

☐

.7-How many other people have contact with your horses? ☐

8- How do you deal with horse faeces in grazed field:

- Remove daily ☐ Others ways ☐
- Remove weekly ☐ not remove ☐

9- The last 6 months did have you kept any animals rather than horses in your premises?

Yes ☐ no ☐

If yes, what animals and how many?

10-What other species share grazing area with your horses (indicate approx. number)?

- Cattle ☐ Goat ☐
- Sheep ☐ Poultry ☐
- Others ☐ None ☐

11-What species are kept on neigh boring premises or grazing:

- Cattle ☐ Horses ☐
- Sheep ☐ Poultry ☐
- Goat ☐ Pigs ☐
- Others ☐ None ☐

12- What other species are kept on or visit your premises that might have contact with your horses:

- Cattle ☐ Sheep ☐
- Dogs ☐ Pigs ☐
- Cats ☐ Poultry ☐
- Others ☐ None ☐

13- What wild animals have you seen in your fields:

- Seagulls ☐ Foxes ☐
- Badges ☐ Others ☐
- Rabbit ☐ None ☐

14-What wild animals have you seen inside your stables:

- Rats ☐ Pigeons ☐
- Mice ☐ Starlings ☐
- Other ☐ None ☐

15-Have any horses showed signs of enteric (gut) disease in the last 6months?

Yes☐ no☐

If yes, which of the following applies to any horses on your premises?

- Diarrhoea ☐
- Colic ☐
- Weight loss ☐
- Others ☐

16-Have you participate in any competition or show in the last 6 months?

Yes ☐ no ☐

17- Have any horses/ponies been treated with antibiotics the last 3-6 months?

Yes ☐ no ☐

If yes, which drugs were used:

Form no ☐

H/form no ☐

Date/ ☐☐ /☐☐/0☐

Field form questionnaire

Horse name/

Owner name/ contact Tel/

General premises details: -

1-What is the size of the farm the horse is kept in ☐
(or just pick one of the following choices)

☐ 0-5acres ☐ 6-15acres ☐ 16-25acres ☐ > 25

2- Is the premise
- **A** include just horses ☐
- **B** horses and other animals ☐

3- Please describe briefly the area surrounding your premises?

☐ Rural ☐ suburban farm ☐ Semi rural ☐ others → Specify ____________

4-What animals kept on neighbouring premises? (list them please)

(For example mention number of farms and the purposes of plus animals species in each one)

Premises name	Purposes/main activities	Animals kept	Number of kept animals

NOTE: -*If the question 2 answer was A answer just section I but if the answer was B answer both sections I and II.

*If you need more space use the back of the sheet to add any extrainformations.

Section I: -

1-How many horses in the yard? ☐

2-What breed are in the yard (please give numbers of each breed in the boxe's).

TB ☐ warm ☐ pony ☐ Arab ☐ others ☐ Specify ________

3-What are the horses used for (tick all that apply)
(and give the number of each if possible)

Breeding ☐ competition ☐
Leisure ☐ others ☐
Showing ☐ specify please________

4-Are they stabled? yes ☐ no ☐ if no specify ________

If yes are they kept in: -

- boxes outside ☐
- inside a bam ☐

5-Do you

a-have your own grazing area ☐

b-share the area with others ☐

If b how many people or premise share this grazing area ☐

6-How much grazing do your horses have access to ☐

7-Do you remove faeces from grazing yes ☐ no ☐

If yes how regularly you do so ________
((e.g.daily, once or twice /week, once/month……etc))

8-Do you keep any pets in your premises? yes ☐ no ☐

If so mention them plus how many please: -

Animal	how many	

9-Has any of your horses (excluding the "case" horse) received medication including antibiotics, (but excluding wormers and vaccination) in the last 6monthes

☐ Yes ☐ no

If yes what for, and list the drugs been given

Section II: - (for premises with other grazed animals)

10-What is the main purposes of the premises ?
e. g.commercial farm, hobby farm.....etc

11-What animals do you keep in? List them plus how many and how often they graze with horses.

Animal	How many	Often	Occasionally	Rare	Never
Cow					
Sheep					
Goat					
Donkey					
Poultry					
Pig					

12-Have any of the animals above received any vet drugs in the last 6 months? yes☐ no☐
If so list the given drugs please:-

Drugs	Species	Date/given	Reason/diagnosed	How long

13-Is horse feed stored in the same place as other animal feed? yes ☐ no ☐

14-Do all your animals drink from

Separate sources ☐

Shared sources ☐

(State source of water) town ☐ stream ☐ pond ☐ tank ☐ others ☐

specify ____________

15-What wild animal do you see commonly in your premises?

- Foxes ☐
- Badgers ☐
- Rabbit ☐

Seagulls ☐

others ☐

specify ____________

16-Do you undertake rodent control? ☐

Please state what do you use ____________

____

____

Owner name: - [ ] Tel/no:- [ ] form no: - [ ]

Antibiotic resistance questionnaire
(Hospital /medical form)

1-Horse's name ______________

2-Sex ☐ stallion ☐ mare ☐ gelding

3- Age ☐☐

4-Breed ______________

5-Case number ☐

6-Date of admission ☐☐ / ☐☐ / 0 4

And date of onset of condition ☐☐ ☐☐ / 0 4

7-Has the horse been seen by a vet in the last 6 months? ☐ Yes ☐ no

If yes what for?

1-
2-
3-

And what treatment has it been given including antibiotics (specify if possible the dose and the route) and for how long (excluding wormers and vaccination)?

8-Reason for current presentation: -
1- ______________
2- ______________
3- ______________

9-What was the diagnosis
1- ______________
2- ______________
3- ______________

10-Was the horses being treated yes☐ no☐

If yes when did the horse start to be treated? ☐☐ / ☐☐ / 0 3

11-When was the first antibiotic dose given? [|] [|] [0|3]

12-Could you list the drugs including antibiotics, which have been given in the following table:

Drug	Dose	Route	Date from	Date to
________			__/__	
________			__/__	
________			__/__	
________			__/__	
________			__/__	

13-What was the out come of the case

☐ A- discharge

☐ B-euthanised

A or B give date [|]/[|]/[0|3]

Appendix 2A

Horse no	Case no	Sample collection number	Addmission/date	Sampling/date	Horse origin	Age	Sex	Breed	Reason for admission	
1	24.769	1	11/10/2001	22/10/2001	Staffs	16	satllion		Right side epistaxis	
		45		24/10/2001		16			Right side epistaxis	
2	24.801	2	20/10/2001	22/10/2001	Lancs	8	Gielding		wounds	
		28		23/10/2001		8			wounds	
		43		24/10/2001		8			wounds	
3	24.799	4	19/10/2001	22/10/2001	Cheshire	5	Gielding	Pony	GI symp	
		16		23/10/2001		5		Pony	GI symp	
		41		24/10/2001		5		Pony	GI symp	
4	24.748	5	09/10/2001	22/10/2001	Lancs	8	stallion	TB	stringhalt-like behaviour	
		25		23/10/2001		8		TB	stringhalt-like behaviour	
5	24.773	7	14/10/2001	22/10/2001	Cheshire	8	stallion		sarcoids	
		22		23/10/2001		8			sarcoids	
6	24.783	8	16/10/2001	22/10/2001	State-on-trent	7	stallion		Foot abnormalities	
		19		23/10/2001		7			Foot abnormalities	
		47		24/10/2001		7			Foot abnormalities	
7	24.798	9	18/10/2001	22/10/2001	Cheshire	7	stallion		Colic	
		34		24/10/2001		7			Colic	
8	24.706	10	01/10/2001	22/10/2001	East Yorkshire	10	Gielding		sarcoids	
		44		24/10/2001		10			sarcoids	
9	24.62	11	11/09/2001	22/10/2001	Wolverhampton	7	Gielding		jugular thrombophlebitis □	
		46		24/10/2001		7			jugular thrombophlebitis □	
10	24.804	12	21/10/2001	22/10/2001	Sheffield	1.6	Gielding	WELSH	Colic	
		32		24/10/2001		1.6		WELSH	Colic	
11	24.78	13	15/10/2001	22/10/2001	Staffs	12	Gielding	TB	facial abnormality	
		42		24/10/2001		12		TB	facial abnormality	
12	24.681	14	26/09/2001	22/10/2001	Stafford	13	stallion		nasal discharge	
		17		23/10/2001		13			nasal discharge	
		49		24/10/2001		13			nasal discharge	
		65		19/11/2001		13			nasal discharge	
		89		27/11/2001		13			nasal discharge	
13	24.774	26	14/10/2001	22/10/2001	Derby	12.5	Gielding	TBX	Blood in urine	
		40		24/10/2001		12.5		TBX	Blood in urine	
14	24.812	18	23/10/2001	23/10/2001	Cheshire	10	Gielding	x bred	Back pain	
		37		24/10/2001		10		x bred	Back pain	
15	24.811	20	22/10/2001	23/10/2001		10	stallion	COB	sarcoids	
		51		24/10/2001		10		COB	sarcoids	
16	24.806	24	22/10/2001	23/10/2001	Lancs	8.5	Gielding	COB	lamness	
		31		24/10/2001		8.5		COB	lamness	
17	24.808	29	22/10/2001	23/10/2001	Staffs	14	Gielding		LH-lamness	
		35		24/10/2001		14			LH-lamness	
18	24.872	52	*02/11/2001*	*19/11/2001*	*Nr. Wolver hampton*	*6*	*Gielding*		*mild lamness(cast)*	→

Appendix 2A

Horse no	Drugs given 24-48 hours preior to sampling date	Antibiotic drugs therapy during hospitalization prior sampling	Dose1	ADD1	Dose2	ADD 2	Dose3	
1	Flunixin	Crystapen	79 mg	iv	0	0	0	
		Crystapen	0	0	0	0	0	
2	Crystapen+Gentamycin	Crystapen+Gentamycin	6 gm	iv	6.8 mg	iv	0	
	Crystapen+Gentamycin	Crystapen+Gentamycin	6 gm	iv	6.8 mg	iv	0	
	Crystapen+Gentamycin	Crystapen+Gentamycin	6 gm	iv	6.8 mg	iv	0	
3	Crystapen+FLUNIXIN	Crystapen+Neo penicillin	6 gm	iv	20 mg	iv	0	
	Crystapen	Crystapen+Neo penicillin	6 gm	iv	0	0	0	
	Crystapen	Crystapen+Neo penicillin	6 gm	iv	0	0	0	
4			0	0	0	0	0	
			0	0	0	0	0	
5	PBZ		1gm	PO	0	0	0	
	PBZ		1gm	PO	0	0	0	
6	PBZ		1gm	PO	0	0	0	
	PBZ		1gm	PO	0	0	0	
	PBZ		1gm	PO	0	0	0	
7	Flunixin+Crystapen	Crystapen	2.7mg	IV	6gm	IV	0	
	CRYSTAPEN	Crystapen	6gm	IV	0	0	0	
8	PBZ		1gm	PO	0	0	0	
	PBZ		1gm	PO	0	0	0	
9	PBZ		1gm	PO	0	0	0	
	PBZ		1gm	PO	0	0	0	
10	Crystapen+Gentamycin+PBZ	Crystapen+Gentamycin	3gm	IV	1980mg	IV	660mg	
	Crystapen+Gentamycin+PBZ	Crystapen+Gentamycin	3gm	IV	1980mg	IV	660mg	
11	PBZ		16gm	PO	0	0	0	
	PBZ		16gm	PO	0	0	0	
12			0	0	0	0	0	
			0	0	0	0	0	
			0	0	0	0	0	
	Baytril	Baytril	25mg	PO	0	0	0	
	Baytril	Baytril	25mg	PO	0	0	0	
13	PBZ		1gm	PO	0	0	0	
	Penicillin, Gentamycin, PBZ, Metronix	Penicillin, Gentamycin, Metronix	4440mg	PO	2930mg	iv	1gm	
14			0	0	0	0	0	
			0	0	0	0	0	
15			0	0	0	0	0	
			0	0	0	0	0	
16			0	0	0	0	0	
			0	0	0	0	0	
17			0	0	0	0	0	
	PBZ, Crystapen	Crystapen	2948mg	iv	9000mg	iv	0	
18		TMS+Gnetamycin+Crystapen	0	0	0	0	0	

Appendix 2A

Horse no	ADD 3	Dose 4	ADD 4	*E. coli*	*Campylobacter*	Resistance *E. coli*	Samples yielded muliple isolates with different resistance proiles	
1	0	0	0	Y		R		
	0	0	0	Y				
2	0	0	0	Y		R		
	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
3	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
	0	0	0	Y		R		
4	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
5	0	0	0	Y		R		
	0	0	0	Y				
6	0	0	0	Y		R		
	0	0	0	Y				
	0	0	0	Y		R		
7	0	0	0	Y		R	Y	
	0	0	0	Y		R		
8	0	0	0	Y		R	Y.	
	0	0	0	Y		R		
9	0	0	0	Y		R		
	0	0	0	Y		R		
10	IV	0	0	Y		R		
	IV	0	0	Y		R	Y	
11	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
12	0	0	0	Y		R		
	0	0	0	Y				
	0	0	0	Y		R		
	0	0	0	Y		R		
	0	0	0	Y		R	Y	
13	0	0	0	Y		R		
	PO	0	iv	Y		R		
14	0	0	0	Y				
	0	0	0	Y		R		
15	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
16	0	0	0	Y				
	0	0	0	Y		R	Y	
17	0	0	0	Y		R		
	0	0	0	Y		R	Y	
18	0	0	0	Y		R	Y	→

Appendix 2A

Horse no	Samples yielded muliple isolates with similar proiles	MDR	Amp	B-lactam drugs	Apra	ChL	Flo	Tet-	TrI	Nal	Cip	ami	Sul	R-phenotype	VREs	Resistance *E. coli* isolates (n=)
1		Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		1
			S	S	S	S	S	S	S	S	S	R	R			0
2			R	S	S	S	S	R	R	S	S	R	R			1
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp.chlo,tet,tri		3
		Y	R	S	S	R	S	R	R	S	S	R	R	amp.chlo,tet,tri	*van-c E.gallinarium*	2
3			R	S	S	S	S	R	R	S	S	R	R		*van-c E.gallinarium*	3
	Y	Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		3
	Y		R	S	S	S	S	R	R	S	S	R	R			3
4		Y	R	S	S	R	S	R	R	R	R	R	R	amp.chlo,tet,tri.nal		2
		Y	R	S	S	R	S	R	R	R	S	R	R	amp.chlo,tet,tri,nal		2
5			S	S	S	S	S	S	R	S	S	R	R			1
			S	S	S	S	S	S	S	S	S	R	R			0
6	Y		S	S	S	S	S	S	R	S	S	R	R			2
			S	S	S	S	S	S	S	S	S	R	R			0
	Y		S	S	S	S	S	S	R	S	S	R	S			2
7			R	S	S	S	S	S	R	S	S	R	R			2
	Y		S	S	S	S	S	R	R	S	S	R	R			2
8		Y	R	S	S	S	S	R	R	R	S	R	R	amp,tet,tri,nal		2
			R	S	S	S	S	R	R	S	S	R	R			1
9	Y		S	S	S	S	S	S	R	S	S	R	R			3
	Y		S	S	S	S	S	S	R	S	S	R	R			3
10	Y		R	S	S	S	S	R	R	S	S	R	R			3
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlor,tet,tri		3
11	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,cho,tet,tri,nal		3
	Y		R	S	S	S	S	R	R	S	S	R	R		*van-c E.gallinarium*	3
12		Y	R	S	S	S	S	R	R	R	S	R	R	amp,ete,tri,nal		1
			S	S	S	S	S	S	S	S	S	S	R			0
			S	S	S	S	S	S	R	S	S	R	R			1
	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amo,chlo,tet,tri,nal		3
	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amo,chlo,tet,tri,nal	*van-c E.gallinarium*	3
13			R	S	S	S	S	R	R	S	S	R	R			1
	Y	Y	R	R	S	R	R	R	R	R	R	R	R	amp,chlo,tet,tri,nal	*van-c E.gallinarium*	3
14			S	S	S	S	S	S	S	S	S	R	R			0
			S	S	S	S	S	S	R	S	S	R	R			1
15			S	S	S	R	S	R	R	S	S	S	S			2
		Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
16			S	S	S	S	S	S	S	S	S	R	R			0
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
17		Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
	Y	Y	R	R	S	R	R	R	R	S	S	R	R	amp,chlo,tet,tri		3
18	Y	Y	R	S	S	R	S	R	R	S	S	S	R	amp,chlo,tet,tri		3

Appendix 2A

Horse no	Case no	Sample collection number	Addmission date	Sampling date	Horse origin	Age	Sex	Breed	Reason for admission	
		79		20/11/2001		6			mild lamness(cast)	
		111		14/01/2002		6			mild lamness(cast)	
		86		27/11/2001		6			mild lamness(cast)	
19	24.922	53	14/11/2001	19/11/2001	lancs	8	stallion		nasal discharge	
		87		27/11/2001		8			nasal discharge	
		92		28/11/2001		8			nasal discharge	
20	24.907	54	09/11/2001	19/11/2001	Cheshire	9	stallion		Depr-pyrexia	
		77		20/11/2001		9			Depr-pyrexia	
		88		27/11/2001		9			Depr-pyrexia	
		100		28/11/2001		9			Depr-pyrexia	
21	24.919	56	13/11/2001	19/11/2001	Harrogate	8	stallion	TB	Resection of dorsal spinous processes □	
		74		20/11/2001		8		TB	Resection of dorsal spinous processes □	
		85		27/11/2001		8		TB	Resection of dorsal spinous processes □	
		94		28/11/2001		8		TB	Resection of dorsal spinous processes □	
22	24.933	59	15/11/2001	19/11/2001	Lancs	5	Gielding	TB	sarcoids	
		76		20/11/2001		5		TB	sarcoids	
23	24.92	63	13/11/2001	19/11/2001	Cheshire	10	stallion	TB	LH-lamness	
		73		20/11/2001		10		TB	LH-lamness	
24	24.95	82	19/11/2001	27/11/2001	Oldham	7	stallion	IDx	wounds x radial fracture	
		99		28/11/2001		7		IDx	wounds x radial fracture	
25	25.159	103	11/01/2002	14/01/2002	West midland	3	Gielding	COB	Colic Like Syndrom	
		115		15/01/2002		3		COB	Colic Like Syndrom	
26	25.114	109	02/01/2002	14/01/2002	West midland	7	Gielding		sarcoids	
		113		15/01/2002		7			sarcoids	
27	25.204	131	30/12/2001	23/01/2002		15	stallion		colic	
		141		24/01/2002		15			colic	
28	25.201	133	19/01/2002	23/01/2002	Swopshire	15	Gielding	WELSH	abdominal pain	
		142		24/01/2002		15		WELSH	abdominal pain	
29	25.206	134	20/01/2002	23/01/2002	Preston	7	stallion		Colic	
		137		24/01/2002		7			Colic	
30	25.208	135	21/01/2002	23/01/2002	Swopshire	7	Gielding		atria fibrillation	
		139		24/01/2002		7			atria fibrillation	
31	25.199	136	19/01/2002	23/01/2002	Swopshire	3	Gielding	DUTCH	present for after surgery	
		140		24/01/2002		3		DUTCH	present for after surgery	
32	24.874	67	04/11/2001	20/11/2001	Staffs	14	Gielding	TBX	lamness(cast)	
		62		19/11/2001		14		TBX	lamness(cast)	
33	24.771	3	13/10/2001	22/10/2001	Wirral	11	Gielding	TB	mild colic	
34	25.158	120	11/01/2002	15/01/2002	Lanes	3	stallion	COB	Colic emergency □	
35	24.884	15	05/11/2001	23/10/2001	Shropshire □□	8	Gielding	TB	LH-lamness	
36	24.826	33	23/10/2002	24/10/2001	*Liverpool*	13	stallion	COB	*gas shadow in the Dorsal hoof wall*	
37	*24.809*	*38*	*22/10/2001*	*23/10/2001*	*Cheshire*	*7*	*stal[illegible]*	*PONY*	*back problem*	→

Appendix 2A

Horse no	Drugs given 24-48 hours preior to sampling date	Antibiotic drugs therapy during hospitalization prior sampling	Dose1	ADD1	Dose2	ADD 2	Dose3
		TMS+Gnetamycin+Crystapen	0	0	0	0	0
		TMS+Gnetamycin+Crystapen	0	0	0	0	0
		TMS+Gnetamycin+Crystapen	0	0	0	0	0
19	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
20	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
	PBZ, TMS	TMS	1gm	PO	0	PO	0
	PBZ, TMS	TMS	1gm	PO	0	PO	0
21	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
	PBZ		1gm	PO	0	0	0
22			0	0	0	0	0
			0	0	0	0	0
23			0	0	0	0	0
			0	0	0	0	0
24	Ceftifur	Ceftifur	1100mg	im	0	0	0
	Ceftifur	Ceftifur	1100mg	im	0	0	0
25			0	0	0	0	0
			0	0	0	0	0
26			0	0	0	0	0
			0	0	0	0	0
27	crystappen, pbz, flunixin	Crystapen+Flunixin	0	0	0	0	0
	crystappen, pbz, flunixin	Crystapen+Flunixin	0	0	0	0	0
28	flunixin, borgal	borgal	2.5mg	iv	2.5mg	iv	0
	borgal 2.m ml iv	fborgal	2mg	iv	0	iv	0
29	Flunixin+ CRYSTAPEN	Crystapen	110mg	iv	6gm	iv	0
	Flunixin+ CRYSTAPEN	Crystapen	110mg	iv	6gm	iv	0
30			0	0	0	0	0
			0	0	0	0	0
31	Flunixin+ CRYSTAPEN	Crystapen	0	iv	6gm	iv	0
	Flunixin+ CRYSTAPEN	Crystapen	0	iv	6gm	iv	0
32	TMS	TMS	15gm	PO	0	0	0
	TMS	TMS	15gm	PO	0	0	0
33		Crystapen	0	0	0	0	0
34			0	0	0	0	0
35			0	0	0	0	0
36	*Ceftifur, PBZ*	*Ceftifur*	1gm	iv	2gm	PO	0
37			*0*	*0*	*0*	*0*	*0*

Appendix 2A

Horse no	ADD 3	Dose 4	ADD 4	*E. coli*	*Campylobacter*	Resistance *E. coli*	Samples yielded muliple isolates with different resistance proiles	
	0	0	0	Y	*C. jejuni*	R		
	0	0	0	Y		R	Y	
	0	0	0	Y		R		
19	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
	0	0	0	Y	*C. jejuni*	R	Y	
20	0	0	0	Y		R	Y	
	0	0	0	Y		R		
	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
21	0	0	0	Y		R		
	0	0	0	Y		R		
	0	0	0	Y		R		
	0	0	0	Y	*C. jejuni*	R		
22	0	0	0	Y		R	Y	
	0	0	0	Y				
23	0	0	0	Y		R		
	0	0	0	Y		R		
24	0	0	0	Y		R	Y	
	0	0	0	Y		R		
25	0	0	0	Y		R		
	0	0	0	Y		R		
26	0	0	0	Y		R		
	0	0	0	Y		R	Y	
27	0	0	0	Y				
	0	0	0	Y		R		
28	0	0	0	Y		R	Y	
	0	0	0	Y				
29	0	0	0	Y		R		
	0	0	0	Y		R		
30	0	0	0	Y				
	0	0	0	Y				
31	0	0	0	Y				
	0	0	0	Y				
32	0	0	0	Y		R	Y	
	0	0	0	Y		R	Y	
33	0	0	0	Y		R	Y	
34	0	0	0	Y				
35	0	0	0	Y		R	Y	
36	0	0	0	Y				
37	0	0	0	Y		R		→

Appendix 2A

Horse no	Samples yielded muliple isolates with similar proiles	MDR	Amp	B-lactam drugs	Apra	ChL	Flo	Tet-	TrI	Nal	Cip	amy	Sul	R-phenotype	VREs	Resistance *E. coli* isolates (n=)
	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		2
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
			R	S	S	S	S	R	R	S	S	R	R		*van-c E.gallinarium*	3
19	Y	Y	R	R	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
	Y	Y	R	S	S	R	S	R	R	R	S	R	R	amp,chlo,tet,tri,nal		3
			R	S	S	S	S	S	R	S	S	R	R			3
20		Y	R	R	S	R	R	R	R	R	R	S	R	amp,chlo,tet,tri,nal		3
	Y		S	S	S	S	S	S	R	S	S	R	R			3
		Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
21			R	S	S	S	S	R	R	S	S	R	R			1
			R	S	S	S	S	R	R	S	S	R	R			1
	Y		R	S	S	S	S	R	R	S	S	S	R			3
	Y		R	S	S	S	S	R	R	S	S	R	R			2
22	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
			S	S	S	S	S	S	S	S	S	R	R			0
23		Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		1
			S	S	S	S	S	R	R	S	S	S	R			1
24		Y	R	R	S	R	S	R	R	R	S	R	R	amp,chlo,tet,tri,nal		3
	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
25	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
26	Y		R	S	S	S	S	R	R	S	S	R	R			3
	Y	Y	R	R	S	R	R	R	R	S	S	S	R	amp,chlo,tet,tri		3
27			S	S	S	S	S	S	S	S	S	R	R			0
		Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
28	Y	Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		3
			S	S	S	S	S	S	S	S	S	R	R			0
29	Y		R	S	S	S	S	R	S	S	S	R	R			3
	Y		R	S	S	S	S	R	S	S	S	R	R			2
30			S	S	S	S	S	S	S	S	S	R	R			0
			S	S	S	S	S	S	S	S	S	R	R			0
31			S	S	S	S	S	S	S	S	S	R	R			0
			S	S	S	S	S	S	S	S	S	R	R			0
32	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
	Y		R	S	S	S	S	R	R	S	S	R	R			3
33		Y	R	S	S	R	R	R	R	S	S	R	R	amp,chlo,tet,tri		3
34			S	S	S	S	S	S	S	S	S	R	R			0
35	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
36			S	S	S	S	S	S	S	S	S	R	R			0
37			R	S	S	S	S	R	R	S	S	[illegible]	R			

Appendix 2A

Horse no	Case no	Sample collection number	Addmission date	Sampling date	Horse origin	Age	Sex	Breed	Reason for admission	
38	24.807	39	22/10/2001	24/10/2001	Manchester	6	Gielding	WELSH	eye problem □	
39	24.827	48	23/10/2001	24/10/2001	Shropshire □□	17	Gielding		suspected Colic	
40	24.939	55	16/11/2001	19/11/2001	Birmengham	5	stallion	PONY	Colic Treatment□	
41	24.935	58	15/11/2001	19/11/2001	Cumbria	9	Gielding	TBX	abdominal discomfort □	
42	25.175	119	15/01/2002	15/01/2002	Wolverhampton	7	Gielding	COB	Colic emergency □	
43	24.852	60	29/10/2001	19/11/2001	Isle of man	6	Gielding		head shaking Behaviour □	
44	24.925	61	14/11/2001	19/11/2001	West kirby	1	Gielding	NDALUCE/	swelling R-s of prepuce □	
45	24.911	64	12/11/2001	19/11/2001	Gwynedd	7	mare	WELSHX	back problem	
46	24.951	66	19/01/2001	20/11/2001	Derby shire	4	stallion	COB	penetration of the food by nails	
47	24.942	69	18/11/2001	20/11/2001	Burton wood	10	stallion	COB	Colic	
48	24.949	70	19/11/2001	20/11/2001	Lancs	9	stallion		lamness	
49	24.944	75	18/11/2001	20/11/2001	Lancs	7	Gielding		Colic surgery	
50	24.956	80	21/11/2001	27/11/2001	Bridge north	9	Gielding		Colic	
51	24.89	81	22/11/2002	27/11/2001	Sheffield	22	stallion	PONY	Colic	
52	24.89	95	22/11/2002	28/11/2001	Sheffield	22	stallion	PONY	Colic	
53	24.975	83	27/11/2001	27/11/2001	Durham	12	Gielding	TB	lamness	
54	25.173	118	14/01/2002	15/01/2002	Shropshire	14	stallion	TB	check up	
55	24.968	84	25/11/2001	27/11/2001	Cheshire	15	Gielding	TB	Colic Symptoms	
56	24.938	90	16/11/2001	28/11/2001	Lancs	17	stallion	PONY	lamness Gamma scan	
57	24.918	91	13/11/2001	28/11/2001	West Midland	19	Gielding		post surgery problems	
58	25.169	117	14/01/2002	15/01/2002	Yorkshire	7	stallion		sarcoid OF of L-eye	
59	25.142	106	09/01/2002	14/01/2002	Lancs	5	Gielding		Gamma scan lamness	
60	25.132	107	07/01/2002	14/01/2002	New castle □upon Tyre □	14	Gielding	TB	check up	
61	25.138	108	09/01/2002	14/01/2002	Oldham	11	Gielding	Arab	Colic	
62	25.126	110	07/01/2002	14/01/2002	Shropshire □□	8	Gielding	TB	check up	
63	25.122	112	05/01/2002	15/01/2002	Cheshire	10	stallion	TB	Infection of the LH	
64	25.154	114	10/01/2002	15/01/2002	Manchester	18	stallion	TBX	Recovery after Colic surgery	→

Appendix 2A

Horse no	Drugs given 24-48 hours preior to sampling date	Antibiotic drugs therapy during hospitalization prior sampling	Dose1	ADD1	Dose2	ADD 2	Dose3	
38	Flunixin		0	IV	0	0	0	
39			0	0	0	0	0	
40			0	0	0	0	0	
41			0	0	0	0	0	
42			0	0	0	0	0	
43			0	0	0	0	0	
44			0	0	0	0	0	
45	PBZ		1gm	PO	0	0	0	
46	Crystapen+Gentamycin		4810mg	IV	5772mg	IV	0	
47	Baytril, Flunixin	Baytril	2.5gr	PO	250mg	IV	0	
48			0	0	0	0	0	
49	Neopenicillin, Flunixin □□	Neopenicillin	4.8gm	IM	240mg	IV	0	
50	Neopenicillin, Flunixin □□	Neopenicillin	4.8gm	IM	240mg	IV	0	
51	TMS	TMS	7.9gm	PO	0	0	0	
52	TMS	TMS	7.9gm	PO	0	0	0	
53			0	0	0	0	0	
54			0	0	0	0	0	
55			0	0	0	0	0	
56			0	0	0	0	0	
57	PBZ	Crystapen+penicillin	0.5gm	IV	0	0	0	
58			0	0	0	0	0	
59			0	0	0	0	0	
60			0	0	0	0	0	
61	flunixin, neopenicillin, PBZ	Neopenicillin	900mg	iv	0	im	1gm	
62			0	0	0	0	0	
63	PBZ,Crystapen+Gentamycin	Crystapen+Gentamycin	1gm	IV	5000gm	IV	3168mg	
64	Ceftifur, flunixin	Ceftifur	1.5gm	iv	300mg	iv	0	→

Appendix 2A

Horse no	ADD 3	Dose 4	ADD 4	*E. coli*	*Campylobacter*	Resistance *E. coli*	Samples yielded muliple isolates with different resistance proiles	
38	0	0	0	Y				
39	0	0	0	Y		R	Y	
40	0	0	0	Y		R		
41	0	0	0	Y		R	Y	
42	0	0	0	Y		R		
43	0	0	0	Y				
44	0	0	0	Y		R		
45	0	0	0	Y		R		
46	0	0	0	Y				
47	0	0	0	Y		R	Y	
48	0	0	0	Y		R		
49	0	0	0	Y				
50	0	0	0	Y		R	Y	
51	0	0	0	Y		R	Y	
52	0	0	0	Y		R		
53	0	0	0	Y		R	Y	
54	0	0	0	Y		R		
55	0	0	0	Y		R	Y	
56	0	0	0	Y		R		
57	0	0	0	Y		R		
58	0	0	0	Y		R		
59	0	0	0	Y				
60	0	0	0	Y		R		
61	PO	0	0	Y		R	Y	
62	0	0	0	Y		R	Y	
63	IV	0	0	Y		R	Y	
64	0	0	0	Y		R		→

Appendix 2A

Horse no	Samples yielded muliple isolates with similar proiles	MDR	Amp	B-lactam drugs	Apra	ChL	Flo	Tet-	TrI	Nal	Cip	amy	Sul	R-phenotype	VREs	Resistance *E. coli* isolates (n=)
38			S	S	S	S	S	S	S	S	S	R	R			0
39			R	S	S	S	S	R	R	S	S	R	R			2
40	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
41	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		3
42	Y	Y	R	S	S	R	S	R	R	R	S	S	R	amp,chlo,tet,tri,nal		2
43			S	S	S	S	S	S	S	S	S	R	R			0
44	Y		R	S	S	S	S	R	R	S	S	R	R			3
45	Y	Y	R	S	S	R	S	R	R	S	S	R	R	amp,chlo,tet,tri		2
46			S	S	S	S	S	S	S	S	S	R	R			0
47	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
48			R	S	S	S	S	S	S	S	S	R	R			1
49			S	S	S	S	S	S	S	S	S	R	R			0
50	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
51	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
52	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
53	Y	Y	R	R	S	R	R	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
54	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
55	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
56	Y	Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		2
57	Y	Y	R	S	S	R	S	R	R	R	R	R	R	amp,chlo,tet,tri,nal		3
58	Y	Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		2
59			S	S	S	S	S	S	S	S	S	R	R			0
60			R	S	S	S	S	R	R	S	S	R	R			1
61		Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		3
62	Y	Y	R	S	S	S	S	R	R	R	R	R	R	amp,tet,tri,nal		3
63			R	S	S	S	S	R	R	S	S	R	R			2
64	Y	Y	R	R	S	R	R	R	R	S	S	S	S	amp,chlo,tet,tri		3

Appendix 2B

Horse/no	Feacal sample collection	Horse name	Source	Date	Age	Sex	Breed	on premises (years)	*E.coli*	R- *E. coli*	
1	143	molly	premise 1	11/02/2002	16	2	thoroughbred	9	Y	R	
	173	molly	premise 1	18/03/2002	16	2	thoroughbred	9	Y		
	232	molly	premise 1	19/04/2002	16	2	thoroughbred	9	Y		
	224	molly	premise 1	11/04/2002	16	2	thoroughbred	9	Y		
2	144	eamon	premise 1	11/02/2002	14	3	thoroughbred	7	Y		
	174	eamon	premise 1	18/03/2002	14	3	thoroughbred	7	Y		
	213	eamon	premise 1	11/04/2002	14	3	thoroughbred	7	Y		
	233	eamon	premise 1	19/04/2002	14	3	thoroughbred	7	Y		
3	145	maxwell	premise 1	11/02/2002	9	3	rish sport hors	7	Y		
	175	maxwell	premise 1	18/03/2002	9	3	rish sport hors	7	Y		
	234	maxwell	premise 1	19/04/2002	9	3	rish sport hors	7	Y		
4	146	ruby	premise 1	11/02/2002	8	3	unknown	5	Y		
	176	ruby	premise 1	18/03/2002	8	3	unknown	5	Y		
	210	ruby	premise 1	11/04/2002	8	3	unknown	5	Y		
	235	ruby	premise 1	19/04/2002	8	3	unknown	5	Y		
5	147	edward	premise 1	11/02/2002	14	3	thoroughbred	12	Y		
	177	edward	premise 1	18/03/2002	14	3	thoroughbred	12	Y		
	230	edward	premise 1	11/04/2002	14	3	thoroughbred	12	Y		
	236	edward	premise 1	19/04/2002	14	3	thoroughbred	12	Y		
6	150	hopie	premise 1	11/02/2002	11	2	unknown	4	Y		
	180	hopie	premise 1	18/03/2002	11	2	unknown	4	Y		
	212	hopie	premise 1	11/04/2002	11	2	unknown	4	Y		
	237	hopie	premise 1	19/04/2002	11	2	unknown	4	Y		
7	152	pickles	premise 1	11/02/2002	25	3	cob	15	Y		
	182	pickles	premise 1	18/03/2002	25	3	cob	15	Y		
	209	pickles	premise 1	11/04/2002	25	3	cob	15	Y		
	238	pickles	premise 1	19/04/2002	25	3	cob	15	Y		
8	153	magic	premise 1	11/02/2002	11	3	new forest	8	Y		
	183	magic	premise 1	18/03/2002	11	3	new forest	8	Y		
	222	magic	premise 1	11/04/2002	11	3	new forest	8	Y		
	239	magic	premise 1	19/04/2002	11	3	new forest	8	Y		
9	155	theo	premise 1	11/02/2002	18	3	irish cob	10	Y	R	
	185	theo	premise 1	18/03/2002	18	3	irish cob	10	Y	R	
	218	theo	premise 1	11/04/2002	18	3	irish cob	10	Y		
	240	theo	premise 1	19/04/2002	18	3	irish cob	10	Y		→

Appendix 2B

Horse/no	SAMPLES YIELDED MULTIPLE DIFFERENT RESISTANCE	SAMPLES YIELDED MULTIPLE SIMILAR RESISTANCE	MDR4	Amp	Apra	Chl	Flo	
1			s	R	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
2			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
3			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
4			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
5			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
6			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
7			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
8			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
9			s	s	s	s	s	
		Y	s	s	s	s	s	
			s	s	s	s	s	→
			s	s	s	s	s	

Appendix 2B

Horse/no	Tet	Tri	Nal	Cip	B-lactam drugs	Amy	Sul	VREs	no of resistance E-coli isolates
1	R	R	s	s	s	s	R		1
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
2	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
3	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
4	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
5	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
6	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
7	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
8	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
9	s	R	s	s	s	s	R		1
	s	R	s	s	s	R	R		2
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0

Appendix 2B

Horse/no	Feacal sample collection	Horse name	Source	Date	Age	Sex	Breed	on premises (years)	*E.coli*	R- *E. coli*	
10	157	blue	premise 1	11/02/2002	18	3	cob	10	Y		
	206	blue	premise 1	19/03/2002	18	3	cob	10	Y		
	241	blue	premise 1	19/04/2002	18	3	cob	10	Y		
11	158	georgy	premise 1	11/02/2002	15	3	welsh cob	9	Y		
	207	georgy	premise 1	19/03/2002	15	3	welsh cob	9	Y		
	214	georgy	premise 1	11/02/2002	15	3	welsh cob	9	Y	R	
	242	georgy	premise 1	19/04/2002	15	3	welsh cob	9	Y		
12	159	pepper	premise 1	11/02/2002	13	3	unknown	7	Y		
	187	pepper	premise 1	18/03/2002	13	3	unknown	7	Y		
	243	pepper	premise 1	19/04/2002	13	3	unknown	7	Y		
13	160	gerry	premise 1	11/02/2002	9	3	unknown	4	Y		
	188	gerry	premise 1	18/03/2002	9	3	unknown	4	Y		
	221	gerry	premise 1	11/04/2002	9	3	unknown	4	Y	R	
14	161	nicky	premise 1	11/02/2002	18	3	unknown	3	Y		
	189	nicky	premise 1	18/03/2002	18	3	unknown	3	Y		
	219	nicky	premise 1	11/04/2002	18	3	unknown	3	Y		
	245	nicky	premise 1	19/04/2002	18	3	unknown	3	Y		
15	162	marcos	premise 1	11/02/2002	23	3	cob	17	Y		
	190	marcos	premise 1	18/03/2002	23	3	cob	17	Y		
	227	marcos	premise 1	11/04/2002	23	3	cob	17	Y		
	246	marcos	premise 1	19/04/2002	23	3	cob	17	Y		
16	164	norman	premise 1	11/02/2002	21	3	cob	11	Y		
	192	norman	premise 1	18/03/2002	21	3	cob	11	Y		
	220	norman	premise 1	11/04/2002	21	3	cob	11	Y		
	247	norman	premise 1	19/04/2002	21	3	cob	11	Y		
17	165	manuel	premise 1	11/02/2002	7	3	welsh pony	7	Y		
	193	manuel	premise 1	18/03/2002	7	3	welsh pony	7	Y		
	229	manuel	premise 1	11/04/2002	7	3	welsh pony	7	Y		
	248	manuel	premise 1	19/04/2002	7	3	welsh pony	7	Y		
18	166	dazzler	premise 1	11/02/2002	10	2	unknown	9	Y		
	194	dazzler	premise 1	18/03/2002	10	2	unknown	9	Y		
	249	dazzler	premise 1	19/04/2002	10	2	unknown	9	Y		
19	167	dexter	premise 1	11/02/2002	11	3	unknown	6	Y		→
	194	dexter	premise 1	18/03/2002	11	3	unknown	6	Y		

Appendix 2B

Horse/no	SAMPLES YIELDED MULTIPLE DIFFERENT RESISTANCE	SAMPLES YIELDED MULTIPLE SIMILAR RESISTANCE	MDR4	Amp	Apra	Chl	Flo	
10			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
11			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
12			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
13			s	s	s	s	s	
			s	s	s	s	s	
	Y		s	s	s	s	s	
14			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
15			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
16			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
17			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
18			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
19			s	s	s	s	s	→
			s	s	s	s	s	

Appendix 2B

Horse/no	Tet	Tri	Nal	Cip	B-lactam drugs	Amy	Sul	VREs	no of resistance E-coli isolates
10	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
11	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	R	s	s	s	R	R		1
	s	s	s	s	s	R	R		0
12	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
13	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	R	s	s	s	s	R		2
14	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
15	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
16	s	s	s	s	s	R	R	*n-c E.gallinariu*	0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
17	s	s	s	s	s	R	s		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
18	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
19	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0

Appendix 2B

Horse/no	Feacal sample collection	Horse name	Source	Date	Age	Sex	Breed	on premises (years)	*E.coli*	R- *E. coli*	
	208	dexter	premise 1	11/04/2002	11	3	unknown	6	Y		
	250	dexter	premise 1	19/04/2002	11	3	unknown	6	Y		
20	168	apsche	premise 1	11/02/2002	11	3	irish cob	6	Y	R	
	196	apsche	premise 1	18/03/2002	11	3	irish cob	6	Y	R	
	251	apsche	premise 1	19/04/2002	11	3	irish cob	6	Y	R	
21	170	seamus	premise 1	11/02/2002	16	3	unknown	8	Y	R	
	198	seamus	premise 1	18/03/2002	16	3	unknown	8	Y		
	252	seamus	premise 1	19/04/2002	16	3	unknown	8	Y		
22	171	dell	premise 1	11/02/2002	9	2	unknown	3	Y		
	199	dell	premise 1	18/03/2002	9	2	unknown	3	Y		
	253	dell	premise 1	19/04/2002	9	2	unknown	3	Y		
23	172	geena	premise 1	11/02/2002	16	3	unknown	8	Y		
	215	geena	premise 1	11/04/2002	16	3	unknown	8	Y		
	254	geena	premise 1	19/04/2002	16	3	unknown	8	Y	R	
24	201	samson	premise 1	18/03/2002	14	3	cob pony	8	Y		
	255	samson	premise 1	19/04/2002	14	3	cob pony	8	Y		
25	202	bruno	premise 1	18/03/2002	12	3	cob	5	Y		
	223	bruno	premise 1	11/04/2002	12	3	cob	5	Y	R	
	256	bruno	premise 1	19/04/2002	12	3	cob	5	Y		
26	204	summer	premise 1	18/03/2002	4	2	unknown	2	Y		
	231	summer	premise 1	11/04/2002	4	2	unknown	2	Y		
	257	summer	premise 1	19/04/2002	4	2	unknown	2	Y		
27	163	SWEEP	premise 1	11/02/2002	9	2	unknown	7	Y		
	191	SWEEP	premise 1	18/03/2002	9	2	unknown	7	Y		
	225	SWEEP	premise 1	18/03/2002	9	2	unknown	7	Y		
28	148	FLISSY	premise 1	11/02/2002	13	3	unknown	11	Y		
	228	FLISSY	premise 1	18/03/2002	13	3	unknown	11	Y		
	178	FLISSY	premise 1	18/03/2002	13	3	unknown	11	Y		
29	179	FRECKLES	premise 1	18/03/2002	20	2	pony welsh	9	Y	R	
	149	FRECKLES	premise 1	11/02/2002	20	2	pony welsh	9	Y	R	
30	181	RYAN	premise 1	18/03/2002	26	3	irish cob	11	Y		
	151	RYAN	premise 1	11/02/2002	26	3	irish cob	11	Y		
31	154	HANNAH	premise 1	11/02/2002	21	2	pony cob	13	Y	R	→
	184	HANNAH	premise 1	18/03/2002	21	2	pony cob	13	Y		

Appendix 2B

Horse/no	SAMPLES YIELDED MULTIPLE DIFFERENT RESISTANCE	SAMPLES YIELDED MULTIPLE SIMILAR RESISTANCE	MDR4	Amp	Apra	Chl	Flo	
			s	s	s	s	s	
			s	s	s	s	s	
20			s	s	s	s	s	
			s	s	s	s	s	
		Y	s	s	s	s	s	
21			s	R	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
22			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
23			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
24			s	s	s	s	s	
			s	s	s	s	s	
25			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
26			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
27			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
28			s	s	s	s	s	
			s	s	s	s	s	
			s	s	s	s	s	
29			s	R	s	s	s	
		Y	s	R	s	s	s	
30			s	s	s	s	s	
			s	s	s	s	s	
31			s	R	s	s	s	→
			s	s	s	s	s	

Appendix 2B

Horse/no	Tet	Tri	Nal	Cip	B-lactam drugs	Amy	Sul	VREs	no of resistance E-coli isolates
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
20	R	R	s	s	s	R	R		1
	s	R	s	s	s	R	R		1
	R	R	s	s	s	s	s		2
21	s	s	s	s	R	R	R		1
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
22	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
23	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	R	s	s	s	R	R		1
24	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
25	s	s	s	s	s	R	R		0
	s	s	s	s	s	s	R		1
	s	s	s	s	s	R	R		0
26	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
27	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
28	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
29	R	s	s	s	s	R	R		1
	s	R	s	s	s	s	R		2
30	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
31	R	R	s	s	s	s	R		1
	s	s	s	s	s	R	R		0

Appendix 2B

Horse/no	Feacal sample collection	Horse name	Source	Date	Age	Sex	Breed	on premises (years)	*E.coli*	R- *E. coli*	
32	186	T.J.	premise 1	18/03/2002	17	3	welsh	9	Y		
	156	T.J.	premise 1	11/02/2002	17	3	welsh	9	Y	R	
33	197	tosca	premise 1	18/03/2002	18	2	cob	9	Y		
	211	tosca	premise 1	18/03/2002	18	2	cob	9	Y		
34	200	harvey	premise 1	18/03/2002	12	3	welsh sec B	7	Y		
	216	harvey	premise 1	18/03/2002	12	3	welsh sec B	7	Y		
35	226	rap	premise 1	18/03/2002	9	2	unknown	5	Y		
36	203	donkey	premise 1	18/03/2002	22	2	unknown	11	Y	R	
37	217	dal	premise 1	18/03/2002	17	2	unknown	12	Y	R	

Appendix 2B

Horse/no	SAMPLES YIELDED MULTIPLE DIFFERENT RESISTANCE	SAMPLES YIELDED MULTIPLE SIMILAR RESISTANCE	MDR4	Amp	Apra	Chl	Flo	
32			s	s	s	s	s	
			s	s	s	s	s	
33			s	s	s	s	s	
			s	s	s	s	s	
34			s	s	s	s	s	
			s	s	s	s	s	
35			s	s	s	s	s	
36		Y	s	s	s	s	s	
37	Y		s	s	s	s	s	

Appendix 2B

Horse/no	Tet	Tri	Nal	Cip	B-lactam drugs	Amy	Sul	VREs	no of resistance E-coli isolates
32	s	s	s	s	s	s	R		0
	s	R	s	s	s	s	R		1
33	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
34	s	s	s	s	s	R	R		0
	s	s	s	s	s	R	R		0
35	s	s	s	s	s	R	R		0
36	s	s	R	R	s	R	R		3
37	s	R	s	s	s	R	R		3

Appendix 2C

	Faecal sample collection/no	Horse name	date	Age	Sex	on premises (YEARS)	ABa last 6 months	*E. coli*	Resistance *E.coli*	
1	332	kevin	11/09/2002	6	3	0.7		Y		
	323	kevin	03/07/2002	6	3	0.7		Y	R	
2	304	penny	03/07/2002	12	3	2		Y	R	
	334	penny	11/09/2002	12	3	2		Y		
3	307	chance	03/07/2002	18	3	4		Y	R	
	336	chance	11/09/2002	18	3	4		Y	R	
4	313	aime	03/07/2002	9	3	3		Y	R	
	339	aime	11/09/2002	9	3	3		Y	R	
5	319	lucy	03/07/2002	9	2	5		Y	R	
	340	lucy	11/09/2002	9	2	5		Y	R	
6	314	max	03/07/2002	8	3	4		Y	R	
	342	max	11/09/2002	8	3	4		Y		
7	343	jack	11/09/2002	8	3	6		Y		
8	322	harry	03/07/2002	17	3	5	penicillin	Y	R	
	348	harry	11/09/2002	17	3	5	penicillin	Y		
9	349	pharamb	11/09/2002	13	3	8	penicillin	Y	R	
10	297	Tara	03/07/2002	15	2	5		Y		
11	298	Pabbs	03/07/2002	16	3	5		Y		
12	299	Clover	03/07/2002	15	3	5		Y		
13	300	Master robin	03/07/2002	17	3	5		Y		
14	301	Dylon	03/07/2002	17	2	5		Y		
15	302	Christy	03/07/2002	17	2	5		Y		
16	303	Soverign	03/07/2002	4	2	2		Y		
17	305	Pinkie	03/07/2002	8	3	3		Y		
18	306	Rusty	03/07/2002	15	3	7		Y		
19	308	Spike	03/07/2002	16	3	7		Y	R	
20	311	Murphy	03/07/2002	18	3	4		Y	R	
21	312	Ilwey	03/07/2002	35	3	5		Y	R	
22	317	Porry	03/07/2002	14	3	5		Y	R	
23	318	Banana	03/07/2002	14	3	3		Y	R	
24	320	Merlin	03/07/2002	12	2	6		Y		
25	321	Roxy	03/07/2002	18	2	6		Y	R	
26	324	Luke	03/07/2002	20	2	6		Y	R	
27	335	Clood	11/09/2002	15	2	5		Y		
28	337	Ella	11/09/2002	11	3	2		Y		
29	338	Robbie	11/09/2002	8	3	3		Y		
30	341	Erin	11/09/2002	12	3	5		Y		
31	344	Newy	11/09/2002	15	3	5		Y		
32	345	Marry	11/09/2002	4	3	2		Y		
33	346	Cracker	11/09/2002	8	3	3		Y	R	
34	347	Rosie	11/09/2002	9	2	3		Y	R	
35	333	oliver	11/09/2002	21	2	4		Y	R	→

Appendix 2C

Samples yielded multiple different resistance	Samples yielded multiple similar resistance	MDR4	Amp	Apr	Chl	Tet	Tri	Nal	cipro	B-lactam drugs	Flo	
			S	S	S	S	S	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
			S	S	S	S	R	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
	R	MDR	R	S	S	R	R	R	R	S	S	
Y			S	S	S	R	R	S	S	S	S	
Y			S	S	S	R	R	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
Y		MDR	R	R	S	R	R	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			R	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
	R		S	S	S	R	R	S	S	S	S	
	R	MDR	R	S	R	R	R	S	S	R	R	
	R		S	S	S	S	R	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
Y			S	S	S	R	R	S	S	S	S	
	R		S	S	S	S	R	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
			S	S	S	S	S	S	S	S	S	
	R		S	S	S	R	S	S	S	S	S	
Y			R	S	S	S	R	S	S	S	S	
			R	S	R	R	R	S	S	R	R	→

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	Resistant genes
1	1	4	PLEH	AMP,CHL,TET,TRI	S	S	®	S	®	S	>256	256	≥256	128		*dfr1,dfr12,catI,tet A ,B,tem*
2	3	5	PLEH	AMP,TRI	S	S	®	®	®	S	>256		≥256			*dfr1,tem*
3	3	6	PLEH	AMP,CHL,FLO,TET,TRI	S	®	®	®	®	S	>256	256	≥256	64		*dfr1,dfr12,tetA,tetB,tem*
4	3	7	PLEH	AMP,TRI	S	S	®	S	®	S	>256		≥256			*dfr1,tem*
5	5	1	PLEH	AMP,NAL,TET,TRI	®	S	®	®	®	®	256		≥256	0.031	4	*tetB,dfr1,tem*
6	5	14	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	256	256	≥256	32		*dfr12,tetB,catI,tem*
7	8	217	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1*
8	8	218	PLEH	AMP,TRI	S	S	®	S	®	S	2		≥256			*dfr1*
9	34	278	PLEH	TRI	S	S	S	S	®	S			≥256			*dfr1*
10	34	219	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1*
11	13	192	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr17,tetB,catI,tem*
12	13	193	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	256	>256	≥256	128	8	*dfr17,tetB,catI,tem*
13	13	194	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	32	8	*dfr17,tetB,catI,tem*
14	89	381	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	16	*dfr17,tetB,catI,tem*
15	89	199	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	128	32	*dfr17,tetB,tem*
16	89	200	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	128	16	*dfr17,tetB,tem*
17	79	382	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	256	>256	≥256	128		*dfr17,tetB,catI,tem*
18	79	383	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	32		*dfr17,tetB,catI,tem*
19	86	271	PLEH	AMP	S	S	®	S	®	S	128					
20	86	2	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	0.031		*dfr1,dfr12,dfr17,tetB,tem*
21	86	218	PLEH	AMP,TRI	S	S	®	S	®	S	2		≥256			*dfr1,tem*
22	92	342	PLEH	AMP,TRI	S	S	®	S	®	S	256		≥256			*dfr1,tem*
23	92	343	PLEH	AMP,TRI	S	S	®	S	®	S	256					*tem*
24	92	325	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	128		*dfr1,tetB,tem*
25	99	3	PLEH	AMP,CHL,TET,TRI	S	S	®	S	®	S	256	128	≥256	64		*dfr12,tetB,catI,tem*
26	99	267	PLEH	AMP,CHL,TET,TRI	S	S	®	S	®	®	128	256	≥256	64		*dfr1,tetB,catI*
27	99	268	PLEH	AMP,CHL,TET,TRI	S	S	®	S	®	®	128	256	≥256	64		*dfr1,tetB,catI*
28	59	132	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	®	256	256	≥256	128	8	*dfr17,tetB,catI,tem*
29	59	23	PLEH	CHL,TET,TRI	S	S	®	S	S	S		64		64		*tetB*
30	59	207	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256		64	16	*dfr17,tetA,tetB,catI,tem*
31	25	367	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	®	®	S	256	256	≥256	64		*dfr1,tetA,catI,tem*
32	25	368	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	256	128	≥256	32		*dfr1,tetA,tem*
33	29	369	PLEH	AMP,CHL,NAL,TET,TR	S	S	®	®	®	S	256	256	≥256	0.062		*dfr1,tetA,catI,tem*
34	29	370	PLEH	AMP,TET,TR	S	S	®	®	®	S	128		≥256	4		*dfr1,tetB,tem*
35	29	371	PLEH	TET,TR	S	S	®	®	®	S			≥256	32		*dfr1,tetB*
36	12	372	PLEH	AMP,TET,TR	S	S	®	®	®	S	128		≥256	256		*dfr1,tetA,tem*
37	12	373	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	64		*dfr1,tetA,tem*
38	12	374	PLEH	AMP,TET,TR	S	S	®	®	®	S	64		≥256	8		*dfr1,tetB,tem*
39	26	375	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	128		*dfr1,tetA,tem*
40	100	376	PLEH	AMP.CHL,TET.TRI	S	S	®	®	®	S	256	256	≥256	32		*dfr1,tetB,catI,tem*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
41	100	377	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	127		*dfr1,tem*
42	100	378	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	64		*dfr1,tetB,tem*
43	94	379	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	64		*dfr1,tetB,tem*
44	94	380	PLEH	AMP,TET,TR	S	S	®	®	®	S	256		≥256	4		*dfr1,tetB,tem*
45	11	11	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1*
46	11	12	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1*
47	11	13	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1*
48	28	15	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	256	256	≥256	256		*dfr12,tetA,catI,tem*
49	28	16	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	>256	256	≥256	64		*dfr1,tetA,catI,tem*
50	28	17	PLEH	TET,TRI	S	S	®	S	®	S			≥256	8		*dfr1,tetB*
51	15	18	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	>256	≥256	128		*dfr12,tetA,catI,tem*
52	15	19	PLEH	TET,TRI	S	S	®	®	®	S			≥256	32		*dfr1,tetB*
53	15	20	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr17,catI,tem*
54	20	21	PLEH	TRI	S	S	®	S	S	S			≥256			*dfr1*
55	20	384	PLEH	CHL,TET,TRI	S	S	®	S	S	S		256				*catI*
56	16	27	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	128		≥256	128	32	*dfr17,tetB,tem*
57	16	28	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		128	4		*dfr1,tetB,tem*
58	16	29	PLEH	AMP,TET,TRI	S	S	®	S	®	S	128			32		*tetA.tem*
59	38	30	PLEH	AMP,TET,TRI	S	S	®	S	®	S	>256		≥256	32		*dfr1,tetB,tem*
60	40	32	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128	16	*dfr17,tetB,catI,tem*
61	40	33	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	®	>256	256	≥256	128	1	*dfr12,tetA,catI,tem*
62	40	34	PLEH	AMP,CHL,FLO,NAL,TET,TRI	®	®	®	®	®	®	>256	256	≥256	64	0.5	*tetB,catI,tem*
63	37	36	PLEH	TRI	S	S	®	®	®	S			≥256			*dfr1*
64	49	37	PLEH	TRI	S	S	®	®	®	S			≥256			*dfr1*
65	44	38	PLEH	AMP,TET,TRI	S	S	®	®	®	S	256		≥256	16		*dfr1,tetB,tem*
66	31	39	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	≥256	128		*dfr12,tetB,catI,tem*
67	31	40	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	256	256	≥256	128		*dfr12,tetA,tetB,catI,tem*
68	31	41	PLEH	TRI	S	S	®	®	S	S						
69	35	42	PLEH	AMP,CHL,FLO,TET,TRI	S	®	®	®	®	S	256	256	≥256	64		*dfr1,tetA,tem*
70	35	43	PLEH	AMP,TET,TRI	S	S	®	®	®	S	>256		≥256	32		*dfr1,tetA,tetB,tem*
71	35	44	PLEH	CHL,FLO,TET,TRI	S	S	®	®	®	S		32	≥256	32		*dfr1,tetA,tetB*
72	48	45	PLEH	AMP,TET,TRI	S	S	®	®	®	®	128			32		*dfr17,tetA,tem*
73	48	46	PLEH	TET,TRI	S	S	®	®	®	S				128		*dfr17,tetB*
74	41	47	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	4		*dfr1,tetB,tem*
75	41	48	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	32		*dfr1,tetB,tem*
76	41	49	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	32		*dfr1,tetA,tetB,tem*
77	43	52	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	>256	256	≥256	64		*dfr1,tetB,catI,tem*
78	32	56	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	128	256	≥256	128		*dfr12,tetB,catI,tem*
79	32	57	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	32		*dfr1,tetb,tem*
80	32	58	PLEH	AMP,TET,TRI	S	S	®	®	®	S	128		≥256	32		*dfr1,tetb,tem*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
81	51	59	PLEH	AMP,TRI	S	S	®	®	®	S	256		128			*dfr1,tem*
82	51	60	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	≥256	128		*dfr1,dfr12,tetB,catl,tem*
83	51	61	PLEH	AMP,TET,TRI	S	S	®	®	®	®	128		128	32		*dfr1,tetA,tem*
84	42	62	PLEH	AMP,TET	S	S	®	®	®	S	128			32		*tetA,tem*
85	42	63	PLEH	AMP,TET	S	S	®	®	®	S	128			32		*tetB,tem*
86	42	64	PLEH	AMP,TET,TRI	S	S	®	®	®	®	256			32		*dfr12,tetB,tem*
87	47	65	PLEH	TRI	S	S	®	®	S	S						
88	47	66	PLEH	TRI	S	S	®	®	S	S						
89	46	67	PLEH	TRI	S	S	®	®	®	S			≥256			*dfr1*
90	46	68	PLEH	TRI	S	S	®	®	®	®			≥256			*dfr1*
91	46	69	PLEH	TRI	S	S	®	®	®	®			128			*dfr1*
92	43	70	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	256	8	≥256	64		*dfr12,tetB,catl,tem*
93	14	71	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	>256	>256	≥256	256		*dfr12,tetA,catl,tem*
94	2	72	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	≥256	64		*dfr1,tetB,catl,tem*
95	10	73	PLEH	AMP,TET,TRI	S	S	®	®	®	S	>256			64		*dfr12,tetB,tem*
96	10	74	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	≥256	64		*tetB,catl,tem*
97	7	76	PLEH	TRI	S	S	®	®	®	®			≥256			*dfr1*
98	56	77	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256			64		*tetB,tem*
99	62	80	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256		≥256	64		*dfr1,dfr12,tetB,tem*
100	62	81	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	128	256	≥256	128		*dfr1,dfr17,tetB,catl,tem*
101	62	82	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	®	®	®	>256	8	≥256	128		*dfr1,dfr12,tetB,catl,tem*
102	58	83	PLEH	AMP,TET,TRI	S	S	®	®	®	®	256			64		*dfr12,tem*
103	58	84	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	®	®	®	>256	8	≥256	128		*dfr1,dfr12,catl,tem*
104	58	85	PLEH	AMP,TET,TRI	S	S	®	®	®	S	>256		≥256	64		*dfr1,dfr12,tem*
105	61	86	PLEH	AMP,TET,TRI	S	S	®	®	®	S	>256			64		*dfr12,tetB,tem*
106	61	87	PLEH	AMP,TET,TRI	S	S	®	®	®	S	>256			128		*dfr12,tetB,tem*
107	61	88	PLEH	AMP,TET,TRI	S	S	®	®	®	S	256		≥256	16		*dfr1,dfr12,dfr17,tetA,tetB,tem*
108	64	89	PLEH	AMP,TET,TRI	S	S	®	®	®	S	256			64		*dfr12,dfr17,tetB,tem*
109	9	90	PLEH	TRI	S	S	®	®	®	S			≥256			*dfr1*
110	64	91	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	128	256		*dfr12,tetB,catl,tem*
111	63	92	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128		*dfr17,tetB,catl,tem*
112	55	93	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	256	0.25	*dfr17,tetB,catl,tem*
113	55	94	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128	8	*dfr17,tetB,catl,tem*
114	55	95	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	256	8	*dfr17,tetA,tetB,catl,tem*
115	54	96	PLEH	AMP,CHL,FLO,TET,TRI	S	®	®	®	®	S	128	256	≥256	64		*dfr1,tem*
116	54	97	PLEH	TRI	S	S	®	®	®	S						
117	54	98	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	S	S	128	>256	≥256	128		*dfr17,tem*
118	52	99	PLEH	CHL,TET,TRI	S	S	®	®	®	®		>256		64		*dfr12,tetB,catl*
119	52	100	PLEH	AMP,CHL,TET,TRI	S	S	®	®	S	S	8	8	≥256	2		*catl,tem*
120	52	101	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	>256	256	≥256	64		*dfr12,tetA,tetB,catl,tem*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
121	65	102	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	S	®	S	>256	256	≥256	128		*dfr1,tetA,tetB,catl,tem*
122	65	103	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	>256	256	≥256	128		*dfr1,dfr17,tetB,catl,tem*
123	65	104	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	®	256	8	≥256			*dfr17,tetB,tem*
124	53	105	PLEH	AMP,CHL,TET,TRI	S	®	®	®	®	S	256	8	≥256	0.125		*dfr1,tetB,tem*
125	53	106	PLEH	AMP,CHL,TET,TRI	S	S	®	S	®	®	256	8	≥256	128		*dfr17,dfr12,tetB,tem*
126	53	107	PLEH	AMP,TRI	S	S	®	S	®	S	2		≥256			*dfr1,tem*
127	67	108	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	128	256	≥256	1		*dfr1,dfr12,tetB,catl,tem*
128	67	109	PLEH	AMP,TET,TRI	S	S	®	®	®	®	128		≥256	32		*dfr12,tetB,tem*
129	67	110	PLEH	AMP,TET,TRI	S	S	®	®	®	S	256		≥256	8		*dfr12,tetB,tem*
130	69	111	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128	32	*dfr17,tetB,catl,tem*
131	69	112	PLEH	TET,TRI	S	S	®	®	®	S			128	32		*dfr1,tetB*
132	69	113	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128		*dfr17,tetB,catl,tem*
133	81	114	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr17,catl,tem*
134	81	115	PLEH	TRI	S	S	®	S	®	S			128			*dfr1*
135	81	116	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1,dfr12*
136	87	117	PLEH	AMP,CHL,NAL,TET,TRI	S	S	S	S	S	S	256	8	≥256	0.5		*tetB,tem*
137	87	118	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256			128		*dfr12,tem*
138	87	119	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	®	®	S	256	8	≥256	1		*dfr1,tetB,tem*
139	74	120	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		128	64		*dfr1,tetB,tem*
140	84	121	PLEH	TRI	S	S	®	®	®	S			128			*dfr1*
141	84	122	PLEH	TRI	S	S	®	S	®	S			128			*dfr1*
142	84	123	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	64	8	*dfr1,dfr17,tetB,catl,tem*
143	83	124	PLEH	AMP,CHL,NAL,TET,TRI	®	S	S	®	®	S	128	>256	≥256	128	8	*dfr17,tetB,catl,tem*
144	83	125	PLEH	AMP,CHL,NAL,TET,TRI	®	®	®	S	®	S	>256	>256	≥256	256		*dfr17,tetA,tetB,catl,tem*
145	83	126	PLEH	AMP,CHL,FLO,TET,TRI	S	®	®	S	®	S	>256	>256	≥256	256		*dfr1,dfr17,tetA,tem*
146	4	127	PLEH	TRI	S	S	®	S	®	S			128			*dfr1*
147	4	128	PLEH	AMP,TET,TRI	S	S	®	S	®	S	>256			64		*tetB,tem*
148	4	129	PLEH	AMP,TRI	S	S	®	S	®	S	>256		64			*dfr1,tem*
149	70	130	PLEH	AMP,TRI	S	S	®	S	®	S	>256					*tem*
150	77	134	PLEH	TRI	S	S	®	S	®	S			64			*dfr1*
151	77	135	PLEH	TRI	S	S	®	S	®	S						
152	77	136	PLEH	TRI	S	S	®	S	®	S			128			*dfr1*
153	80	137	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	256	>256	≥256	128	8	*dfr17,tetB,catl,tem*
154	80	138	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128	8	*dfr17,tetB,catl,tem*
155	80	139	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	S	128	>256	64	128		*dfr1,tem*
156	90	146	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256	>256	≥256	128	8	*dfr17,tetB,shv*
157	90	147	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256	>256	≥256	128		*dfr17,tetB,tem*
158	95	148	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr17,tetB,catl,tem*
159	95	149	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128		*dfr17,tetB,catl,tem*
160	95	150	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128		*dfr1,dfr17,tetB,catl,tem*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
161	91	151	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr1,dfr17,tetB,catl,tem*
162	91	152	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr1,dfr17,tetB,catl*
163	91	153	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128		*dfr17,catl,tem*
164	73	154	PLEH	TET,TRI	S	S	S	S	S	S			64	1		*dfr1,tetB*
165	85	155	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	64		*dfr1,dfr17,tetB,tem*
166	85	156	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	64		*dfr1,dfr17,tem*
167	85	157	PLEH	AMP,TET,TRI	S	S	S	S	S	S	256			0.5		*tetA*
168	88	158	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	S	>256	16	≥256	64		*dfr1,dfr17,tetA,tem*
169	88	159	PLEH	TRI	S	S	®	S	®	S			≥256			*dfr1,dfr17*
170	88	160	PLEH	AMP,TET,TRI	S	S	®	S	®	S	>256		64	64		*dfr1,tetB,tem*
171	82	161	PLEH	TET,TRI	S	S	®	®	®	®				128		*dfr12*
172	82	162	PLEH	TET,TRI	S	S	®	®	®	®				64		*dfr12,tetB*
173	82	163	PLEH	AMP,CHL,NAL,TET,TRI	S	®	®	®	®	S	>256	<256	≥256	128		*dfr1,tetB,catl,tem*
174	9	164	PLEH	TRI	S	S	®	S	®	S						*dfr1*
175	115	167	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	>256	>256	≥256	128	8	*dfr17,tetB,catl,tem*
176	115	168	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	128	>256	≥256	128	8	*dfr12,dfr17,tetB,catl,tem*
177	115	169	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	128	>256	≥256	128	32	*dfr12,dfr17,tetA,tetB,catl,tem*
178	114	170	PLEH	AMP,CHL,FLO,TET,TRI	S	®	S	S	®	S	256	256	≥256	64		*dfr1,tetA,tetB,tem*
179	114	171	PLEH	AMP,CHL,FLO,TET,TRI	S	®	S	S	®	S	256	256	≥256	64		*dfr1,tetA,tetB,tem*
180	114	172	PLEH	AMP,CHL,FLO,TET,TRI	S	®	S	S	®	S	>256	256	≥256	64		*dfr1,tetA,tetB,tem*
181	113	173	PLEH	AMP,CHL,FLO,TET,TRI	S	®	S	S	®	S	256	256	≥256	64		*dfr1,tetB,tem*
182	113	174	PLEH	NAL,TRI	S	S	S	S	®	S			≥256			*dfr1*
183	113	175	PLEH	NAL,TRI	S	S	S	S	®	S						*dfr1*
184	112	176	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256			128		*tetB,tem*
185	112	177	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256		≥256	128		*dfr1,dfr12,tetB,tem*
186	111	178	PLEH	AMP,CHL,TET,TRI	S	S	®	®	®	®	>256	256	≥256	128		*dfr1,dfr12,tetA,catl,tem*
187	111	179	PLEH	AMP,TET,TRI	S	S	®	S	®	S	>256		≥256	32		*dfr17,tetA,tem*
188	111	180	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	16		*dfr17,tetb,tem*
189	110	181	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	S	256		≥256	128		*dfr17,tem*
190	110	182	PLEH	AMP,TET,TRI	S	S	®	S	®	®	>256		≥256	32		*dfr9,dfr17,tetB,tem*
191	110	183	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	256		*dfr17,tetB,tem*
192	109	184	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256			128		*dfr12,tetB,tem*
193	109	185	PLEH	AMP,TET,TRI	S	S	®	®	®	®	>256			128		*dfr12,tetA,tem*
194	109	186	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	16		*dfr17,tetB,tem*
195	108	187	PLEH	AMP,TET	S	S	®	S	®	S	32			0.016		*tetA,tem*
196	108	188	PLEH	AMP,TET,TRI	S	S	®	S	®	S	256		≥256	64		*dfr17,tetB,tem*
197	108	189	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	256		*dfr17,tetA,tem*
198	107	190	PLEH	AMP,TRI	S	S	®	S	®	S	>256		≥256			*dfr17,tem*
199	103	195	PLEH	AMP,CHL,NAL,TET,TRI	S	S	®	S	®	S	128	128	≥256	128	32	*dfr1,dfr17,tetB,catl,tem*
200	103	196	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	256		*dfr17,tetB,catl,tem*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
201	103	197	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	®	128	256	≥256	128		*dfr17,tetB,catI,tem*
202	119	210	PLEH	AMP,CHL,NAL,TET,TRI	S	S	S	S	®	S	64	4	≥256	64		*dfr1*
203	119	211	PLEH	AMP,CHL,NAL,TET,TRI	S	S	S	S	®	S	64	4	≥256	64		*dfr1,tetB*
204	118	212	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	256	≥256	64	8	*dfr17,tetB,catI,tem*
205	118	213	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	64	>256	≥256	64		*dfr17,catI,tem*
206	118	214	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	64	>256	≥256	64		*dfr17,tetB,catI,tem*
207	117	215	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	64		*dfr17,tetB,tem*
208	117	216	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	128		*dfr17,tem*
209	133	220	PLEH	AMP,NAL,TET,TRI	®	S	®	S	®	S	256		≥256	256		*dfr17,tem*
210	133	221	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	64		*dfr17,catI,tem*
211	133	222	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	64		*dfr17,tetA,catI,tem*
212	134	223	PLEH	AMP,TET	S	S	®	S	®	S	>256			16		*tem*
213	134	224	PLEH	AMP,TET	S	S	®	S	®	S	>256			32		*tetA,tem*
214	134	225	PLEH	AMP,TET	S	S	®	S	®	S	256			32		*tetA,tem*
215	137	226	PLEH	AMP,TET	S	S	®	S	®	S	>256			32		*tetA,tem*
216	137	227	PLEH	AMP,TET	S	S	®	S	®	S	>256			64		*tem*
217	141	231	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	>256	≥256	64	8	*dfr17,catI,tem*
218	141	232	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	®	®	S	64	>256	≥256	64	8	*dfr17,catI,tem*
219	141	233	PLEH	AMP,CHL,NAL,TET,TRI	®	S	®	S	®	S	64	256	≥256	64		*dfr17,tetA,catI,tem*
220	143	234	Premises A	AMP,TET,TRI	S	S	S	S	®	S	>256		≥256	64		*dfr1,tetA,tem*
221	149	235	Premises A	TRI	S	S	S	S	®	S			≥256			*dfr1*
222	149	236	Premises A	TRI	S	S	S	S	®	S			≥256			*dfr1*
223	154	237	Premises A	TET,TRI	S	S	S	S	®	S			≥256	128		*dfr1*
224	155	238	Premises A	TRI	S	S	S	S	®	S			≥256			*dfr1*
225	156	239	Premises A	TRI	S	S	S	S	S	S			≥256			*dfr1*
226	168	240	Premises A	TRI	S	S	S	S	®	S			≥256			*dfr1*
227	170	241	Premises A	AMP	S	S	®	®	®	®	128					
228	179	242	Premises A	AMP,TET	S	®	®	®	®	S	256			128		
229	203	243	Premises A	NAL	®	S	®	®	®	®					0.016	
230	203	244	Premises A	NAL	®	S	®	®	®	®					0.016	
231	203	245	Premises A	NAL	®	S	®	®	®	®					0.016	
232	214	246	Premises A	TRI	S	S	®	S	®	S			≥256			*dfr1*
233	217	247	Premises A	TRI	S	S	®	S	®	S			≥256			*dfr1*
234	217	248	Premises A	TRI	S	S	®	S	®	S			≥256			*dfr1*
235	217	249	Premises A	TRI	S	S	®	S	®	S			≥256			*dfr1*
236	221	250	Premises A	TRI	S	S	S	S	®	S			64			*dfr1*
237	221	251	Premises A	TRI	S	S	S	S	®	S			≥256			*dfr1*
238	223	252	Premises A	NAL	S	S	S	S	®	S						
239	251	254	Premises A	TET,TRI	S	S	S	S	S	S				1		
240	251	255	Premises A	TET,TRI	S	S	S	S	®	S			≥256	32		*dfr1*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
241	254	256	Premises A	TRI	S	S	®	S	®	S			128			*dfr1*
242	196	257	Premises A	TRI	S	S	®	S	®	S			64			*dfr1*
243	185	258	Premises A	TRI	S	S	®	S	®	S			64			*dfr1*
244	185	259	Premises A	TRI	S	S	®	S	®	S			128			*dfr1*
245	323	313	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
246	323	314	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
247	322	315	Premises B	AMP,APRA,TET,TRI	S	S	®	®	®	®	128		≥256	64		*dfr1*
248	322	316	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
249	322	317	Premises B	AMP,TRI	S	S	®	S	®	S	128		≥256			*dfr1*
250	321	318	Premises B	TET,TRI	S	S	®	S	®	S			≥256	32		*dfr1*
251	321	319	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
252	321	320	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
253	324	321	Premises B	TRI	S	S	®	®	S	S			≥256			*dfr1*
254	324	322	Premises B	TRI	S	S	®	®	S	S			≥256			*dfr1*
255	324	323	Premises B	TRI	S	S	®	®	S	S			≥256			*dfr1*
256	311	327	Premises B	TET,TRI	S	S	S	S	®	S			≥256	64		*dfr1*
257	311	328	Premises B	TET,TRI	S	S	S	S	®	S			≥256	64		*dfr1*
258	311	329	Premises B	TRI	S	S	S	S	®	S			128			*dfr1*
259	304	260	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
260	304	261	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
261	304	262	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
262	319	263	Premises B	TRI	S	S	®	S	®	S			128			*dfr1*
263	319	281	Premises B	TET,TRI	S	S	S	S	®	S			≥256	32		*dfr1*
264	340	264	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
265	340	282	Premises B	TET,TRI	S	S	S	S	®	S			≥256	64		*dfr1*
266	308	330	Premises B	TRI	S	S	S	S	®	S			128			*dfr1*
267	308	331	Premises B	TRI	S	S	S	S	®	S			128			*dfr1*
268	308	332	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
269	307	333	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
270	307	334	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
271	307	335	Premises B	TRI	S	S	S	S	®	S			64			*dfr1*
272	318	336	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
273	318	337	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
274	318	338	Premises B	TRI	S	S	S	S	®	S			64			*dfr1*
275	317	339	Premises B	TRI	S	S	S	S	®	S						*dfr1*
276	317	340	Premises B	TRI	S	S	S	S	®	S			≥256			*dfr1*
277	314	347	Premises B	TRI	S	S	®	®	®	S			≥256			*dfr1*
278	314	348	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
279	314	349	Premises B	TRI	S	S	®	S	®	S						*dfr1*
280	312	350	Premises B	AMP,CHL,FLO,TET,TRI	S	®	®	S	®	S	256	128	64	64		*dfr1*

Appendix 3

Isloates no	Faecal culture coll-no	Culture coll-no	Source	Resistance profile	Cip	B-lactams	Str	Spe	Sul	Gen	Amp / MIC	Chl/ MIC	Tri/ MIC	Tet/ MIC	Cip/ MIC	*Resistant genes*
281	312	351	Premises B	AMP,CHL,FLO,TET,TRI	S	®	®	S	®	S	256	256	≥256	64		*dfr17*
282	312	352	Premises B	AMP,CHL,FLO,TET,TRI	S	®	®	S	®	S	256	256		64		*dfr1*
283	313	353	Premises B	TRI	S	S	S	S	®	S						
284	313	354	Premises B	TRI	S	S	®	®	®	S			≥256			
285	313	355	Premises B	TRI	S	S	®	S	®	S			≥256			
286	333	356	Premises B	AMP,CHL,FLO,TET,TRI	S	®	®	S	®	S	>256	256	≥256	64		
287	336	357	Premises B	TRI	S	S	®	S	®	S						*dfr1*
288	339	358	Premises B	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	256	16	*dfr17*
289	339	359	Premises B	AMP,NAL,TET,TRI	®	S	®	S	®	®	256		≥256	256	8	*dfr17*
290	339	360	Premises B	AMP,NAL,TET,TRI	®	S	®	S	®	S	256		≥256	256	8	*dfr17*
291	346	361	Premises B	TET	S	S	®	S	®	S				128		
292	346	362	Premises B	TET	S	S	®	S	®	S				32		
293	347	363	Premises B	TRI	S	S	®	S	®	S						
294	347	364	Premises B	TRI	S	S	®	S	®	S			≥256			*dfr1*
295	347	365	Premises B	AMP,TRI	S	S	®	S	®	S	>256		≥256			*dfr1*
296	349	366	Premises B	AMP,CHL,FLO,NAL,TET,TRI	S	S	S	S	®	S	>256	32	≥256	64		*dfr1*

Figure1. Examples of different PCR positive isolates to different resistant genes (random selections of 2-3 isolates in each example)

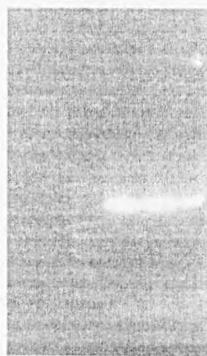
Dfr1 (254bp)

Dfr12 (485bp)

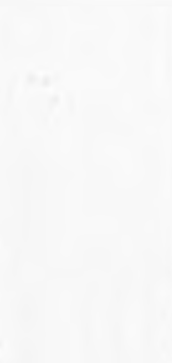
Dfr 7-17 (195bp)

catI (585bp)

tetA (210bp)



tetB (659bp)



tem (971bp) and *shv* (885bp)

Appendix 4A

Horse Name/case number	Sample time	Culture collection number	Sex	Breed	Age	Add/date □	Onset/date	Complications	Treatment starting date	ABs tretament starting date	
Merlin 29115	1st \	181	G	TBX	11	02/11/2004	02/09/2004	Colic	02/11/2004	02/11/2004	
	2nd \	184									
	3rd \	187									
	4th \	194									
	5th \	214									
	6th \	233									
Bruno 27246	1st \	2	G	Welsh	20	26/02/2003	02/02/2003	Colic	02/02/2003	27/02/2003	
	2nd \	7									
	3rd \	11									
	4th \	27									
	5th \	71									
	6th \	122									
Freddie 28094	1st \	95	G	Sheltand	20	08/03/2003	08/03/2003	Large-colon tursion	08/03/2003	08/03/2003	
	2nd \	96									
	3rd \	99									
	4th \	101									
	5th \	123									
	6th \	196									→
Riley 28162	1st \	106	G	TBX	4	12/08/2003	09/08/2003	Colic	12/08/2003	12/08/2003	
	2nd \	109									
	3rd \	114									
	4th \	132									
	5th \	136									
	6th \	198									
Ellish 27282	1st \	28	M	X breed	10	05/03/2003	04/03/2004	Colic	05/03/2003	05/03/2003	
	2nd \	18									
	3rd \	35									
	4th \	67									
	5th \	76									
	6th \	134									
Charlie 28280	1st \	29	M	Cob	12	05/03/2003	05/03/2003	Twisted large colon	05/03/2003	05/03/2003	
	2nd \	34									
	3rd \	38									
	4th \	65									
	5th \	72									
	6th \	127									

Appendix 4A

Antibiotic name	Sample time	Original sheet number	Resistant E. coli	Amp	B-lactam Drugs	Apr	Chl	Flo	Nal	Cip	Tet	Tri	MDR	VREs	Resistant isolates (n=)
Neopenicillin	1st \	181		S	S	S	S	S	S	S	S	S			0
	2nd \	184	R	®	S	S	S	S	®	S	®	®	MDR	*E. gallinarum-vanC-1*	3
	3rd \	187	R	®	S	S	S	S	®	S	®	®	MDR		3
	4th \	194		S	S	S	S	S	S	S	S	S			0
	5th \	214		S	S	S	S	S	S	S	S	S			0
	6th \	233		S	S	S	S	S	S	S	S	S			0
Neopenicillin	1st \	2		S	S	S	S	S	S	S	S	S			0
	2nd \	7	R	®	S	®	®	®	®	®	®	®	MDR		3
	3rd \	11	R	®	S	S	S	S	®	®	®	®	MDR		3
	4th \	27	R	®	S	S	®	®	®	®	®	®	MDR		3
	5th \	71		S	S	S	S	S	S	S	S	S			0
	6th \	122		S	S	S	S	S	S	S	S	S			0
Neopenicillin	1st \	95		S	S	S	S	S	S	S	S	S			0
	2nd \	96		S	S	S	S	S	S	S	S	S			0
	3rd \	99		S	S	S	S	S	S	S	S	S			0
	4th \	101	R	®	S	S	S	S	S	S	®	®			2
	5th \	123	R	®	S	S	S	S	S	S	S	®			1
	6th \	196	R	S	S	S	S	S	S	S	S	®			1
Neopenicillin	1st \	106	R	®	S	S	S	S	S	S	S	S			3
	2nd \	109	R	®	S	S	S	S	S	S	®	®			3
	3rd \	114	R	S	S	S	S	S	S	S	®	®			3
	4th \	132	R	S	S	S	S	S	S	S	S	®			3
	5th \	136	R	S	S	S	S	S	S	S	®	®			3
	6th \	198	R	®	S	S	S	S	S	S	®	®			3
Neopenicillin	1st \	28		S	S	S	S	S	S	S	S	S			0
	2nd \	18		S	S	S	S	S	S	S	S	S			0
	3rd \	35	R	®	S	S	®	®	®	®	®	®	MDR		3
	4th \	67	R	S	S	S	S	S	S	S	S	®			1
	5th \	76		S	S	S	S	S	S	S	S	S			0
	6th \	134		S	S	S	S	S	S	S	S	S			0
Neopenicillin	1st \	29	R	®	S	S	S	S	S	S	S	S			1
	2nd \	34	R	®	®	S	®	®	®	®	®	®	MDR		3
	3rd \	38	R	®	S	S	®	®	®	®	®	®	MDR		3
	4th \	65	R	S	S	S	S	S	S	S	®	®			3
	5th \	72	R	®	S	S	S	S	S	S	®	®			1
	6th \	127		S	S	S	S	S	S	S	S	S			0

Appendix 4B

Horse Name/case number	Samplin time	Culture collection/ no	Sex	Breed	Age	Add/date □	Onset/date	Condition	Treatment starting/date	
Native D 28548	1	138	G	TB	7	20/10/03	19/10/03	thorn penetration	20/10/2003	
	2	142								
	3	147								
	4	150								
	5	169								
	6	229								
Harvey 28779	1	153	G	TBX	16	12/01/2003	12/01/2003	WOUNDS	12/01/2003	
	2	156								
	3	161								
	4	158								
	5	175								→
	6	231								
Bella 27276	1	14	M	TB	13	03/04/2003	Autumn 2002	lame/proplem	03/04/2003	
	2	23								
	3	30								
	4	64								
	5	73								
	6	128								
Monty 28928	1	167	G	TB	5	01/07/2004	01/07/2004	WOUNDS	01/07/2004	
	2	170								
	3	171								
	4	172								
	5	232								
	6	247								

Appendix 4B

No	ABs treatament starting date	Antibiotics thearpy	Sample time	Original sheet number	Resistant *E. coli*	Amp	B-lactam Drugs	Apr	Chl	Flo	Nal	Cip	
1	20/10/2003	Ceftifur,TMPs	1st \	138	R	®	S	S	S	S	S	S	
2			2nd \	142	R	®	®	S	®	S	®	®	
3			3rd \	147	R	®	®	S	®	S	®	®	
4			4th \	150	R	®	®	S	®	S	®	®	
5			5th \	169	R	®	S	S	S	S	®	®	
6			6th \	229		S	S	S	S	S	S	S	
7	12/01/2003	Ceftifur,Baytril	1st \	153	R	S	S	S	S	S	S	S	
8			2nd \	156	R	®	®	S	®	S	®	®	
9			3rd \	161	R	®	®	S	®	S	®	®	
10			4th \	158	R	®	®	S	®	S	®	®	
11			5th \	175	R	®	®	S	®	S	®	®	→
12			6th \	231		S	S	S	S	S	S	S	
13	03/04/2003	Metronidazole	1st \	14	R	S	S	S	S	S	S	S	
14			2nd \	23	R	S	S	S	S	S	S	S	
15			3rd \	30	R	S	S	S	S	S	S	S	
16			4th \	64	R	S	S	S	S	S	S	S	
17			5th \	73	R	S	S	S	S	S	S	S	
18			6th \	128		S	S	S	S	S	S	S	
19	01/07/2004	Ceftifur,TMPs	1st \	167		S	S	S	S	S	S	S	
20			2nd \	170	R	®	®	S	®	S	®	®	
21			3rd \	171	R	®	®	S	®	S	®	®	
22			4th \	172	R	®	®	S	®	S	®	®	
23			5th \	232		S	S	S	S	S	S	S	
24			6th \	247		S	S	S	S	S	S	S	

Appendix 4B

No	Tet	Tri	MDR	VREs/genes	Resistant isolates (n =)
1	S	®		*E. gallinarum-vanC-1*	3
2	®	®	MDR		2
3	®	®	MDR		3
4	®	®	MDR		3
5	®	®	MDR		1
6	S	S			0
7	S	®			3
8	®	®	MDR		3
9	®	®	MDR	*E. gallinarum-vanC-1*	3
10	®	®	MDR		3
11	®	®	MDR	*E. gallinarum-vanC-1*	3
12	S	S			0
13	S	®			2
14	S	®			3
15	S	®			3
16	S	®			3
17	S	®			3
18	S	S			0
19	S	S			0
20	®	®	MDR		3
21	®	®	MDR		3
22	®	®	MDR	*E. gallinarum-vanC-1*	3
23	S	S			0
24	S	S			0
					50

Appendix 4C

Horse Name/case number	non-GI-	Culture collection /no	Sex	Breed	Age	Add/date □	Complications	Treatment starting date	Sample time	
Lucre 27260	1	1	G	TBX	7	28/02/03	Nasal danmage	23/02/04	1st \	
	2	4							2nd \	
	3	6							3rd \	
	4	20							4th \	
	5	62							5th \	
	6	126							6th \	
Dazzler 27313	1	40	M	TB	2	11/03/2003	Umbilical hernia	non	1st \	
	2	43							2nd \	
	3	45							3rd \	
	4	50							4th \	
	5	68							5th \	
	6	130							6th \	
Dundee 28773	1	152	M	Warm	9	02/12/2003	Lameness condition	non	1st \	
	2	155							2nd \	
	3	162							3rd \	→
	4	163							4th \	
	5	174							5th \	
	6	230							6th \	
Kit 27278	1	9	M		4	04/03/2003	suspect-Colic	05/03/2003	1st \	
	2	24							2nd \	
	3	17							3rd \	
	4	70							4th \	
	5	77							5th \	
	6	125							6th \	
Trooper 27127	1	137	G	Warm	9	31/01/03	suspected-Colic	27/01/03	1st \	
	2	143							2nd \	
	3	145							3rd \	
	4	149							4th \	
	5	166							5th \	
	6	226							6th \	

Appendix 4C

Original sheet number	Resistant *E. coli*	Amp	B-lactam Drugs	Apr	Chl	Flo	Nal	Cip	Tet	Tri	MDR	VREs	Resistant isolates (n=)
1		®	S	S	S	S	S	S	S	S			0
4	R	®	S	S	S	S	®	®	®	®	MDR		2
6	R	®	S	S	S	S	®	®	®	®	MDR		1
20	R	®	S	S	®	®	®	®	®	®	MDR		3
62	R	®	S	S	®	®	®	®	®	®	MDR		3
126		S	S	S	S	S	S	S	S	S			0
40		S	S	S	S	S	S	S	S	S			0
43		S	S	S	S	S	S	S	S	S			0
45	R	®	S	S	S	S	S	S	S	S			2
50	R	®	S	S	S	S	®	®	®	®	MDR		3
68	R	®	S	S	S	S	®	S	®	®	MDR		3
130		S	S	S	S	S	S	S	S	S			0
152		S	S	S	S	S	S	S	S	S			0
155	R	®	S	S	S	S	S	S	S	S			1
162	R	®	S	S	S	S	S	S	S	S		*E. gallinarum-vanC-1*	1
163	R	®	®	S	®	S	S	S	®	®	MDR	*E. gallinarum-vanC-1*	1
174	R	S	S	S	S	S	S	S	®	®		*E. gallinarum-vanC-1*	3
230		S	S	S	S	S	S	S	S	S		*E. gallinarum-vanC-1*	0
9		S	S	S	S	S	S	S	S	S			0
24		S	S	S	S	S	S	S	S	S			0
17		S	S	S	S	S	S	S	S	S			0
70		S	S	S	S	S	S	S	S	S			0
77		S	S	S	S	S	S	S	S	S			0
125	R	S	S	S	S	S	S	S	S	®			1
137	R	®	S	S	®	S	S	S	S	®		*E. gallinarum-vanC-1*	3
143	R	®	®	S	®	S	®	S	®	®	MDR	*E. gallinarum-vanC-1*	3
145	R	®	®	S	®	S	S	S	®	®	MDR	*E. gallinarum-vanC-1*	3
149	R	®	®	S	®	S	S	S	®	®	MDR	*E. gallinarum-vanC-1*	3
166		S	S	S	S	S	S	S	S	S			0
226		S	S	S	S	S	S	S	S	S			0

Appendix 5

no	Resistance isloates collection	Faecal sample collection	Source	Amp/ MICsug/ml	B-lactamase drugs	Apra	Chl MICsug/ml	Flo	Tet MICsug/ml	
1	4	4	NON GI-	R/128	S	S	S	S	R/256	
2	5	4	NON GI-	S	S	S	S	S	S	
3	6	6	NON GI-	R/128	S	S	S	S	R/256	
4	17	20	NON GI-	R/64	S	S	R/128	S	R/128	
5	18	20	NON GI-	R/64	S	S	R/128	S	R/64	
6	19	20	NON GI-	R/128	S	S	R/>256	R	R/256	
7	54	45	NON GI-	R/>256	S	S	S	S	S	
8	55	45	NON GI-	R/>256	S	S	S	S	S	
9	58	50	NON GI-	R/256	S	S	S	S	R/64	
10	59	50	NON GI-	R/128	S	S	S	S	R/256	
11	60	50	NON GI-	R/128	S	S	S	S	R/>256	
12	84	62	NON GI-	S	S	S	S	S	S	
13	85	62	NON GI-	R/64	S	S	R/256	R	R/256	
14	86	62	NON GI-	S	S	S	S	S	S	
15	94	68	NON GI-	R/256	S	S	S	S	R/128	
16	95	68	NON GI-	R/256	S	S	S	S	R/128	
17	96	68	NON GI-	R/256	S	S	S	S	R/128	
18	186	137	NON GI-	S	S	S	S	S	S	→
19	187	137	NON GI-	R/<256	S	S	R/256	S	S	
20	188	137	NON GI-	S	S	S	S	S	S	
21	197	143	NON GI-	R/256	R	S	R/>256	S	R/256	
22	198	143	NON GI-	R/256	R	S	R/>256	S	R/256	
23	199	143	NON GI-	R/256	R	S	R/>256	S	R/256	
24	203	145	NON GI-	R/256	R	S	R/256	S	R/128	
25	204	145	NON GI-	R/>256	R	S	R/256	S	R/256	
26	205	145	NON GI-	R/256	R	S	R/256	S	R/128	
27	214	149	NON GI-	R/128	S	S	R/>256	S	S	
28	215	149	NON GI-	R/256	R	S	R/256	S	R/256	
29	216	149	NON GI-	R/256	S	S	R/256	S	S	
30	225	155	NON GI-	R/256	S	S	S	S	S	
31	235	162	NON GI-	R/>256	S	S	S	S	S	
32	236	163	NON GI-	R/128	R	S	R/256	S	R/128	
33	249	174	NON GI-	S	S	S	S	S	R/>256	
34	250	174	NON GI-	S	S	S	S	S	R/>256	
35	251	174	NON GI-	S	S	S	S	S	R/>256	
36	266	226	NON GI-	S	S	S	S	S	S	
37	7	7	GI+	R/128	S	S	S	R	R/64	
38	8	7	GI+	R/>256	S	R	S	S	R/64	
39	9	7	GI+	R/256	S	S	S	S	R/64	
40	11	11	GI+	R/128	S	S	S	S	R/128	

Appendix 5

no	Tri MICsug/ml	Nal	Cip MICsug/ml	MDR	Str	Spe	Sul	Gen	RESISTANT GENES IDENTIFIED	Conjugation exp.
1	R>256	R	R/4	MDR	®	S	®	®	*dfr7-17,tetA,tem*	
2	R>256	S	S		®	S	®	S	*dfr1*	
3	R>256	R	R/8	MDR	®	S	®	®	*dfr7-17,tetA,tem*	
4	R>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
5	R>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
6	R>256	R	R/4	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
7	S	S	S		®	S	®	S	*tem*	Transconjugants
8	S	S	S		®	S	®	S	*tem*	Transconjugants
9	R/>256	R	R/2	MDR	®	S	®	S	*dfr7-17,tetA,tetB,tem*	
10	R/>256	R	R/4	MDR	®	S	®	®	*dfr7-17,tetA,tem*	
11	R/>256	R	R/8	MDR	®	S	®	®	*dfr7-17,tetA,tem*	
12	R/>256	S	S		S	S	®	S	*dfr1*	
13	R/>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
14	R/>256	S	S		S	S	®	S	*dfr1*	
15	R/>256	R	R/2	MDR	®	S	®	S	*dfr7-17,tetB,tem*	
16	R/>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetB,tem*	
17	R/>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetB,tem*	
18	R/>256	S	S		®	S	®	S	*dfr1*	
19	R/>256	S	S		®	S	®	S	*dfr1,dfr12,tem,catI*	
20	R/>256	S	S		®	S	®	S	*dfr1*	
21	R/>256	R	R/16	MDR	®	®	®	®	*tetA,tem,catI*	
22	R/>256	R	R/8	MDR	®	®	®	®	*tetA,catI*	
23	R/>256	R	R/4	MDR	®	®	®	®	*tetA,tem,catI*	
24	R/>256	R	R/8	MDR	®	®	®	®	*dfr1,dfr12,tetA,catI*	
25	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem,catI*	Transconjugants
26	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem*	Transconjugants
27	R/>256	S	S		®	®	®	®	*dfr12,tem,catI*	
28	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem,catI*	Transconjugants
29	R/>256	S	S		®	®	®	S	*dfr12,tem,catI*	
30	S	S	S		®	®	®	S	*tem*	
31	S	S	S		®	®	®	®	*tem*	Transconjugants
32	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem*	Transconjugants
33	R/>256	S	S		®	®	®	S	*dfr1,tetA*	
34	R/>256	S	S		®	®	®	S	*dfr1,tetA*	
35	R/>256	S	S		®	®	®	S	*dfr1,tetA*	
36	S	S	S		®	S	®	S		
37	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tetB,tem*	
38	R/>256	R	R/8	MDR	®	®	®	®	*dfr12,dfr7-17,tetA,tetB,tem0*	
39	R/>256	R	R/8	MDR	®	®	®	®	*dfr12,dfr7-17,tetA,tetB,tem0*	
40	R/>256	R	R/16	MDR	®	®	®	S	*dfr7-17,tetA,tetB,tem*	

Appendix 5

no	Resistance isloates collection	Faecal sample collection	Source	Amp/ MICsug/ml	B-lactamase drugs	Apra	Chl MICsug/ml	Flo	Tet MICsug/ml	
41	12	11	GI+	R/256	S	S	S	S		
42	13	11	GI+	R/>256	S	S	S	S	R/128	
43	25	27	GI+	R/128	S	S	R/>256	S	R/128	
44	26	27	GI+	R/128	S	S	R/256	R	R/256	
45	27	27	GI+	R/128	S	S	R/>256	S	R/256	
46	28	29	GI+	R/>256	S	S	S	S	S	
47	40	34	GI+	R/256	R	S	R/>256	R	R/256	
48	41	34	GI+	R/256	S	S	R/>256	S	R/256	
49	42	34	GI+	R/>256	S	S	S	S	R/128	
50	43	35	GI+	R/256	S	S	R/>256	R	R/128	
51	44	35	GI+	R/4	S	S	R/8	S	R/128	
52	45	35	GI+	R/2	S	S	S	S	R/128	
53	49	38	GI+	R/64	S	S	R/>256	S	R/128	
54	50	38	GI+	R/64	S	S	R/>256	R	R/256	
55	51	38	GI+	R/256	S	S	R/>256	S	R/256	
56	90	65	GI+	S	S	S	S	S	R/128	
57	91	65	GI+	S	S	S	S	S	R/128	
58	92	65	GI+	S	S	S	S	S	R/128	
59	97	67	GI+	S	S	S	S	S	S	
60	98	72	GI+	R/>256	S	S	S	S	S	
61	136	101	GI+	R/>256	S	S	S	S	S	→
62	137	101	GI+	R/128	S	S	S	S	R/128	
63	147	106	GI+	R/>256	S	S	S	S	S	
64	148	106	GI+	R/>256	S	S	S	S	S	
65	149	106	GI+	R/>256	S	S	S	S	S	
66	150	109	GI+	R	S	S	S	S	R/256	
67	151	109	GI+	R	S	S	S	S	R/128	
68	152	109	GI+	R/>256	S	S	S	S	R/256	
69	161	114	GI+	S	S	S	S	S	R/128	
70	162	114	GI+	S	S	S	S	S	R/>256	
71	163	114	GI+	S	S	S	S	S	R/128	
72	172	123	GI+	R/8	S	S	S	S	S	
73	177	132	GI+	S	S	S	S	S	S	
74	178	132	GI+	S	S	S	S	S	S	
75	179	132	GI+	S	S	S	S	S	S	
76	183	136	GI+	S	S	S	S	S	R/128	
77	184	136	GI+	S	S	S	S	S	S	
78	185	136	GI+	S	S	S	S	S	S	
79	269	184	GI+	R/>256	S	S	S	S	R/256	

Appendix 5

no	Tri MICsug/ml	Nal	Cip MICsug/ml	MDR	Str	Spe	Sul	Gen	RESISTANT GENES IDENTIFIED	Conjugation exp.
41	R/256	R	R/4	MDR	®	®		®	*dfr1,dfr12,dfr7-17,tetA,tetB,tem*	
42	R/>256	R	R/4	MDR	®	®	®	®	*dfr1,dfr12,dfr7-17,tetA,tetB,tem*	
43	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,catI*	
44	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,catI*	
45	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
46	S	S	S		®	®	®	S	*tem*	Transconjugants
47	R/>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
48	R/>256	R	R/2	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
49	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tetB,tem*	
50	R/>256	R	R/8	MDR	®	®	®	S	*tetA,shv*	
51	R/>256	R	R/8	MDR	®	S	®	S	*tetA,shv*	
52	R/>256	R	R/16	MDR	®	S	®	S	*tetA,shv*	
53	R/>256	R	R/4	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
54	R/>256	R	R/8	MDR	®	S	®	S	*dfr7-17,tetA,tem,catI*	
55	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
56	R/>256	S	S		®	S	®	S	*dfr1,tetB*	
57	R/>256	S	S		®	S	®	S	*dfr1,tetB*	
58	R/>256	S	S		®	S	®	S	*dfr1,tetB*	
59	R/>256	S	S		S	S	S	S		
60	R/>256	S	S		®	S	®	S	*dfr7-17*	Transconjugants
61	R/>256	S	S		®	®	®	®		Transconjugants
62	R/>256	S	S		®	®	®	®	*dfr1,dfr12,dfr7-17,tetA*	
63	S	S	S		®	®	®	®	*tem*	Transconjugants
64	S	S	S		®	®	®	®	*tem*	Transconjugants
65	S	S	S		®	®	®	®	*tem*	Transconjugants
66	R>256	S	S		®	®	®	®	*tetA,tem*	
67	R>256	S	S		®	®	®	®	*tetA,tem*	
68	R>256	S	S		®	®	®	®	*tetA,tem*	
69	R>256	S	S		®	®	®	®	*dfr1,tetA*	
70	R>256	S	S		®	®	®	®	*dfr1,dfr7-17,tetA*	
71	R>256	S	S		®	®	®	®	*dfr1,dfr7-17,tetA*	
72	R>256	S	S		®	®	®	®	*dfr1*	Transconjugants
73	R>256	S	S		®	®	®	®	*dfr1,dfr7-17*	
74	R>256	S	S		®	®	®	®	*dfr1,dfr7-17*	
75	R>256	S	S		®	®	®	®	*dfr7-17*	
76	R>256	S	S		®	®	®	®	*dfr7-17,tetA*	
77	R>256	S	S		®	®	®	®	*dfr1,dfr7-17*	
78	R>256	S	S		®	®	®	®	*dfr7-17*	
79	R>256	R	R/16	MDR	®	®	®	S	*dfr12,dfr7-17,tetA,tem*	

Appendix 5

no	Resistance isloates collection	Faecal sample collection	Source	Amp/ MICsug/ml	B-lactamase drugs	Apra	Chl MICsug/ml	Flo	Tet MICsug/ml	
80	270	184	GI+	R/>256	S	S	S	S	R/256	
81	271	184	GI+	R/>256	S	S	S	S	R/256	
82	278	187	GI+	R/>256	S	S	S	S	R/256	
83	279	187	GI+	R/>256	S	S	S	S	R/256	
84	280	187	GI+	R/>256	S	S	S	S	R/256	
85	294	198	GI+	R/>256	S	S	S	S	S	
86	295	198	GI+	R/>256	S	S	S	S	R/128	
87	296	198	GI+	R/>256	S	S	S	S	R/256	
88	296	196	GI+	S	S	S	S	S	S	
89	14	14	NON-GI +	S	S	S	S	S	S	
90	15	14	NON-GI +	S	S	S	S	S	S	
91	21	23	NON-GI +	S	S	S	S	S	S	
92	22	23	NON-GI +	S	S	S	S	S	S	
93	23	23	NON-GI +	S	S	S	S	S	S	
94	29	30	NON-GI +	S	S	S	S	S	S	
95	30	30	NON-GI +	S	S	S	S	S	S	
96	31	30	NON-GI +	S	S	S	S	S	S	
97	87	64	NON-GI +	S	S	S	S	S	S	
98	88	64	NON-GI +	S	S	S	S	S	S	
99	89	64	NON-GI +	S	S	S	S	S	S	
100	99	73	NON-GI +	S	S	S	S	S	S	→
101	100	73	NON-GI +	S	S	S	S	S	S	
102	101	73	NON-GI +	S	S	S	S	S	S	
103	189	138	NON-GI +	R/8	S	S	S	S	S	
104	190	138	NON-GI +	S	S	S	S	S	S	
105	191	138	NON-GI +	S	S	S	S	S	S	
106	195	142	NON-GI +	R/256	R	S	R/>256	S	R/256	
107	196	142	NON-GI +	R/128	R	S	R/>256	S	R/256	
108	209	147	NON-GI +	R/256	R	S	R/>256	S	R/256	
109	210	147	NON-GI +	R/256	R	S	R/>256	S	R/256	
110	211	147	NON-GI +	R/>256	S	S	S	S	R/256	
111	217	150	NON-GI +	R/>256	S	S	S	S	R/128	
112	218	150	NON-GI +	R/128	R	S	R/>256	S	R/256	
113	219	150	NON-GI +	S	S	S	S	S	S	
114	222	153	NON-GI +	S	S	S	S	S	S	
115	223	153	NON-GI +	S	S	S	S	S	S	
116	224	153	NON-GI +	S	S	S	S	S	S	
117	226	156	NON-GI +	R/>256	R	S	R/>256	S	R/>256	
118	227	156	NON-GI +	R/>256	R	S	R	S	R/128	

Appendix 5

no	Tri MICsug/ml	Nal	Cip MICsug/ml	MDR	Str	Spe	Sul	Gen	RESISTANT GENES IDENTIFIED	Conjugation exp.
80	R>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem*	
81	R>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem*	
82	R>256	R	R/16	MDR	®	®	®	S	*dfr7-17,tetA,tem*	
83	R>256	R	R/4	MDR	®	®	®	S	*dfr7-17,tem*	
84	R>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tem*	
85	R>256	S	S		®	®	®	S	*tem*	Transconjugants
86	R>256	S	S		®	®	®	S	*dfr1,tem*	
87	R>256	S	S		®	®	®	S	*dfr1,tem*	
88	S	S	S		®	S	®	S	*dfr1*	
89	R/>256	S	S		S	S	®	S	*dfr1*	
90	R/>256	S	S		®	®	®	S	*dfr1*	
91	R/>256	S	S		®	®	®	S	*dfr1*	
92	R/>256	S	S		®	®	®	S	*dfr1*	
93	R/>256	S	S		®	®	®	S	*dfr1*	
94	R/>256	S	S		®	®	®	S		
95	R/>256	S	S		®	®	®	S		
96	R/>256	S	S		®	®	®	S		
97	R/>256	S	S		®	®	®	S	*dfr1*	
98	R/>256	S	S		®	S	®	S	*dfr1*	
99	R/>256	S	S		S	S	S	S	*dfr1*	
100	R/>256	S	S		®	S	®	®	*dfr1*	
101	R/>256	S	S		®	S	®	®	*dfr1*	
102	R/>256	S	S		®	S	®	®	*dfr1,7-17*	
103	S	S	S		®	®	®	S	*tem*	Transconjugants
104	R/>256	S	S		®	®	®	®		
105	R/>256	S	S		®	®	®	®		
106	R/>256	R	R/4	MDR	®	®	®	®	*dfr12,tetA,tem,catI*	
107	R/>256	R	R/8	MDR	®	®	®	®	*tetA,tem,catI*	
108	R/>256	R	R/8	MDR	®	®	®	®	*dfr7-17,tetA,tem,catI*	
109	R/>256	R	R/16	MDR	®	®	®	®	*dfr7-17,tetA,tem,catI*	
110	R/>256	R	R/2	MDR	®	®	®	®	*dfr12,dfr7-17,tetA,tetB,tem,*	
111	R/>256	S	S		®	®	®	S	*dfr12,tetA,tetB,tem*	
112	R/>256	R	R/4	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
113	R/>256	S	S		®	®	®	S	*dfr1*	
114	R/>256	S	S		®	®	®	S	*dfr7-17*	
115	R/>256	S	S		®	®	®	S	*dfr1*	
116	R/>256	S	S		®	®	®	S	*dfr7-17*	
117	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
118	R/>256	S	S		®	®	®	®	*dfr1,dfr12,tetA,tem,catI*	

Appendix 5

no	Resistance isloates collection	Faecal sample collection	Source	Amp/ MICsug/ml	B-lactamase drugs	Apra	Chl MICsug/ml	Flo	Tet MICsug/ml	
119	228	156	NON-GI +	R/128	R	S	R	S	R/256	
120	229	158	NON-GI +	R/256	R	S	R/>256	S	R/256	
121	230	158	NON-GI +	R/128	R	S	R/>256	S	R/256	
122	231	158	NON-GI +	R/128	R	S	R/>256	S	R/>256	
123	232	161	NON-GI +	R/>256	R	S	R/256	S	R/256	
124	233	161	NON-GI +	R/256	R	S	R/256	S	R/128	
125	234	161	NON-GI +	R/256	R	S	R/>256	S	R/256	
126	237	170	NON-GI +	R/128	R	S	R/>256	S	R/256	
127	238	170	NON-GI +	R/128	R	S	R/>256	S	R/256	
128	239	170	NON-GI +	R/>256	R	S	R/>256	S	R/256	→
129	240	171	NON-GI +	R/128	R	S	R/>256	S	R/256	
130	241	171	NON-GI +	R/256	R	S	R/>256	S	R/256	
131	242	171	NON-GI +	R/128	R	S	R/>256	S	R/256	
132	243	172	NON-GI +	R/128	R	S	R/>256	S	R/256	
133	244	172	NON-GI +	R/256	R	S	R/>256	S	R/256	
134	245	172	NON-GI +	R/128	R	S	R/>256	S	R/>256	
135	252	175	NON-GI +	R/128	S	S	R/>256	S	R/256	
136	253	175	NON-GI +	R/256	R	S	R/>256	S	R/>256	
137	254	175	NON-GI +	R/128	R	S	R/>256	S	R/>256	
138	235	169	NON-GI +	R/128	S	S	S	S	R/>256	

Appendix 5

no	Tri MICsug/ml	Nal	Cip MICsug/ml	MDR	Str	Spe	Sul	Gen	RESISTANT GENES IDENTIFIED	Conjugation exp.
119	R/>256	R	R/8	MDR	®	®	®	®	*dfr7-17,tetA,tem,catI*	
120	R/>256	R	R/4	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
121	R/>256	R	R/16	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
122	R/>256	R	R/4	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
123	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem,catI*	
124	R/>256	S	S	MDR	®	®	®	®	*dfr1,dfr12,tetA,tem,catI*	
125	R/>256	R	R/4	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
126	R/>256	R	R/2	MDR	®	®	®	®	*dfr7-17,tetA,tem*	
127	R/>256	R	R/16	MDR	®	®	®	®	*dfr7-17,tetA,tem*	
128	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem*	
129	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
130	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
131	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
132	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
133	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
134	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
135	R/>256	R	R/8	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
136	R/>256	R	R/16	MDR	®	®	®	S	*dfr7-17,tetA,tem,catI*	
137	R/>256	R	R/8	MDR	®	®	®	S	*dfr12,dfr7-17,tetA,tem,catI*	
138	R/>256	R	S	MDR	®	S	®	S	*dfr7-17,tem*	