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An Assessment of Non-Antibiotic Approaches to Mastitis Control in the Dry Period and Their Impact on Intramammary Infection Dynamics.

by

Jonathan Neil Huxley

A Dissertation Submitted to the University of Bristol in Accordance with the Requirements of the Degree of Doctor of Philosophy (PhD) in the Faculty of Medicine.

Department of Clinical Veterinary Science, September 2002.

Word Count 50,784.

ABSTRACT

Mastitis is an important disease causing health, productivity and welfare problems in dairy cattle. Over the past 40 years, control programs have successfully revolved around prophylactic and therapeutic antibiotic usage. This has led to a substantial decrease in the prevalence of subclinical mastitis and the suggestion that antibiotic prophylaxis should be reduced.

This thesis describes a study that compared the efficacy of a non-antibiotic internal teat sealer containing bismuth subnitrate to antibiotic dry cow therapy (DCT). In cows uninfected at drying off, the teat sealer was significantly better than antibiotic DCT at preventing new dry period intra-mammary infections caused by *Escherichia coli*, all *Enterobacteriaceae* species and all major pathogens. Animals that received antibiotic DCT suffered numerically more cases of clinical mastitis during the dry period and next lactation. Significantly fewer *Corynebacterium bovis* intra-mammary infections (IMI) were cured in the teat sealer group during the dry period.

A novel method of speciation based on endonuclease restriction analysis of the 16S rRNA gene sequence was successfully developed and utilized to differentiate *C. bovis* for other *Corynebacterium* species. A novel lipophilic *Corynebacterium* species, named "*C. langfordii*", was identified, typed by 16S rRNA gene sequencing and described.

Re-analysis of the study database demonstrated for the first time that quarters infected with *C. bovis* during the dry period were significantly less likely to acquire a major pathogen IMI. It appeared that IMI with *C. bovis* "protected" quarters from infection with mastitis pathogens.

In vitro studies on solid and in liquid media demonstrated that metabolic products of *C. bovis* could inhibit the growth of some mastitis pathogens. The inhibitory factor was partially and almost completely inactivated by heating to 100°C and treatment with Proteinase K respectively. Production of an inhibitory factor (possibly a bacteriocin) is proposed as one explanation for the protective effect demonstrated *in vivo*.

"If I have seen further it is by standing on the shoulders of giants".

Sir Isaac Newton, 1675.

"The great and inherited development of the udders of cows in countries where they are habitually milked, in comparison with the state of these organs in other countries, is another instance of the effect of use".

Charles Darwin, "The Origin of Species", 1859.

ACKNOWLEDGEMENTS

I would like to thank Eithne Comerford for convincing me that a PhD was the right move and Dr. Chris Helps and Martin Green for their expert technical assistance.

The teat sealer study was made possible with the help and support of the farmers and farm staff on sixteen dairy farms in the South West of England. Their constant warm welcome and enthusiasm for the product and the concept kept me going when things were difficult.

Sincere thanks go to Dr. Andrew Bradley and Professor Phillip Dufus for their guidance and supervision throughout.

I will always remain eternally grateful to my parents David and Gill for providing me with a truly happy, balanced and secure childhood. Nothing was ever too much trouble and where appropriate they allowed me to learn by my own mistakes, which taught me the invaluable skill of trusting my own judgment.

My enthusiasm for and everything I learnt about cattle and how to be around them was acquired from Dad, an "expert" dairyman and farmer.

I would like to acknowledge the financial support of the Cross Vetpharm Group, Dublin who gave me free hand to explore this engrossing and under investigated area of veterinary science.

Last but by no means least, Helen, my wife. Thanks Hel for your support from inception to these rather difficult last few months, all my love.

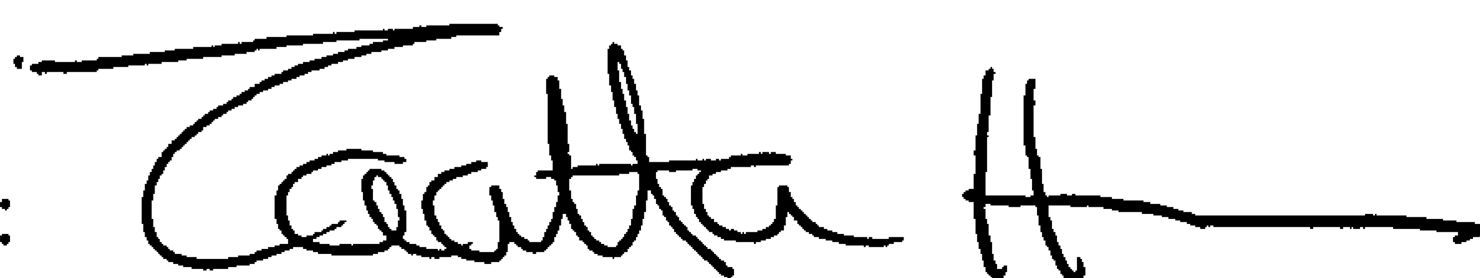
August 2002

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulation of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

Signed:  Date: 17/12/02

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LIST OF ABBREVIATIONS

BMSCC	bulk milk somatic cell count
bp	base pairs
CI	confidence interval
DCT	dry cow therapy
DNA	deoxyribonucleic acid
dNTP	2'deoxyribonucleoside 5'-triphosphate
EDTA	Ethylene diamine tetra acetic acid
EU	European union
Ig	immunoglobulin
IMI	intramammary infection
LRS	likelihood ratio statistic
MMB	milk marketing board
MW	molecular weight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear neutrophils
PMTD	post milking teat disinfection
ppm	parts per million
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SCC	somatic cell count
SE	standard error
sp.	species (singular)
spp.	species (plural)
subsp.	subspecies
TAE	tris-acetate buffer
Taq	*Thermux aquaticus*
Tris	hydroxymethylmethylamine
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER 1: INTRODUCTION.

1.1. BOVINE MASTITIS – A BACKGROUND TO THE UK SITUATION

1.1.1. Definitions

Mastitis can be defined as inflammation of the mammary gland. The word is derived from the Greek, "mastos" ("breast") and "itis" ("inflammation").

Clinical mastitis is inflammation of the mammary gland causing clinically visible signs *e.g.* milk clots, udder heat, pain or swelling. **Subclinical mastitis** is inflammation of the mammary gland detectable only by means other than visible signs *e.g.* positive milk culture, elevation of somatic cell count (SCC, a measure of the nucleated cells, principally leucocytes present in milk), changes in milk conductivity (Chamings *et al.* 1984; Hillerton and Walton 1991), pH (Holdaway *et al.* 1996) enzyme levels (Holdaway *et al.* 1996), composition (Holdaway *et al.* 1996) or acute phase proteins (Eckersall *et al.* 2001).

1.1.2. Aetiology

Bovine mastitis can be caused by bacteria, yeasts, fungi, mycoplasmas, algae and viruses. Other aetiologies include external physical injury and the presence within the gland of foreign bodies or irritant substances. In the UK, bacterial causes of mastitis predominate; over 130 microbial species have been isolated from the bovine mammary gland (Watts 1988). Bacterial causes of mastitis have classically been divided into two categories, contagious and environmental. A third category of mastitis caused by "minor" pathogens is recognized but often not reported because of their limited clinical significance.

1.1.2.1. "Contagious" Mastitis

Contagious mastitis pathogens are adapted to live within the bovine mammary gland *e.g. Streptococcus agalactiae* and *Staphylococcus aureus.* They classically cause chronic intramammary infections (IMI), characterised by persistent high somatic cell counts and intermittent low-grade clinical mastitis. The udder of infected animals is the principle reservoir of infection *e.g. S. agalactiae*, although *S. aureus* can also be

isolated from the skin of the teat and udder and from other sites around the cow. Transmission between cows occurs mainly during the milking process when infected milk from carrier animals comes into contact with the teats of other cows. Once established *S. aureus* IMI can be very difficult to cure (bacteriologically) because the organism is highly resistant to intramammary antimicrobial therapy (Tyler and Baggot 1992).

1.1.2.2. "Environmental" Mastitis

Environmental mastitis is caused by organisms present within the environment, which opportunistically invade the mammary gland and cause disease *e.g. Streptococcus uberis* and members of the genus *Enterobacteriaceae* especially *Escherichia coli.* It is associated with acute bouts of clinical mastitis with transient but not prolonged elevations in SCC.

E. coli can cause per acute peri-parturient clinical mastitis leading to systemic illness, severe udder damage, toxaemia and in the worst cases death. Classically chronic infection was not thought to play an important role in disease, although recent work has demonstrated that it can occur (Bradley and Green 2000).

S. uberis, although principally considered to have an environmental origin (especially from deep littered loose housing), is increasingly being recognised as a cause of chronic IMI which can be difficult to cure bacteriologically.

1.1.2.3. "Minor Pathogens"

Minor pathogens *e.g. Corynebacterium bovis* and the coagulase negative *Staphylococci* usually cause a low-grade subclinical mastitis with mild elevation of SCC; they are rarely associated with clinical disease. *C. bovis* behaves as a contagious pathogen, spreading from cow to cow during the milking process. It is probably the most infectious organism of the bovine mammary gland (Pankey *et al.* 1985). There is evidence to suggest that quarters infected with minor pathogens may be less prone to IMI with other pathogenic bacteria (Section 1.3.8.).

1.1.3. Economic Considerations

Mastitis, along with lameness and infertility remains among the top three diseases causing health, productivity and welfare problems in UK dairy cattle. Booth estimated

that mastitis alone costs the UK dairy industry £83 million per year with an additional cost of £10 million in antibiotic and cell count penalties (Booth 1997). More recently the costs of a mild, severe and fatal case of clinical mastitis have been estimated at £113, £332 and £1418 respectively, equating to a cost for an average case of £137 (Kossaibati 2000). The size of the UK national dairy herd and the mean annual incidence of clinical mastitis have recently been estimated at 2.35 million cows (Brigstocke 2000) and 41.6 cases per 100 cows per year (Bradley and Green 2001). At a cost per average case of £137, this equates to a current cost to the industry of £134 million for clinical mastitis alone.

1.1.4. Historical Perspective

The UK dairy industry has undergone huge changes since the end of the World War II, which was the catalyst for the widespread introduction of "industrial" dairy farming and the move away from self sufficient holdings and small scale milk production. In the 1940s the average herd size was approximately 15 cows (Booth 1997), rising to 30 in 1970 and 71 in 1995 (Anon 2002). In 1973 the average dairy cow produced 3975l of milk. By 2000 this had reached an average of 5915l (Anon 2002). Over a similar time period the number of registered milk producers in the UK has fallen from over 100,000 in 1970, to the current figure of less than 30,000 (Anon 2002). The UK dairy industry is continuing to undergo a prolonged period of consolidation; cows in larger herds on fewer farms are producing more milk and yet overall UK production has remained relatively stable at between 14 and 15 million litres per year (Anon 2002).

1.1.5. Incidence and Aetiology of Clinical Mastitis

The incidence and aetiology of clinical mastitis has changed substantially over the last 50 years. Based on interpretation of the literature, Booth (1997) estimated that the annual incidence was approximately 140 and 74 cases per 100 cows per year in the 1940s and 1977 respectively. In 1980, 1981 & 1982, the incidence was reported to be 54.6, 49.8 & 41.2 cases per 100 cows per year (Wilesmith *et al.* 1986). More recently the incidence was calculated at 43.4 (Kossaibati *et al.* 1998) and 41.6 (Bradley and

Green 2001) cases per 100 cows per year between 1994 and 1996 and 1997 and 1998 respectively.

The fall in the incidence of clinical disease has also been accompanied by changes in mastitis aetiology (Table 1.1). Implementation of the five-point plan (Section 1.1.7.1) has led to a marked decrease in clinical mastitis caused by contagious bacteria, particularly *S. aureus* and *S. agalactiae* and to a lesser extent *S. dysgalactiae*. Consequently, the most frequently reported causes of clinical mastitis at present are the environmental organisms *i.e. E. coli* and *S. uberis*, which tend to be poorly controlled by management strategies such as the five point plan (at the time the plan was developed the prevalence of these organisms was low, so it was not designed with these pathogens in mind).

Table 1.1: Causes of Clinical Mastitis in the UK National Herd by Proportion of Diagnoses, between 1942 and 2002.

Causal Pathogen	1942-43[1]	1960[2]	1975-78[3]	1978[4]	1982[5]	1996[6]	1997-98[7]	1999-2002[8]
S. aureus	17	22	29	16	14.8	20	5.3	3
S. dysgalactiae	10	9	}	11	8.9	8	4.7	4
S. agalactiae	44	4	} 18	5	1.4	4	0	0
S. uberis	4	12	}	15	16.9	19	12.8	37
E. coli / Coliforms	2	10	11	20	18.3	26	40.9	23
Minor pathogens					4.3		6.0	
Mixed Growth			13		5.0		6.5	
Contaminated			6		8.4		0.9	
No Growth			18		14.9		15.1	14

[1](Jones 1998), [2](Jones 1998), [3](Pearson and Mackie 1979), [4](Jones 1998), [5](Wilesmith et al. 1986), [6](Jones 1998), [7](Bradley and Green 2001), [8](Milne et al. 2002).

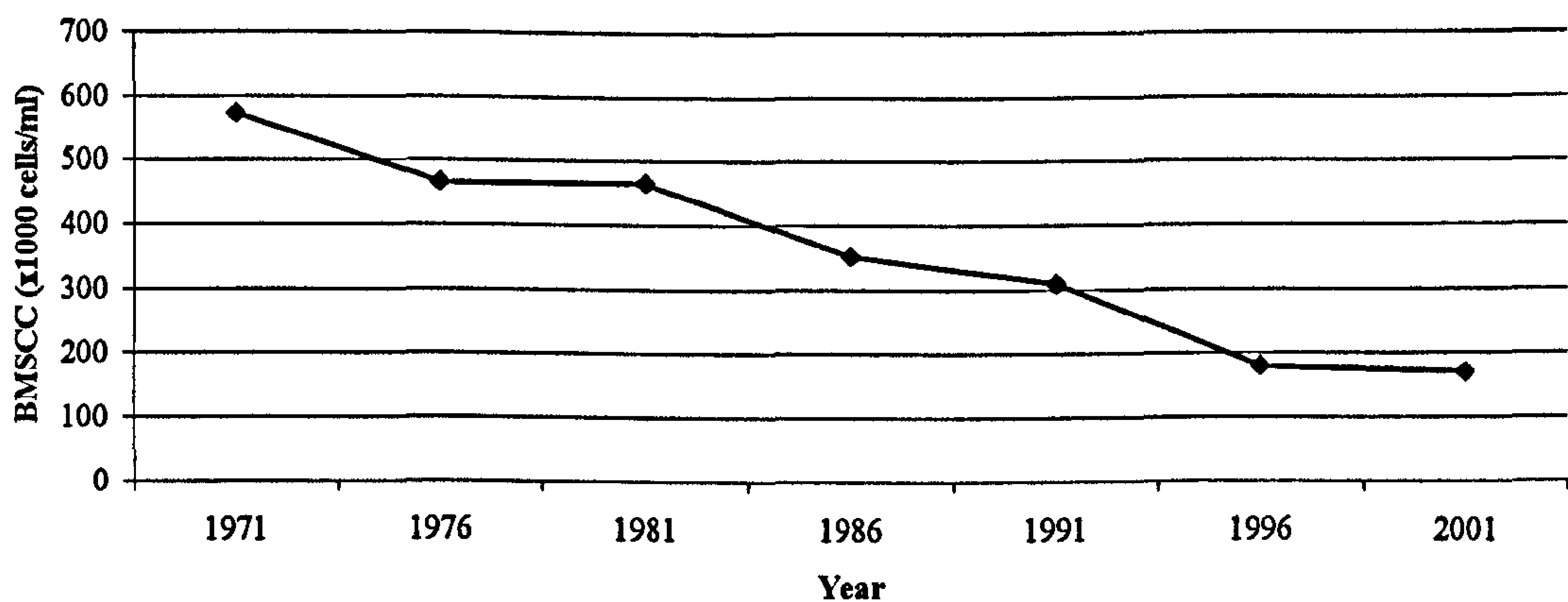
1.1.6. Bulk Milk Somatic Cell Count (BMSCC)

SCC is elevated in the milk of infected quarters; the degree of elevation is dependant on the causal organism and the duration of infection. The SCC of bulk tank milk is representative of all cows being milked at that time and is therefore indicative of the prevalence of subclinical IMI present within those animals. Subclinical IMI are

caused predominantly by the contagious organisms and thus BMSCC can be used as a guide to the level of subclinical contagious mastitis present within a herd.

The Milk Marketing Board (MMB) began monitoring the mean BMSCC of the national herd in 1971. The BMSCC of the national herd has been falling gradually since that time (Figure 1.1)

Figure 1.1: BMSCC of the UK National Dairy Herd (1971 – Present)



(1971 – 1996 (Booth 1988; Booth 1997); 2001,National Milk Records, Personal Communication)*
** A subset of the national herd which record SCC with National Milk Records only.*

1.1.7. Reasons for Change

The reasons for these changes in incidence and aetiology over the last 40 years are many and varied but the three principal driving forces are outlined below. The end result of these changes has been a reduction in the prevalence of contagious mastitis and consequently a dramatic reduction in the number of cows with IMI at the end of lactation.

1.1.7.1. The Five Point Plan

The "Five Point Plan" was developed in the late 1960s at the National Institute for Research in Dairying, Reading and the Central Veterinary Laboratory, Weybridge (Neave *et al.* 1966; Neave *et al.* 1969; Kingwill *et al.* 1970). The plan became the blue print worldwide for the control of clinical and subclinical contagious mastitis and has remained so to the present day.

The five points of the plan and their role in the control of contagious mastitis are:

- Post milking teat disinfection (PMTD) – Dipping or spraying teats in / with disinfectant solutions at the end of milking kills pathogenic bacteria (principally contagious organisms transferred from infected animals) left on the teat skin and distal streak canal by the milking process.
- Prompt treatment of clinical cases – Rapid treatment and cure of clinical cases minimises the degree of environmental and milking machine contamination by pathogenic bacteria, thereby decreasing the challenge to other uninfected cows.
- Regular milking machine maintenance – The milking machine is one of the key vectors for the cow to cow spread of contagious bacteria and can be responsible for propelling bacteria through the teat sphincter. Regular maintenance limits its effect by ensuring optimum function.
- Cull chronically infected cows – Chronically infected cows contaminate the environment and milking machine during intermittent periods of bacterial shedding in milk. Culling them decreases the level of challenge to other members of the herd.
- Routine treatment of all cows with antibiotic dry cow therapy – Cures existing IMI present at the end of lactation and prevents the acquisition of new IMI during the dry period.

1.1.7.2. Deregulation of the UK Milk Buying Market

Historically, all UK milk was sold and marketed centrally through a monopoly operator, the Milk Marketing Board (MMB). In November 1994 the MMB was disbanded allowing a number of companies to operate in the market place. Milk buyers are free to impose penalties and/or bonuses for producers who can meet specific quality and production targets. Currently many milk buyers in the UK pay bonuses to farmers who can produce milk with a BMSCC of below 150,000 cells/ml; financial incentives such as these have further increased the pressure on the UK dairy industry to control and eliminate subclinical contagious mastitis.

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1.1.7.3. European Union Milk Hygiene Directive 92/46

European Union (EU) Milk Hygiene Directive 92/46 came into force in the UK in May 1995. This new legislation made it illegal to sell bulk milk with a three month geometric mean SCC of greater than 400,000 cells/ml, for human consumption. The legislation was designed to standardise and rationalise the quality of EU milk production. It had the side effect of effectively creating a ceiling on the level of subclinical contagious mastitis a herd could have and still legally supply milk for human consumption.

1.1.8. The Dry Period

The dry period is the six to ten week interval between the last milking at the end of lactation and the first milking after the next calving, when milk is not removed from the mammary gland. The risk of acquiring a new IMI (particularly environmental infections) during this period is high (Oliver and Mitchell 1983; Smith *et al.* 1985; Todhunter *et al.* 1991; Bradley and Green 2000). The periods of highest risk are the first trimester (during the involution period from milk production to "steady state") and the last trimester (during the transition period from "steady state" to colostrogenesis and calving) (Smith *et al.* 1985). The second trimester (steady state) of the dry period is particularly resistant to new IMI because the concentration of leucocytes and non-specific factors such as lactoferrin increase as the secretion volume within the udder drops and a keratin plug forms in the teat sphincter preventing the ingress of bacteria.

Recently it has been demonstrated that coliform organisms acquired in the late dry period can lay quiescent within the gland and recrudesce to cause clinical mastitis many days into the next lactation (Bradley and Green 2000). Preventing the acquisition of new infections during the dry period (especially the late dry period) can therefore impact on the number of cases of clinical mastitis during the next lactation.

1.1.9. Udder Defence Mechanisms During the Dry Period

With the exception of anatomical defence mechanisms, specifically the keratin plug, mammary immunology is not a focus of the work in this thesis. A brief outline of mammary immunology with specific reference to the dry period is outlined below, for

a more detailed description of mammary immunology and its function during the dry period the reader is referred elsewhere (Nickerson 1985; Nickerson 1989; Sordillo *et al.* 1997; Kehrli and Harp 2001)

1.1.9.1. Anatomical Defences – The Keratin Plug

During the dry period, the mammary gland is separated from the external environment by the formation of a keratin "plug" which seals the teat sphincter at the end of lactation. Keratin contains long chain fatty acids, which can inhibit the growth of mastitis pathogens (Hogan *et al.* 1987).

A functional keratin plug was present within 16 days of drying off in one study (Comalli *et al.* 1984). More recently however, it has been reported that approximately 50% and five percent of teats were still 'open' (had an 'inadequate' keratin plug) after seven and fifty days of the dry period, respectively. Furthermore, 97% of clinical dry period IMI occurred in 'open' quarters (Williamson *et al.* 1995).

Nickerson reports a study in which quarters were treated with DCT by either full (syringe cannula fully inserted through teat sphincter) or partial (syringe cannula inserted into the first one or two millimetres of the teat sphincter) insertion of the tube cannula. Quarters treated by full insertion acquired significantly more dry period IMI. The author postulated that artificial dilation of the teat sphincter and removal of keratin decreases resistance to IMI (Nickerson 1987). Also of note is the finding of Williamson *et al* (1995), which demonstrated that cows treated with antibiotic DCT had significantly more quarters with a functional keratin plug during the first four weeks of the dry period compared to cows that received no treatment. The authors postulate that antibiotic DCT may be killing bacteria that prevent the formation of the keratin plug (possibly by producing lipolytic enzymes) which are normally present within the teat sphincter.

Increasing the susceptibility of quarters to new infection by removal of keratin has also been demonstrated during lactation (Capuco *et al.* 1992). It is likely that complete occlusion of the teat sphincter during the dry period with keratin is an important udder defence mechanism. Once the keratin plug has formed, it prevents the ingress of bacteria into the udder and appears to be a highly efficient method of preventing dry period IMI.

1.1.9.2. Innate Soluble Defences

Lactoferrin is a major component of mammary secretions during the dry period. Lactoferrin act bacteriostatically by binds iron making it unavailable for bacterial growth. Its effects are most pronounced against *E. coli*; some *S. aureus*, *S. uberis* and *S. agalactiae* isolates are not inhibited (Rainard 1986). During lactation the activity of lactoferrin is limited because it becomes diluted in milk, this is not the case during the dry period, consequently concentration and hence activity are elevated. Also during the dry period, levels of bicarbonate are higher, which promotes activity (Nickerson 1985), and levels of citrate are lower. Citrate competes with lactoferrin for iron binding, when citrate levels are lower lactoferrin activity increases (Sordillo *et al.* 1987). During colostrogenesis, lactoferrin levels drop and citrate levels rise reducing lactoferrins bacteriostatic effects.

Complement is a collection of immunologically active proteins that are thought to aid in causing bacterial lysis. Levels of complement are low during lactation, but elevated during IMI, the dry period and colostrogenesis (Sordillo *et al.* 1997).

The role of other soluble defenses such as lysozyme and the lactoperoxidase/thiocyanate/H_2O_2 in the bovine udder remains unclear; levels are likely to be elevated during the dry period (Sordillo *et al.* 1997).

1.1.9.3. Cellular Defence Mechanisms

The principle leucocytes present in milk are polymorphonuclear neutrophils (PMN) and macrophages. The most numerous cells in the health gland are macrophages, where as PMNs predominate if the gland becomes infected (Nickerson 1985). The concentration of leucocytes present within the gland rises during the dry period (Nickerson 1985), principally because they are not diluted by milk production. The mammary environment is also more conducive to leucocyte function at this time because the concentration of fat and casein drops (Sordillo and Nickerson 1988).

Lymphocytes are composed of two main classes; T lymphocytes are principally responsible for regulating other immune cells and function and B lymphocytes are responsible for producing antibody in response to antigen presented to them. The numbers of B lymphocytes increases in secretion from the non-lactating gland and in colostrum (Nickerson 1985).

1.1.9.4. Specific Soluble Defences

Immunoglobulins (Ig) produced by antigen activated B lymphocytes make up the specific soluble defense mechanisms. Four classes are active within the mammary gland: IgG_1, IgG_2, IgA and IgM; IgG_1, IgG_2 and IgM act as bacterial opsins and IgA aids bacterial agglutination, prevents multiplication and neutralizes toxin (Sordillo *et al.* 1997). During lactation, levels of Ig are low in the healthy gland, however levels are elevated during IMI. During the dry period, levels of Ig slowly increase reaching a peak during colostrogenesis (Sordillo *et al.* 1997).

1.1.10. Summary

The period since the end of World War II has seen huge changes in the structure and management of dairy farming in the UK. The introduction of control plans, deregulation of milk buying and EU regulations have led to a substantial drop in the level of subclinical and clinical contagious mastitis present within the national herd. Environmental mastitis pathogens (particularly *E. coli*) have been less well controlled; they have become relatively more important as the prevalence of contagious mastitis has fallen. The mammary gland is particularly prone to infection during the first and last trimesters of the dry period.

1.2. DRY COW THERAPY

1.2.1. Dry Cow Therapy - Background

Intramammary infusion of penicillin based antibiotic "pastes" for the treatment of clinical mastitis was described as long ago as 1946 (Edwards and Brownlee 1946). In the late 1940's the first successful use of intramammary antibiotics formulations in "oily" bases for the protection of dry cows against IMI with *Arcanobacterium pyogenes* were described (Pearson 1950; Pearson 1951).

Slow release long acting antibiotic dry cow preparations infused aseptically into the quarter at drying off went on to became a corner stone of the Five Point Plan.

Antibiotic dry cow therapy has two principle goals:

- The cure of existing IMI present within the gland at the time of drying off, by exposing the causal bacteria to levels of antibiotic above the minimum inhibitory concentration of that pathogen for a prolonged period.
- Preventing the acquisition of new IMI during the dry period by exposing invading bacteria to an antibiotic environment too hostile to allow colonization of the gland.

1.2.2. Dry Cow Therapy – The Current UK Situation

Antibiotic dry cow therapy (DCT) is designed for aseptic infusion into the udder, after the last milking, at the end of lactation. It is formulated to release antibiotic both rapidly and then slowly to maintain levels for prolonged periods. As the prevalence of contagious mastitis has fallen, the number of quarters infected at drying off has been reduced dramatically in most herds; in these cows the only role for DCT is to prevent the acquisition of new IMI during the dry period.

Antibiotic DCT was originally developed to control contagious mastitis which was the predominant problem at that time, consequently its action has largely been targeted against Gram-positive bacteria. Action against Gram-negative pathogens has been considered a beneficial side effect of the incorporation of some antibiotics into the formulation, rather than a design prerequisite. As previously discussed (Section 1.1.8.) preventing the acquisition of Gram-negative infections during the late dry period could reduce the number of cases of clinical mastitis during the next lactation, however currently few antibiotic dry cow tubes provide adequate protection against Gram-negative infection during this period.

Recently, there has been pressure to reduce antibiotic usage in food animal production systems because of worries over the development of antibiotic resistance in human pathogens. Additionally, consumer interest in organic farming systems has led to renewed interest in management styles that reduce antibiotic usage. The use of "prophylactic" antibiotic treatments has come under particular scrutiny. In the past, antibiotic DCT was justified (as a treatment), because of the high prevalence of IMI present on most farms. As previously discussed this is no longer the case in many herds. The rationale for infusing large doses of long acting antibiotics into cows not

carrying IMI is becoming increasingly questionable and only justifiable if no alternatives for preventing the acquisition of new dry period IMI are available.

1.2.3. Alternatives to Antibiotic DCT - Teat Sealants

The keratin plug (Section 1.1.9.1.) can take a number of weeks to form and in some animals it never forms. Comalli *et al* (1984) demonstrated that the teat canal lumen cross sectional area was significantly greater on day seven after drying off because of a reduction in thickness of the epithelium and stratum granulosum layers. The keratin plug then forms to fill the teat sphincter. Teat sealants that mimic the functions of the keratin plug (especially prior to its formation at drying off and after its breakdown during colostrogenesis) could represent an alternative to antibiotic DCT (for preventing the acquisition of new dry period IMI) if they can limit the entry of mastitis pathogens during the dry period.

Materials designed to "seal" the teat from the external environment have been used for many years. Anecdotally many substances have been tried including grease, Stockholm tar, impervious tapes and spray on "artificial skins" developed for human medicine. The problem with all these methods has been an inability to provide adequate coverage and persistence because of the constant abrasions of the teats between the hind legs and the ground when lying and during rising to standing. Two products, one an internal and one an external teat sealant have however shown some promise.

1.2.3.1. External Teat Sealants

External seals based on liquid acrylic latex applied as a post milking dip that dried to form a film on the teats of milking cows was investigated in the late 1970s. When applied after milking to two teats only, a significant reduction in the *S. aureus* and coliform new IMI rate was demonstrated compared to negative control quarters (Farnsworth *et al.* 1980). The dip was shown to reduce bacterial contamination of the teat by 90%; bacterial growth under the seal was inhibited compared to non-dipped control animals (Farnsworth *et al.* 1981). The ability of the product to prevent new IMI during the dry period was also assessed. It was applied daily during the first and last weeks of the dry period to two teats only. All trial cows also received antibiotic dry cow therapy. No difference in the number of new dry period coliform,

staphylococcal or streptococcal IMI was observed (McArthur *et al.* 1984). Long-term persistence of aqueous based latex product was a problem, necessitating frequent re-application.

The persistence of external sealants was recently improved for dry period use following the development of a product containing a polymer in a solvent base. In mid dry period cows, average teat end cover was demonstrated for 8.5 days for the solvent-based product versus 4.1 days for the original seal (Hemling *et al.* 2000). In another study mean duration of teat end cover was 6.3 ± 0.1 days on mid dry period cows and late gestation heifers. The duration of cover was increased on long compared to short teats (7.0 ± 0.4 *cf.* 6.0 ± 0.2) and on teats with raised and rough teat end lesions (10.1 ± 0.9 days) (Leslie *et al.* 1999). Recent work from the UK has demonstrated that applying the sealant at lower temperatures leads to longer persistence. Sealant applied at 2°C (8.0 days) stayed on significantly longer than that applied at 10°C (6.9 days), 20°C (6.8 days) or 30°C (6.4 days) (Creasey *et al.* 2002).

Compared to antibiotic DCT alone, when applied at drying off and one week pre-calving in combination with antibiotic DCT, the solvent based polymer sealant was shown to reduce the number of new dry period IMI caused by *S. aureus*, environmental *Streptococci* and coliforms (Lim *et al.* 2000) and major pathogens, environmental *Streptococci* and coagulase negative *Staphylococci* (Timms 2000).

In another trial employing a split udder design, quarters were randomly assigned to one of four treatment groups: antibiotic DCT; polymer sealant; antibiotic DCT plus polymer sealant; no treatment. When sampled two weeks after drying off quarters that only received polymer sealant had significantly fewer IMI caused by coagulase negative *Staphylococci*, major pathogens and all pathogens, compared to the no treatment group (Timms 2000). There were no statistically significant differences in the number of IMI in the groups that received antibiotic DCT, polymer sealant or antibiotic DCT plus sealant.

The solvent-based polymer seal used in these studies was released without efficacy data onto the UK market during 1998 (Dry*Flex*, Delaval). It is recommended as an adjunct to antibiotic DCT, for application at drying off and ten days before the predicted calving date in an attempt to ensure teat end coverage during the two risk periods. Data sheet recommendations suggest that pre-calving cows should be observed daily and re-dipped as necessary if coverage with the seal is inadequate prior

to calving. Provisional data from one recently completed UK study on two farms in South West England using this product suggests that the sealant does protect quarters against infection with major pathogens (compared to a no treatment control group) providing that the sealant is properly used and adequate teat coverage is maintained after drying off and before calving (A. J. Bradley, Personal Communication, 2002).

The application protocol advocated for the external teat sealant available in the UK is complex and time consuming. Using the external sealant will increase costs further as it is currently only recommended as an adjunct to antibiotic DCT, which is already considered expensive by many UK dairy farmers (Personal Observation).

1.2.3.2. Internal Teat Sealers

Development and evaluation of an internal "teat sealer" based on 25% bismuth subnitrate (Section 1.2.3.3.) in a paraffin/wax base began in Ireland in 1972. Infused into quarters at drying off, it was designed to sink to the base of the teat and cover the internal aspect of the teat sphincter, preventing new IMI by acting as a physical barrier to the ingress of bacteria. A study employing a split udder design exposed teats to solutions containing *S. aureus* and *S. dysgalactiae* by weekly challenge dipping. The seal was shown to significantly ($P < 0.005$) reduce the number of new IMI acquired during the dry period, compared to negative (no treatment) control quarters (Meaney 1976; Meaney 1977). Using a similar experimental design, on a small number of cows (25), it was demonstrated that the internal teat seal was as good as a long acting cloxicillin based antibiotic dry cow tube at preventing new IMI during the dry period (Meaney 1993).

Work continued on this teat sealer formulation but only in combination with a short acting antibiotic infused into the quarter immediately before the teat sealer (Meaney 1993). A dry cow product (Osmonds Teatseal, Cross Vetpharm Group; 600mg cloxicillin in an aqueous base followed by 37% bismuth subnitrate in a 7g tube) based on this work has been commercially available in Ireland since the 1970s. At the time of the original development little interest was expressed in non-antibiotic dry cow preparations because of the rapidly expanding interest and market for long acting antibiotic formulations (Meaney 2000).

The internal sealer was reformulated (65% bismuth subnitrate with Alugel 30 DF and heavy liquid paraffin) and evaluated under New Zealand field conditions during 1996.

528 cows uninfected at drying off were selected for inclusion in the trial based on last recorded cell count (<200,000/ml) and negative bacteriological examination (duplicate samples in the last week of lactation). Employing a split udder design quarters were randomly assigned to receive teat sealer, teat sealer plus a cloxicillin dry cow tube, a cephalonium dry cow tube or no treatment (negative control). Compared to negative control quarters, quarters in each of the three treatment groups had significantly fewer clinical IMI during the dry period, fewer new IMI at calving and fewer cases of clinical mastitis during the first five months of the next lactation. Differences among the three treatment groups were not significant (Woolford *et al.* 1997; Woolford *et al.* 1998).

Lacticin, a broad-spectrum bactericidal bacteriocin produced by *Lactococcus lactis* subspecies *lactis,* has recently been added to the teat sealer and its efficacy assessed *in vitro* and *in vivo.* Using a plate inhibition test, teat sealer plus lacticin inhibited the growth of *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *S. aureus in vitro* (Ryan *et al.* 1999). *In vivo,* quarters were infused with either a teat sealer alone or a teat sealer plus lacticin and challenge inoculated with *S. dysgalactiae* directly into the teat sinus. Quarters receiving the teat sealer plus lacticin acquired significantly fewer IMI caused by the test organism (61% *cf.* 6%) (Ryan *et al.* 1999). The addition of non antibiotic naturally occurring protein bacteriocins which are broken down in the stomach if consumed represents an extremely interesting and potentially very useful addition to the teat sealer already in use.

1.2.3.3. Bismuth Subnitrate

Bismuth in its pure form is a silver white heavy metal with atomic number 83. It was discovered in 1450 and occurs naturally as a free element, bismuth oxide, bismuth sulfide and in lead ores. Annual worldwide consumption is approximately 4500 tonnes. Uses include industrial, laboratory, cosmetic and pharmaceutical. Bismuth is non toxic unless absorbed in high quantities. Its relative safety is demonstrated in some of its uses. Bismuth has recently replaces lead as a safer alternative for use in shot gun cartridges, bismuth subsalicylate is a common component of antacids, antidiarrheal and anti gastric ulcer drugs and bismuth compounds are used in the cosmetics industry.

Bismuth subnitrate is a white powder that is poorly absorbed if taken orally. Medical and scientific uses include packing cavities after ear, nose and throat surgery,

the treatment of gastro-intestinal disorders because of it similar properties to bismuth subsalicylate and as an electron microscope stain (Albershein and Killias 1963).

1.2.4. Summary

Teat sealants (especially internal seals) may offer a viable alternative to long acting antibiotic preparations for the prevention of new IMI during the dry period. They will not cure existing IMI and are therefore only of use in quarters not infected at drying off. Two types of seal are currently available, an external polymer seal available in the UK, only recommended as an adjunct to dry cow therapy and an internal seal based on bismuth subnitrate available in New Zealand and Ireland.

1.3. *CORYNEBACTERIUM BOVIS*

1.3.1. Introduction

C. bovis is a member of the genus *Corynebacterium*. The genus was first used to describe the causative agent of the important human disease diphtheria (*C. diphtheriae*) (Aubel *et al.* 1997). The genus name is often considered synonymous with the term coryneform, which is a descriptive term for the microscopic morphology of members of the group. Coryneform organisms are aerobic, asporogenous, non-partially-acid fast, irregularly shaped, Gram-positive rods (Funke *et al.* 1997), which often demonstrate a characteristic "club" shape. Although the genus "*Corynebacterium*" is the most important member of the coryneform grouping it also contains species from many other genera including *Turicella, Arcanobacterium, Actinomyces, Arthobacter, Brevibacterium* and *Dermobacter*. *Corynebacterium* species occupy a broad range of environments; they have been isolated from habitats ranging from soil and plant material, to food, marine, animal and human sources (Collins *et al.* 2001). *C. bovis* is almost exclusively isolated from bovine sources (Funke *et al.* 1997), primarily the mammary gland, although it has been isolated from the preputial skin of bulls (Corbeil *et al.* 1985).

1.3.2. Characteristics

C. bovis belongs to a sub-grouping of the genus whose principal characteristic is that their growth *in vitro* is significantly enhanced by the addition of lipid to synthetic media. These species are referred to as "lipophilic" (Funke *et al.* 1997). On brain heart agar incubated aerobically at 37°C for 48 hours, *C. bovis* produces no or poor growth (tiny, barely visible white/translucent colonies, Figure 3.2). On blood agar incubated under identical conditions, it produces fine (< 1mm) white colonies, but often only after 72 hours growth (Personal Observation). However if like other members of the sub-group, it is grown on media supplemented with Tween 80 (Cobb 1963) (one of a series of detergents that contains a polyethoxysorbate oleate composed of a variety of fatty acids with 14 to 18 carbons (Coyle and Lipsky 1990)) growth is much more luxuriant. After 48 hours aerobic incubation at 37°C on brain heart agar supplemented with one percent Tween 80 v/v, *C. bovis* produces large (approximately two millimetre), round, smooth, slightly convex colonies with a creamy white colour (Figure 3.2). On blood agar supplemented with Tween 80 colonies are similar to those produced on brain heart agar and are non-haemolytic.

All members of the genus are non-motile and catalase positive (Coyle and Lipsky 1990). *C. bovis* is oxidative. Acid is produced from glucose, but not from maltose, sucrose, mannitol or xylose. *C. bovis* does not hydrolyse urea or esculin, nitrate is not reduced. Alkaline phosphatase and β-galactosidase activity is detected; production of pyrazinamidase is variable (Funke *et al.* 1997).

1.3.3. Lipophilic *Corynebacterium* Species

There are currently ten recognized lipophilic *Corynebacterium* species (*C. accolens*, *C. afermentans* subsp. *lipophilum*, *C. bovis*, group F-1, group G-2, *C. jeikeium*, *C. lipophiloflavum*, *C. macginleyi*, *C. mastitidis* and *C. urealyticum*) and three species whose status within the genus has never been confirmed ("*C. genitalium*", "*C. pseudogenitalium*" and "*C. tuberculostericum*").

1.3.4. Location of Infection

The primary site of *C. bovis* infection within the bovine mammary gland is thought to be the teat canal. Large numbers of organisms were demonstrated within the central

5mm section of the teat canal, in close association with the keratin layer, after natural infection (Black *et al.* 1972) and in the distal third of the teat canal following artificial infection (Pankey *et al.* 1985). The numbers of organisms present appears to fall rapidly around Furstenberg's rosette (the proximal end of the teat sphincter) (Black *et al.* 1972; Pankey *et al.* 1985), although *C. bovis* has been demonstrated adhered to epithelial cells in this area (Frost *et al.* 1977). *C. bovis* was not found (Pankey *et al.* 1985) or cultured (Black *et al.* 1972) from epithelial surfaces above Furstenberg's rosette (*i.e.* the teat sinus). However, other authors found inflammatory changes effecting the teat cistern and Furstenberg's rosette of 16 of 19 quarters naturally infected with *C. bovis*. In 13 of these quarters inflammation was also seen in the mammary parenchyma and there were degenerative changes to the ductal and alveolar epithelium (Ngatia *et al.* 1991).

Of quarters which yield growth of *C. bovis* in strip samples, 75% (Pankey *et al.* 1985), 49% (Honkanen-Buzalski and Bramley 1984) and 25% (Black *et al.* 1972) of teat puncture samples (samples collected through the teat wall) were also culture positive, although the number of colony forming units per millilitre of milk were much lower in teat puncture samples.

It appears that the primary site of *C. bovis* infection is the teat canal although infection can exist above Furstenberg's rosette in the teat cistern and higher up the gland. The ability to survive and cause disease above the teat sphincter may be associated with strain differences in pathogenicity.

1.3.5. Pathogenicity

C. bovis is principally considered a pathogen of limited significance within the bovine mammary gland, primarily causing subclinical disease (Black *et al.* 1972; Bramley *et al.* 1976; Honkanen-Buzalski *et al.* 1984). Some authors even consider it should be more properly termed a commensal (Brooks and Barnum 1984). Despite its limited pathogenicity it is highly infectious (Bramley *et al.* 1976). In fact in artificial infection studies it was demonstrated to be more infectious than the classic contagious pathogens *S. aureus* and *S. agalactiae* (Pankey *et al.* 1985). The prevalence of *C. bovis* IMI in 18 cows quarter sampled on a weekly basis, rose from 18.6% in the first week after calving, to 65.5% between weeks two and eight, and peaked at 92.2% in weeks 21 to 24 (Forbes 1970). Interestingly of quarters that became infected only four

self cured during lactation. The author concluded that *C. bovis* is both extremely infectious and able to persist.

C. bovis has occasionally been reported as a cause of opportunistic infections in humans (Vale and Scott 1977) and has recently been described as the etiological agent of hyperkeratotic dermatitis of athymic nude mice (Scanziani *et al.* 1997; Duga *et al.* 1998).

1.3.5.1. Effect on SCC

All bacterial infections of the mammary gland induce a leucocytosis and therefore an elevation of the SCC of the effected quarter. *C. bovis* is classically described as a minor pathogen because disease is largely confined to a subclinical form and the increase in SCC associated with IMI is small. The increase in quarter SCC between *C. bovis* positive and bacteriologically negative quarter has been demonstrated to be 28,000 cells/ml (33,400 cells/ml in *C. bovis* positive quarters *cf.* 5,600 cell/s ml in bacteriologically negative quarters) (Ngatia *et al.* 1991), 38,000 cells/ml (52,481 *cf.* 14,791) (Lam *et al.* 1997), 40,000 cells/ml (119,000 *cf.* 79,000) (LeVan *et al.* 1985), 46,000 cells/ml (171,300 *cf.* 125,400) (Brooks *et al.* 1983), 67,000 cells/ml (151,000 *cf.* 84,000) (Honkanen-Buzalski *et al.* 1984) and 156,000 cells/ml (195,000 *cf.* 38,900) (Erskine *et al.* 1987) for naturally occurring infections. For experimentally induced infections the difference in quarter SCC before and after infection has been demonstrated as 15,000 cells/ml (145,900 *cf.* 130,900) (Brooks and Barnum 1984), 44,000 cells/ml (50,100 *cf.* 6,300) (Sordillo *et al.* 1989) and 109,000 cells/ml (238,900 *cf.* 129,600) (Pankey *et al.* 1985).

The median, arithmetic and geometric means of these previously reported SCC are remarkably consistent for both naturally occurring and experimentally induced IMI (43,000, 63,000 and 52,000 cells/ml versus 44,000, 56,000 and 42,000 cells/ml) and would suggest that the induced leucocytosis, elevates quarter SCC by approximately 50,000 cells/ml.

However, it would appear that the link between quarter prevalence of *C. bovis* (and the associated elevation in quarter SCC) and herd BMSCC are complex. In one UK herd, over a five year period, where the prevalence of *C. bovis* increased from below 20% to greater than 70% because of the failure of an exit race teat spray the herd BMSCC remained remarkable stable at approximately 250,000 cells/ml (Hillerton *et al.* 1995). A recent nine year study in the USA on 893 herds concluded

that for every one percent increase in the prevalence of *C. bovis* infection at the cow level, the BMSCC was elevated by 1,091 cells/ml, after all other factors had been taken into account (Wilson *et al.* 2001).

1.3.5.2. Effect on Level of Production

Quarters subclinically infected with mastitis pathogens or with elevated SCC produce less milk (King 1978; Jones *et al.* 1984) which has lower than normal levels of milk solids (King 1978). Quarters infected with *C. bovis* have a mildly elevated SCC and it is therefore logical to assume that infection will affect overall yield and/or the yield of fat and protein.

A non significant, four percent reduction in daily yield between 53 *C. bovis* infected and contralateral uninfected quarters has been reported although the authors did not find any difference in the composition of fat and protein (LeVan *et al.* 1985). Pociecha (1989) reported a significantly lower milk fat content in cows colonized with *C. bovis*, although other milk constituents were not effected. Conversely, Brooks and Barnum (1984), reported no effect on fat, protein, lactose or daily quarter yield after experimentally induced infection in ten quarters of five cows and no effect on daily yields comparing cows free of IMI to those with at least one naturally infected quarter on 74 Canadian farms (2250 cows) (Brooks *et al.* 1983).

The literature suggests that if quarters infected with *C. bovis* do suffer a reduction in overall yield and concentration of milk constituents, the effect is likely to be small and of limited significance.

1.3.5.3. C. bovis as a Cause of Clinical Disease

C. bovis and *Corynebacterium* species have occasionally been reported as the cause of both individual cases of clinical mastitis and "herd outbreaks" of disease. Over a two year period in one US research herd, Morin and Constable (1998) concluded 22% of clinical mastitis was caused by *Corynebacterium* species. Disease was however significantly milder in cases caused by *Corynebacterium* species compared to those caused by other organisms. Hillerton *et al* (1995) reported an apparent outbreak where up to 50% of clinical mastitis cases over a period of a few months yielded *C. bovis* in pure culture. Cobb and Walley (1962) and Counter (1981) both reported outbreaks of clinical mastitis caused by organisms diagnosed as *C. bovis*. In both cases clinical disease was reproducible after artificial infection of uninfected quarters

with organisms isolated from clinical mastitis cases. More recently, Robinson and Harwood (1998) reported an outbreak associated with an "atypical" *C. bovis* that showed more robust growth on blood agar.

In cases where *C. bovis* is described as a cause of clinical disease most authors discuss the possibility that the coryneforms present in mastitic samples may in fact be an incidental finding in cases where the causal major pathogen has not been isolated. In many herds a high proportion of milk samples from clinically normal animals are coryneform positive, it can be expected that the same proportion of clinical mastitis samples in these herds will also be coryneform positive and thus unrelated to the clinical disease.

1.3.5.4. Variations in Pathogenicity

Anderson *et al* (1985) have previously demonstrated differences in the pathogenicity of two strains of *C. bovis* (one from a clinical and one from a subclinical case of mastitis) in a mouse mammary gland model. Eight days after artificial infection, one out of five glands inoculated with the subclinical strain was infected and none contained abscesses, whereas of those infected with the clinical strain, four of five glands were infected and six of another ten glands contained abscesses. Based on histopathological examination the authors concluded that the ability of *C. bovis* to bind to milk fat globule membranes and thus liberate the essential fatty acid required for growth might be an important determinant of pathogenicity.

Other authors have also considered that the availability of lipid is important for growth determinant. Black and Marshall (1972) demonstrated that the addition of lipid extracted from teat canal keratin to artificially growth media increased the rate of *C. bovis* growth. They concluded that teat canal keratin fulfils the lipid growth requirement of *C. bovis* and thus explains its predominance within the teat canal.

1.3.6. Prevalence of *C. bovis* in Dairy Herds

Data on the prevalence of *C. bovis* in lactating cows from around the world and over the last 30 years prove remarkably consistent and suggest that approximately 20 to 30 percent of quarters are infected at any one time. In the largest study of its type on 74 herds in Canada, Brooks *et al* (1983) concluded the prevalence of *C. bovis* was 19.9%, 36.2% and 85.6% at the quarter, cow and herd levels respectively. Other authors have

demonstrated the quarter prevalence to be 23.2% (three herds) in France (Rainard and Poutrel 1988), 15.7% (three herds) in Australia (Hassan *et al.* 1999), 47.2% (seven herds) in Holland (Lam *et al.* 1997), 13.4% (one herd) in the USA (Hommez *et al.* 1999) and 21% (six herds) in the UK (Robinson *et al.* 1983).

1.3.7. Control of *C. bovis*

Despite the high prevalence of *C. bovis* in many herds its limited pathogenicity has meant that few specific recommendations for its control have been formulated. It is however one of the few truly "contagious" organisms of the bovine mammary gland and as such measures designed to control *S. aureus* and *S. agalactiae* have also proved extremely effective against *C. bovis.*

1.3.7.1. Dry Cow Therapy

Dry cow therapy is an efficient method of eliminating existing *C. bovis* IMI. Cure rates of 67.9% and 65.0% (Pankey *et al.* 1985), 96.3%, 96.0%, 92.6% and 73.8% (Honkanen-Buzalski *et al.* 1984), 100%, 100% and 94.1% (Harmon *et al.* 1986) and 75% (Schukken *et al.* 1993) have been reported with a wide range of dry cow antibiotic formulations.

Comparing Canadian herds that practiced PMTD and DCT to those that only practiced PMTD, Brooks *et al* (1983), showed a reduction in *C. bovis* prevalence at both the quarter (11.5% *cf.* 16.2%) and cow (22.0% *cf.* 31.7%) levels.

1.3.7.2. Post Milking Teat Disinfection

PMTD eliminates any contagious pathogens (including *C. bovis*) from the teat surface and distal teat orifice, which may have been transferred there from infected cows during the milking process. It effectively prevents much of the cow to cow transmission which would otherwise occur.

Lam *et al* (1997) conducted a field study on seven farms in Holland, in which PMTD was discontinued on half the teats of all cows. The prevalence of *C. bovis* IMI was significantly lower at drying off (47.2% *cf.* 63.6%) and calving (26.2% *cf.* 36.81%) in dipped quarters. During lactation, the incidence of new (28.5 *cf.* 50.2 cases per 10,000 quarter days at risk) and duration of existing (186.7 *cf.* 236.8 days) *C. bovis* infections was significantly decreased in dipped quarters. Comparing Canadian

herds that practiced PMTD and DCT to those that only practiced DCT, Brooks *et al* (1983), demonstrated a reduction in prevalence at both the quarter (11.5% *cf.* 35.9%) and cow (22.0% *cf.* 60.7%) levels.

Although it is impossible to isolate the effect of each control measure, Bramley *et al* (1976) investigated the affects of introducing routine DCT and PMTD onto 30 farms with a total of 2000 cows. Within three years the quarter prevalence of *C. bovis* had dropped from 47 to five percent.

1.3.8. Protective Affect

The role of *C. bovis* in IMI dynamics is a contentious one. As long ago as 1972 it was suggested that *C. bovis* may play a role in protecting quarters against subsequent infection with other pathogens (Black *et al.* 1972). Since that time the literature has produced much conflicting data on the subject. Papers can broadly be divided into two categories: studies employing artificial infection models; those based on the study of natural infections under field conditions.

Studies employing an experimental design based on artificial infection models have concluded that quarters infected with *C. bovis* are less likely to become infected with *S. aureus* (Linde *et al.* 1980; Brooks and Barnum 1984; Pankey *et al.* 1985; Schukken *et al.* 1999). However no effect was demonstrated against *S. uberis* (Doane *et al.* 1987) and *S. agalactiae* (Brooks and Barnum 1984). In another study the authors concluded quarters infected with *C. bovis* were actually at increased risk of becoming infected with *S. agalactiae* (Pankey *et al.* 1985).

Studies employing an experimental design based on natural infections have concluded that quarters infected with *C. bovis* were less likely to become infected with *S. uberis* (Lam *et al.* 1997), *S. agalactiae* (Rainard and Poutrel 1988) and all major pathogens (Black *et al.* 1972; Pociecha 1989; Lam *et al.* 1997) and were partially protected against *S. aureus* (Zadoks *et al.* 2001). In other studies no protective effect was demonstrated against *S. aureus* (Viseslava and Vera 1989; Berry and Hillerton 2002) *S. agalactiae* (Viseslava and Vera 1989), *S. uberis* (Zadoks *et al.* 2001; Berry and Hillerton 2002), *E. coli* (Hogan *et al.* 1988; Berry and Hillerton 2002) and major pathogens (Honkanen-Buzalski *et al.* 1984). Conversely, other authors have concluded that quarters infected with *C. bovis* were at significantly greater risk of suffering concurrent infection with environmental Streptococci (Hogan

et al. 1988) and *S. aureus*, *S. agalactiae*, other Streptococci and all major pathogens (Brooks *et al.* 1983).

Recently, Green *et al* (2002) have investigated the effect of infection with *Corynebacterium* species in and around the dry period on the incidence of clinical mastitis. Quarters infected with *Corynebacterium* species in the late dry period or the week following calving were at significantly less risk of suffering clinical mastitis during the next lactation. Interestingly however, quarters infected with *Corynebacterium* species at drying off were at significantly greater risk of suffering clinical mastitis during the next lactation.

Unlike univariate statistical analysis, which compares two variables in isolation, multivariate analysis of data sets allows the comparison of variables whilst taking into account the effects of other confounding factors. It is a much more powerful statistical tool, especially in complex biological situations where the interrelationship between variables is not straightforward. Only two studies in the literature have employed logistic regression analysis; they demonstrated a protective effect against both natural (Zadoks *et al.* 2001) and artificial (Schukken *et al.* 1999) *S. aureus* challenge.

The size of a study in terms of absolute numbers of cows (and quarters) and the number of herds involved can also influence the quality and significance of the results produced. Of the two, an increase in the number of farms included would be widely regarded of great consequence to the power of a study, providing reasonable numbers of cows are recruited from each farm. Results of natural infection studies, in descending order of number of herds (and then cows), from the literature are outlined in Table 1.2.

Drawing definite conclusions based on data from the literature is difficult because of the conflicting nature of the evidence. More studies based on naturally occurring infections demonstrate a protective effect, however the largest of these studies demonstrates a significant increase in risk and the second largest no difference in risk, associated with *C. bovis* IMI. Of the two studies employing logistic regression analysis both demonstrated protection against *S. aureus*. Three of the five studies based on artificial infection models demonstrated a protective effect, one demonstrated no effect and one demonstrated increased risk against *S. agalactiae* and decreased risk against *S. aureus*. On balance the author concludes that the literature is

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suggestive of a protective effect associated with *C. bovis* infection (especially against *S. aureus*) although the evidence is far from conclusive.

Table 1.2: Effect of *C. bovis* Infection on Subsequent Infection with Other Pathogens

Number of Herds	Number of Cows	Effect of *C. bovis* infection of subsequent infection with other pathogens
70	2250	Increase risk of infection with all major pathogens, *S. aureus*, *S. agalactiae* and other Streptococci [1].
55	3700	No effect [2].
7	400	Protective against all major pathogens and *S. uberis* [3].
4	290	No effect [4].
3	200	Partially protective against *S. aureus* [5].
3	122	Protective against *S. agalactiae* [6].
2	100	No effect [7].
1	150	Increased risk of infection with environmental Streptococci [8].
1	70	Protective against all major pathogens [9].
Not Stated		Protective against all major pathogens [10].

[1] *(Brooks et al. 1983)*, [2] *(Honkanen-Buzalski et al. 1984)*, [3] *(Lam et al. 1997)*, [4] *(Berry and Hillerton 2002)*, [5] *(Zadoks et al. 2001)*, [6] *(Rainard and Poutrel 1988)*, [7] *(Viseslava and Vera 1989)*, [8] *(Hogan et al. 1988)*, [9] *(Black et al. 1972)*, [10] *(Pociecha 1989)*.

1.3.9. Mode of Protective Affect

If quarters infected with *C. bovis* are protected against subsequent infection with more pathogenic organisms, a number of possible explanations for this effect have been postulated, including competitive growth, antagonism, induced leucocytosis or an increase in the immunity of the host (Black *et al.* 1972).

1.3.9.1. Modulation of Host Immunity

1.3.9.1.1. Induction of Leucocytosis

SCC is a measure of the nucleated cells, principally leucocytes, present in milk and is therefore a gauge of the degree of inflammation present in a quarter. Quarters with higher SCC were at significantly lower risk of become infected following artificial infection with *S. aureus* (Schukken *et al.* 1999) and *Aerobacter aerogenes* (Schalm *et al.* 1964). More recently it has been demonstrated that quarters with very low SCC (<20,000 cells/ml) are at increased risk of suffering clinical mastitis compared to quarters with higher SCC (20,000 – 60,000 cells/ml) (Green 2000). Interestingly the increase in SCC in quarters infected with *C. bovis* (approximately 50,000 cells/ml

higher than those that are bacteriologically negative, Section 1.3.5.1.) is in the middle of this 20,000 – 60,000 cells/ml band.

Polyethylene coils (approximately 12cm long, producing coils 3cm in diameter) introduced into the teat cistern have been used to induce a leucocytosis within the gland, in an attempt to "protect" the quarter from infection with major pathogens. Increases in SCC of 100,000 (Corlett *et al.* 1984), 73,000 & 41,000 (Brooks and Barnum 1982) and 226,000 cells/ml (Paape *et al.* 1981)) between treated and control quarters have been reported. Quarters containing coils were significantly less likely to become infected with *S. agalactiae* (Brooks and Barnum 1982) and *S. aureus* (Paape *et al.* 1981; Brooks and Barnum 1982) following artificial challenge.

It is apparent that elevation of SCC does lead to some degree of protection against IMI, however, Shukken *et al* (1999) concluded that because SCC was included in the logistic regression model used to analyze their results, the protective effect conferred by *C. bovis* infection was partly independent of SCC. In another study, Lam *et al* (1997) demonstrated that IMI with coagulase negative staphylococci elevated quarter SCC more than IMI with *C. bovis*, but infection was associated with less protective effect. They concluded that the protective effect of *C. bovis* is likely to be due to other mechanisms as well as an increase in SCC.

Whilst the elevation of SCC caused by IMI with *C. bovis* is likely to play some role in the protection of quarters against infection with other organisms it would appear likely that it is not the only mechanism in operation.

1.3.9.1.2. Up Regulation of Other Non-Specific Host Defence Mechanisms

The potential role that *C. bovis* may play in the up regulation of non-specific host defenses is unclear because little or no information is available in the literature. It can however be postulated that *C. bovis* IMI may be responsible for an increase in soluble defense mechanisms *e.g.* lysosyme and lactoferrin, an alteration in cell type present within the mammary gland or an up regulation of cell activity *e.g.* speed of migration into the gland or the efficiency of killing. Variation in the efficiency of phagocytosis and killing during infection with other organisms has been demonstrated. During chronic infection the efficiency with which polymorphonuclear cells kill *S. aureus* varies up to 10,000 fold and the percentage of cells that phagocytose fluorescent latex beads ranges between 15 and 80 percent (Daley *et al.* 1991).

1.3.9.2. Bacterial Interference

Established *C. bovis* infections may protect quarters against subsequent infection with other organisms by interfering with their ability to either invade or multiply within the quarter. As long ago as the 1870s, Pasteur and Joubert reported antagonistic interactions between different species of bacteria (Tagg *et al.* 1976). It is now known that bacteria can produce a wide range of molecules and substances that can inhibit the growth of and in some circumstances kill other bacteria, these include: toxins; bacteriolytic enzymes *e.g.* lysostaphin, phospholipase A and haemolysins; bacteriophages and defective bacteriophages; metabolic by-products *e.g.* ammonia, organic acids and hydrogen peroxide; antibiotic like substances *e.g.* bacitracin, gramicidin and valinomycin; bacteriocins (Jack *et al.* 1995).

1.3.10. Bacteriocins

The most widely studied inhibitory factors produced by bacteria are bacteriocins. Bacteriocins are bactericidal proteins or protein containing molecules produced by some strains and species of bacteria (Tagg *et al.* 1976; Jack *et al.* 1995). They are produced extracellularly, have a bactericidal mode of action and are usually plasmid mediated (Jack *et al.* 1995). Production of these bacteriocins is widespread (Tagg *et al.* 1976); many tend to have a narrow spectrum of activity confined to members of the same or closely related species (Kato *et al.* 1984), although bacteriocins produced by Gram-positive bacteria are often active against a wide range of Gram-positive organisms (Jack *et al.* 1995).

Many members of the *Corynebacterium* genus including *C. glutamicum* (Karabekov *et al.* 1984; Patek *et al.* 1986; Carnio *et al.* 1999), *C. mediolanum* (Kato *et al.* 1984), *C. ulcerans* (Abrehem and Zamiri 1983), *C. nebraskense*, *C. michiganense*, *C. insidiosum*, *C. oortii* and *C. iranicum* (Gross and Vidaver 1978) and *C. pseudodiphtheriticum*, *C. striatum*, *C. lilium*, *C. renale* and *C. variabilis* (Carnio *et al.* 1999) have been shown to produce bacteriocins. Recently, one of two strains of *C. bovis* isolated from the surface of German cheeses was shown to produce a bacteriocin that demonstrated inhibitory activity against *Listeria monocytogenes* (Carnio *et al.* 1999). However, the only work of this type specifically pertaining to *C. bovis* and mastitis pathogens demonstrated that the growth rates of *S. aureus* and *S. agalactiae*

were not inhibited by growth metabolites of *C. bovis* cultured in liquid media (Hogan *et al.* 1987).

1.3.11. Differentiating and Diagnosing *C. bovis* from Other Coryneform Organisms

Identification of members of the *Corynebacterium* genus to the species level is problematic (Funke *et al.* 1997). Many and varied techniques have been employed to differentiate members of the genus which almost certainly reflects the fact that none are definitive. Accurate identification of *C. bovis* is vital if pathogenicity (or protection) is to be correctly attributed to this organism. Classification and identification of *C. bovis* has been poorly described in some papers and may explain some of the variation in results reported by previous authors.

1.3.11.1. "Traditional" Approached to Identification

"Traditional" approaches to the identification of bacteria have employed techniques such as enzymic reactions, carbohydrate fermentation profiles, cell and colony morphology and Gram stain (Funke *et al.* 1997). Using these methods only 76% of 202 coryneform isolates could be identified to the species level (Vongraevenitz *et al.* 1994).

Antimicrobial susceptibility patterns vary between species, however the technique has proved of little worth for differentiating members of the *Corynebacterium* genus because of variations between strains of the same species (Riegel *et al.* 1994; Vongraevenitz *et al.* 1994). Similarly, analysis of cellular fatty acid profiles, although allowing organisms to be grouped, proved poor at discriminating between species (Bernard *et al.* 1991; Vongraevenitz *et al.* 1991). It was recommended as an adjunct to classification only (Bernard *et al.* 1991).

Two commercially available identification systems are available for coryneform organisms employing these traditional techniques. API Coryne (bioMérieux sa, Lyon, France) uses 20 enzyme and carbohydrate fermentation reactions to differentiate isolates. When assessed the system could only identify 84.2 (Gavin *et al.* 1992), 65.7 (Soto *et al.* 1994) and 35.4 percent (Funke *et al.* 1997) of isolates to the species level. However using additional tests the correct species was diagnosed in 88.1, 87.5 and 90.5% of cases respectively. The Biolog system (Biolog,

Hazelwood, USA) uses 95 test substrates in a 96 well format. When tested, it correctly identified 60.3% of 174 isolates to the correct genus or species level; 32.2% were incorrectly identified and it provided no identification for the remaining 7.5% of isolates (Lindenmann *et al.* 1995).

Both systems employ a database of standard results for type strains of a species to which they compare the reactions of test cultures. They are therefore limited by the fact that they can only identify test cultures of species present within the database and they rely on all test cultures displaying identical or similar reaction to the type strain for that species.

1.3.11.2. Modern Molecular Techniques

More recently the rapid expansion in molecular biology has lead to the development of a number of novel diagnostic techniques based particularly on advances in molecular genetics.

One of the first techniques to be employed was DNA-DNA hybridization. This method has been used both to investigate the relationship between recognized members of the genus (Riegel *et al.* 1994; Riegel *et al.* 1995) and as an aid to the differentiation and identification of novel *Corynebacterium* species (Riegel *et al.* 1992; Brennan *et al.* 2001; Renaud *et al.* 2001).

In a similar fashion rRNA gene restriction fragment patterns (ribotyping) have been successfully used to help characterize other novel *Corynebacterium* species (Riegel *et al.* 1993; Riegel *et al.* 1993). More recently this method was suggested as a useful identification method for the genus after detailed analysis of 26 species demonstrated the credibility of the technique and created a reference database (Bjorkroth *et al.* 1999).

SDS Page analysis of whole cell protein has also been employed as a method for differentiating *Corynebacterium* species; it was successfully utilized to group four strains of an unknown species (Sjoden *et al.* 1998). Later the method was exploited to differentiate two novel species by comparing their profiles to a known database of recognized genus members (Collins *et al.* 1999; Collins *et al.* 2001). The principal reason why none of these techniques has become more widely used is the success of methods that exploit the 16S rRNA gene sequence.

The 16S rRNA gene is responsible for the production of part of the small subunit of the prokaryotic ribosome. Its molecular biological value stems from its small size (~1500 base pairs (bp)) and the fact that large parts of the sequence are highly conserved although small numbers of base variations do occur, primarily within hypervariable regions (Stackebrandt and Goebel 1994). This is especially true for the *Corynebacterium* genus, in which much higher levels of variability occur compared with *e.g.* the *Staphylococcus* genus (Vaneechoutte *et al.* 1995).

Methods which exploit the 16S rRNA gene sequence include targeted probes, endonuclease restriction analysis, analysis of the intragenic spacer region and sequencing. 16S rRNA targeted probes which are designed only to bind to members of the target genus or species have been successfully used to identify 100% of *Corynebacterium* isolates to the genus level and *C. diphtheriae* and *C. jeikeium* to the species level (Hou *et al.* 1997). Cross reactivity with other members of the genus was minimal. Aubel *et al* (1997) successfully developed a method for differentiating members of the *Corynebacterium* genus based on the length of the 16S-23S rRNA gene spacer region. They conclude that the method is relatively easy to perform and a useful tool for microbiologists. The technique has since been used effectively to differentiate a novel species from a number of other recognized genus members (Renaud *et al.* 2001).

Restriction analysis of the 16S rRNA gene sequence has previously been used to differentiate members of the *Corynebacterium* genus. It relies on the principal that restriction of DNA sequences will result in fragments that differ in quantity and size, depending on the number of times any given piece of DNA contains the specific target sequence for the endonuclease restriction enzyme used. Using the enzymes *Alu* I, *Cfo* I and *Rsa* I allowed the differentiation of 20 of 26 *Corynebacterium* species studied (Vaneechoutte *et al.* 1995). The authors concluded that the technique represented a method of screening large collections of strains in order to select those which needed differentiation with more complex techniques *e.g.* 16S rRNA sequencing or DNA-DNA hybridization. More recently, Wattiau *et al* (2000) used *Hpa* I and *Pst* I to differentiate a new species from three known closely related *Corynebacterium* species based on their different restriction patterns.

Determination of the nearly complete 16S rRNA gene sequence and phylogenetic analysis has rapidly become the "reference" method of differentiating

between and identifying new *Corynebacterium* species (Watts *et al.* 2000) since it was successfully used by Ruimy *et al* (1995) and Pascual *et al* (1995) to investigate and clarify the genus. These papers form the basis of much of the work carried out since on the phylogeny of the genus. Sequencing the 16S rRNA gene has become a prerequisite when proposing novel species and has been used in all recent publication on the subject (Brennan *et al.* 2001; Collins *et al.* 2001; Collins *et al.* 2001; Renaud *et al.* 2001). Although an extremely powerful tool for the identification of organisms, 16S rRNA gene sequencing remains expensive and out with the capabilities of most laboratories. Recently a commercial package has become available which sequences the first 500bp of the 16S rRNA gene sequence and compares it with a database to allow species identification (MicroSeq 500 16S bacterial sequencing kit, Perkin-Elmer Biosystems, Foster city, USA). When tested however, the system could only identify 64.3% of *Corynebacterium* isolates (Tang *et al.* 2000).

Recently Watts *et al* (2000) used a number of techniques to investigate 212 isolates held in laboratory collections as *C. bovis*. Based on presumptive techniques alone (*C. bovis* was considered to be a Gram-positive, catalase positive, non-spore forming bacillus, whose growth was enhanced by the addition of Tween 80 to the media), 49.1% of these isolates were demonstrated to be species other than *C. bovis* (a large proportion of these (24.1%) were non-lipophilic *Corynebacterium* species). The identity of 50 isolates presumptively identified as *C. bovis* were confirmed by sequencing the 16S rRNA gene; the Biolog and API Coryne test systems correctly identified 54 and 88% of them respectively. One isolate (ATCC 13722) held in a type culture collection was demonstrated not to be *C. bovis*. The authors concluded that if traditional approaches to diagnosis are used the minimum characteristics required to identify *C. bovis* are Gram stain, cell morphology, catalase and ß-galactosidase production, nitrate reduction and demonstration of growth stimulation after the addition of Tween 80 to growth media.

CHAPTER 2: EVALUATION OF THE EFFICACY OF AN INTERNAL TEAT SEALER DURING THE DRY PERIOD, UNDER UK FIELD CONDITIONS.

2.1. INTRODUCTION

The five-point plan developed in the 1960s has drastically reduced the prevalence of contagious mastitis pathogens in the UK dairy herd over the last 40 years (Section 1.1.5. and 1.1.6.). More recently the pressure to reduce BMSCC because of EU regulation and financial bonuses paid by milk buyers has reduced their prevalence further (Section 1.1.7.). Consequently the number of animals subclinically infected with major mastitis pathogens at drying off in the UK has declined dramatically. In these animals, dry cow therapy is not required for the treatment of existing IMI, it is only required to prevent new IMI during the dry period. Teat sealants may represent a viable alternative to conventional antibiotic DCT if methods for the accurate selection of animals not infected at the end of lactation can be identified.

An internal teat sealer based on bismuth subnitrate has been used in Ireland in combination with a short acting antibiotic tube since the 1970s. A reformulation of the product containing 65% bismuth subnitrate in Alugel 30 DF and heavy liquid paraffin was launched onto the market in New Zealand in 1996. In a field trial employing a split udder design, the sealant was equivalent to conventional antibiotic dry cow therapy and better than a no treatment negative control at preventing IMI during the dry period and clinical mastitis in early lactation in animals not infected with major pathogens at drying off (Woolford *et al.* 1998).

Whilst the work conducted in New Zealand provides a detailed description of how the product functions in New Zealand it can only ever afford an insight into the potential efficacy of the teat sealer under UK field conditions. The New Zealand dairy industry has a very different structure to that of the UK. Cows are managed more extensively, almost exclusively at pasture, in a low input, low output system. Cows tend to be small Friesian type (often cross bred) animals, in contrast to the much larger Holstein/Friesian that now predominates in the UK. The most important cause of clinical mastitis during the dry period and early lactation in New Zealand is *S.*

uberis (Williamson *et al.* 1995); little clinical mastitis caused by *E. coli* is reported (A.J. Bradley 2002, Personal Communication). In comparison, although *S. uberis* is a major cause of clinical mastitis in the UK, one recent study reported *E. coli* as the most prevalent organism isolated from clinical mastitis cases (Bradley and Green 2001) and in 2000, *E. coli* was the most common organism isolated from clinical mastitis samples submitted to the Veterinary Laboratories Agency in England and Wales (Anon 2001). Therefore although the study results produced in New Zealand provide data supporting the potential value of a teat sealer based on bismuth subnitrate, further work is required to demonstrate its efficacy under UK field conditions.

Over the last 40 years, whilst the prevalence and aetiology of mastitis pathogens has changed substantially, the advocated control measures have remained relatively unchanged. Developments in the field of DCT have been limited to the occasional introduction of either a new antibiotic or the reformulation of an existing product. The aim of the study outlined in Chapter 2 was to assess the ability of an internal teat sealer containing bismuth subnitrate to prevent new IMI during the dry period compared to a positive control antibiotic tube containing cephalonium (Cepravin Dry Cow, Schering-Plough Ltd) in cows not infected with major pathogens at drying off, under UK field conditions. If successful, the product could represent a significant and unique advance in the range of dry cow products available in the UK and assist in the drive to reduce prophylactic antibiotic usage in food producing animals.

2.2. MATERIALS & METHODS

2.2.1. Herd Selection

Sixteen herds were selected on the basis of location (South West England), 12 month geometric mean BMSCC (<200,000 cells/ml), regular cow level SCC sampling of all milking cows (monthly or alternate monthly), good farm records, length of average dry period (an average of approximately 56 days) and likelihood of compliance with and willingness to adhere to the study protocol. Herds with BMSCC below 200,000 cells/ml were selected to maximize the number of cows eligible for inclusion in the

study because in these herds the number of subclinically infected animals was likely to be lower. Herds were not selected on the basis of previous clinical mastitis incidence or aetiology.

A calculation to determine the number of quarters / cows needed to provide adequate statistical power to demonstrate equivalence between the two treatment groups was performed using Epi-Info version 6.04b (CDC, Atlanta). An estimation of the new dry period infection rate was conservatively estimated at ten percent based on recently produced regional data (Bradley and Green 2000). An increase or decrease of two and a half percent points (*i.e.* 7.5 – 12.5%) in the estimated new dry period infection rate was considered biologically significant. To find a statistically significant difference between the treatments with a power of eighty percent it was calculated necessary to recruit 1000 quarters (250 cows) to each group. Therefore the target study size was 500 cows.

2.2.2. Herd Management

In 15 herds, cows were managed at pasture during the summer months and in either covered cubicles or straw yards during the winter. One herd (Farm L) practised an extended grazing system. Cows were kept at pasture and only housed on covered straw yards during the wettest part of winter, typically for two months (January and February). Dry cows on all farms were kept at pasture during the summer months and in covered stalls or straw yards during the winter. Periparturient cows were kept at pasture or in loose boxes. All herds practised post milking teat disinfection (either dipping or spraying) and blanket antibiotic dry cow therapy. Mastitis vaccines were not used on any farm. Cow level SCC were recorded monthly on 15 farms and on alternate months on one farm (Farm N).

2.2.3. Cow Level Selection Criteria

Animals were selected for inclusion in the study using the following cow level selection criteria:

1. No signs of clinical disease – Clinically healthy animals were selected to minimise the danger of unrelated disease confounding study results.

2. Four functional quarters and teats free of disease *e.g.* black spot, hyperkeratosis – A normal mammary system was specified to minimise the effects of disease on the susceptibility of quarters to new IMI which could confound study results.
3. No antibiotic or anti-inflammatory treatment during the previous 30 days – The study was designed to compare an antibiotic with a non-antibiotic treatment. Recent antibiotic or anti-inflammatory treatment for other conditions could have confounded study results particularly if the therapeutic agent administered partitioned to the udder.
4. Predicted dry period of greater than or equal to 51 days – 51 days was the minimum dry period for the positive control antibiotic treatment.
5. All routine cow level SCC </= 200,000 cells/ml (Dohoo and Leslie 1991; Dohoo 2001) during the preceding lactation *i.e.* no SCC > 200,000 cells/ml at any monthly recording during the lactation ending when the cow was presented to the author for drying off - Historical criteria specified to select cows unlikely to be infected with major pathogen IMI.
6. No cases of clinical mastitis recorded during the preceding lactation *i.e.* during the lactation ending when the cow was presented to the author for drying off - Historical criteria specified to select cows unlikely to be infected with major pathogen IMI.

2.2.4. Enrolment Procedure

The author visited herds on a weekly basis. Farms provided details of and made available all cows to be dried off during that week. Clinical mastitis and individual SCC records for the preceding lactation, predicted calving date information and medicine records were examined to exclude animals not meeting the specified cow enrolment criteria. Eligible cows were visually inspected in an unconfined area (*e.g.* the collecting yard) and then brought into the milking parlour. Animals were inspected when confined, the lungs and heart were auscultated, the temperature was taken per rectum and the teats and mammary gland were palpated and examined. Animals deemed unhealthy or suffering from teat or mammary gland abnormalities were excluded from the study. Remaining animals, which met all selection criteria, were enrolled onto the study.

2.2.5. Randomization Procedure

During the first weekly visit to each farm, the enrolment sheets of all cows deemed eligible for inclusion in the study were shuffled to randomise their order. The first cow was allocated to one of two treatment groups (Antibiotic DCT or Teat sealer) by the toss of a coin. Cows were then alternately allocated to the treatment groups in the order in which their enrolment sheet occurred. The last treatment administered on each farm was recorded. During each visit thereafter treatments were assigned alternately (recommencing from the last recorded treatment), employing the order randomisation method outlined above.

This method of randomisation was employed to ensure that the two groups were temporally matched even when low numbers of cows were being enrolled, thus ensuring that the environmental exposure between the two treatment groups was similar.

The herdspersons were not made aware of which animals received which treatment. All animals deemed ineligible for inclusion in the study received antibiotic DCT.

2.2.6. Dry Cow Products

2.2.6.1. Teat Sealer

An internal teat sealer with no antibacterial properties containing 65% w/w bismuth subnitrate in a paraffin base; 4g product in a plastic tube with a mid length nozzle for aseptic infusion into the teat cistern (Teatseal, Cross Vetpharm Group Ltd, Ireland).

2.2.6.2. Antibiotic Dry Cow Therapy

250mg cephalonium in a plastic tube with a mid length nozzle (Cepravin Dry Cow, Schering-Plough Ltd, UK).

2.2.7. Bacteriological and SCC Sampling Protocol at Drying Off

After allocation to a treatment group, duplicate quarter microbiological and single SCC milk samples were collected aseptically from all enrolled cows, following the protocol described in standard operating procedure 4, Figure AI.IV (Appendix I, Page 199). This set of samples was denoted "Drying Off Screening Samples". The quarter

microbiological sample that was collected second was submitted for culture, the sample that was collected first was stored at minus 20°C and only cultured if microbiological results from the second sample suggested it was contaminated. In this case the quarter was considered infected with any organisms isolated in both samples.

2.2.8. Product Administration

Immediately after sampling, before the teat was infused with the allocated treatment (Antibiotic DCT or teat sealer), the teat end was scrubbed with 70% ethanol and allowed to dry. Teat sealer was deposited into the teat cistern and allowed to sink back to the base of the teat. Antibiotic DCT was infused into the teat cistern and massaged up into the quarter after occlusion of the teat sphincter by gentle pinching. All teats were dipped in a solution containing 2800 mg/kg available chlorine (Agrisept, Pharmacia Animal Health, UK). Study animals were marked by the application of two strips of yellow "tail tape" to the central one third of the tail. Cows were released from the parlour and confined to a loafing yard for at least half an hour after sampling and product administration to allow closure of the teat sphincter.

Cows which did not meet the enrolment criteria were not included in the study. They received antibiotic DCT treatment administered following an identical protocol to that outlined above with the exception that aseptic milk samples were not collected. They took no further part in the study.

2.2.9. Transportation and Storage of Samples

All collected milk samples were kept in a cool box during transportation to the laboratory. Samples for microbiological analysis were frozen (-20°C) and samples for SCC analysis were refrigerated (+2°C - +8°C) immediately upon arrival.

2.2.10. Management of Dry Cows

After drying off, dry cows were managed according to normal farm practice. Farm staff were requested to make no allowances for study animals and to ensure that all dry cows were treated identically. Farm staff visually inspected individual animals at least once daily, particular attention was paid to inspection of the mammary gland. Any minor health problems were notified to the author at the weekly visit. Serious

problems were notified to the author immediately to allow appropriate action. Any cases of clinical or suspected clinical mastitis during the dry period were sampled according to a standard protocol (Figure AI.VII). At calving study cows were managed according to normal farm practices. The date and outcome of calving was recorded on the appropriate data capture form by farm staff.

2.2.11. Bacteriological and SCC Sampling Protocol after Calving

Samples for bacteriological and SCC analysis were collected from all study animals after calving and before the first machine milking. This set of samples was denoted "Calving Screening Samples". Farm staff were trained in the aseptic collection of milk samples, according to the protocol outlined in Figure AI.IV (Appendix I), prior to the start of the study. Farm staff collected calving samples, unless the author's weekly visit coincided with the period between calving and first machine milking.

Calving samples collected by herd persons were frozen (microbiological samples) or refrigerated (SCC samples) on farm and collected at the weekly visit. Frozen and refrigerated samples were transported back to the laboratory on dry ice and in cool boxes respectively. Calving samples collected by the author were transported and stored as previously described (Section 2.2.9.).

2.2.12. Clinical Mastitis Sampling Strategy

Clinical mastitis was monitored during the dry period and for the first 100 days of the next lactation. Herd persons were trained in the identification, grading and aseptic sampling of clinical mastitis. Clinical mastitis was identified in quarters on the basis of the presence of abnormal secretion and/or udder changes (*e.g.* pain, heat, swelling). Clinical mastitis severity was graded on a three point scale: Grade 1 – Milk changes only (*e.g.* clots), Grade 2 – Milk and udder changes (*e.g.* heat, swelling), Grade 3 – Systemic signs (*e.g.* depression, pyrexia, anorexia). Secretion samples were collected, prior to the administration of treatment, using a protocol similar to that used for collection of screening samples (Figure AI.IV, Appendix I). Samples were frozen on farm immediately after collection and returned to the laboratory on dry ice.

2.2.13. Bacteriology

Each week, frozen screening and clinical mastitis samples were submitted to the Langford Veterinary Laboratories Agency for bacteriological analysis. Ten microlitres of secretion was inoculated onto sheep blood agar and Edward's agar. One hundred microlitres of secretion was inoculated onto MacConkey agar to enhance the detection of the *Enterobacteriaceae* (Bradley and Green 2000). Plates were incubated at 37°C and read at 24 and 48 hours. Organisms were identified and quantified using standard laboratory techniques (Quinn *et al.* 1994). All isolates identified in screening and clinical mastitis samples were returned from the laboratory to the author on purity plates. Isolates were uniquely identified and stored at -80°C in a commercially available "bead" storage system.

2.2.14. SCC Analysis

Each week SCC samples were submitted to ON MeRiT Laboratories, Newbury, UK, for analysis by the Fossomatic method (Anon 1981), (Anadis SCC 300, France).

2.2.15. Additional Data Collection

Parity, last recorded yield, drying off date, calving date, parenteral antibiotic and anti-inflammatory treatments and withdrawal of any cow from the study as a result of death, sale or culling throughout the dry period and first 100 days of lactation were recorded for all study animals.

2.2.16. Standard Operating Procedures

All procedures carried out during the trial were conducted in accordance with SOP's designed before the study began (Appendix I).

1. SOP 1 - Identification of Dairy Cows used in Field Study
2. SOP 2 - Clinical Examination of Dairy Cows used in Field Study
3. SOP 3 - Observation of Dairy Cows used in Field Study during the Dry Period
4. SOP 4 - Collection of Quarter Milk Samples for Bacteriological and Somatic Cell Count Analysis
5. SOP 5 - Aseptic Administration of Antibiotic Dry Cow Therapy by Intramammary Infusion at Drying Off

6. SOP 6 - Aseptic Administration of Non-Antibiotic Dry Cow Therapy by Intramammary Infusion at Drying Off
7. SOP 7 - Collection of a Milk Sample from a Dairy Cow in the Case of Clinical Mastitis

2.2.17. Data Capture Forms

All data collected during the study was recorded contemporaneously on appropriate data capture forms designed prior to the start of the study.

1. Form A – Farm Enrolment
2. Form B – Product Usage Register
3. Form C – Cow Enrolment
4. Form D – Calving Screening Sample
5. Form E – Clinical Mastitis Case Sample
6. Form F – Concurrent Treatment
7. Form G – Cow Withdrawal
8. Form H – SOP Training Sheet

Data capture forms were duplicated, filed by cow and farm, and stored in separate locations prior to entry into data manipulation software.

2.2.18. Data Collation and Statistical Analysis

Data were collated using Excel and Access 2000 (Microsoft Corporation). All data was inputted into electronic forms in Access directly from data capture forms. Data entry was checked once at the time of entry. A complete audit of the entire data base was conducted at the end of the study once all data had been collected and inputted. In total all data entered into the database was checked manually for errors at least twice.

Data were analysed using Stata version 6 (Stata Corporation, Texas), Epi-Info version 6.04b (CDC, Atlanta) and Egret version 2.0.3 (Cytel Software Corporation, Cambridge, MA). The χ^2 test was used to compare proportions. The Mann-Whitney test was used to compare non-parametric data. The two-sampled t-test was used to compare parametric data. The significance probability was set at *P* </= 0.05 for a two tailed test. All farms were used in all analyses except farm S which was excluded from analysis of last recorded yield, as this data was not recorded on that farm.

In collaboration with Martin Green (University of Warwick), unconditional logistic regression was used to model the occurrence of new IMI and the cure of existing IMI at the quarter level in order to control for potential confounding factors. A strategy was employed as described by Kleinbaum (1994). Outcome variables, which tended towards significance ($P < 0.25$) on univariate analysis, were considered for modeling (Hosmer and Lemeshow 1989). Models were fitted with the following outcome variables: i. New major pathogen IMI at calving; ii. New enterobacterial IMI at calving; iii. New *E. coli* IMI at calving; iv. New minor pathogen IMI at calving; v. All major pathogen cure; vi. All minor pathogen cure; and vii. *Corynebacterium* spp. cure. Treatment group was used as an exposure variable and cow identity was fitted as a random effect to account for the clustering of quarters within cows. The confounding influence of herd, parity, last recorded yield, month of drying off, month of calving, quarter SCC at drying off and dry period length were assessed. Confounders were left in the model when there was judged to be a biological improvement in the model as calculated by either the likelihood ratio statistic (LRS) (significance probability set at 0.05 for a two tailed test) or a narrowing of the confidence interval of product (Kleinbaum 1994). Interactions between covariates were examined and left in the model when there was a significant improvement in the model according to the LRS (Kleinbaum 1994). Similarly, the effect of treatment group was deemed significant when its inclusion significantly improved the model.

2.2.19. Definition of Terms used for Analysis

2.2.19.1. Diagnosis of an IMI

Isolation of an organism was considered an IMI. A sample was considered "contaminated" if more than three organisms were isolated. In this case the duplicate microbiological sample was cultured and the infection status of the quarter was based on the presence of same organisms in both samples.

2.2.19.2. IMI: New Infection During the Dry Period

An organism identified at calving, in a quarter not infected with that organism at drying off. A quarter could therefore become infected with more than one organism during the dry period.

2.2.19.3. IMI: "Cure" During the Dry Period

An organism present at drying off and not identified in the same quarter at calving. A quarter could therefore cure more than one organism during the dry period.

2.2.19.4. IMI: "No Cure" During the Dry Period

An organism present at drying off and subsequently identified in the same quarter at calving. A quarter could therefore remain infected with more than one organism during the dry period.

2.2.19.5. IMI: Cause of Clinical Mastitis

When an organism was isolated in pure growth it was considered to be the cause of clinical mastitis. A sample was a "mixed growth" if there was growth of two or three known mastitis pathogens. A sample was considered "contaminated" when more than three organisms were isolated.

2.2.19.6. Clinical Mastitis – Quarter Case

A single recorded occurrence of clinical mastitis in a quarter, including all multiple quarter and recurrent cases. Recurrent cases were classified as cases recurring in the same quarter greater than five days after the end of the last clinical episode (Bradley and Green 2001)and caused by the same pathogen.

2.2.19.7. Clinical Mastitis – Cow Case

A cow experiencing a clinical mastitis event, regardless of the number of quarters affected or the number of times each quarter was affected if the case of mastitis was caused by the same pathogen. Synonymous with the proportion of cows effected with each pathogen. A cow suffering cases of mastitis caused by more than one pathogen was considered to have suffered multiple cow cases.

2.2.19.8. Minor Pathogen IMI

IMI caused by coagulase negative staphylococci, *Corynebacterium* species and *Micrococcus* species.

2.2.19.9. Major Pathogen IMI

IMI caused by all microbiological isolates recognised as capable of inducing mastitis in cattle, excluding minor pathogens.

2.2.19.10. Enterobacterial IMI

IMI caused by any member of the family *Enterobacteriaceae*, a taxonomic group of organisms similar to but not entirely synonymous with the collection of organisms often referred to as "Coliforms".

2.2.19.11. Parity

Parity number refers to the lactation the animal entered when calving occurred at the end of the dry period in the study (*i.e.* a first lactation animal dried off during the study was assigned parity number two). Parity groups two to four were assessed separately, parities five and greater were assessed as one group due to the smaller number of cows enrolled onto the study in these lactations.

2.3. RESULTS

Sixteen herds were selected for inclusion in the study. Fifteen herds were recruited in the autumn of 1999, one further herd (farm B) was recruited in spring 2000 because most of the farms originally enrolled had reached the end of their calving seasons and the target study size (500 animals) had not been reached.

2.3.1. Herd Parameters

The 16 herds enrolled were between 100 and 350 cows in size; average 305 day adjusted milk yields ranged from 4731 to 8458l and 12 month geometric mean BMSCC ranged between 51,000 and 163,000 cells/ml (Table 2.1).

Table 2.1: Number of Cows, Average Milk Yield, BMSCC and Number of Cows Enrolled on the 16 Herds Involved in the Study

Farm Reference Letter	Number of Cows in Herd	Average 305 Day Adjusted Milk Yield (l)	BMSCC (cells/ml)	Number of Cows Enrolled onto Study
B	134	6254	51,000	22 (44)†
F	188	4731	148,000	15
G	100	6440	87,000	20
H	106	6278	101,000	32
J	135	7671	115,000	10
K	284	7615	143,000	62
L	163	5861	94,000	48
N	350	6999	119,000	80
Q	207	8458	120,000	57
R	136	6374	102,000	17
S	183	6700*	120,000	26
T	153	5008	131,000	16
V	224	7651	91,000	26
W	322	6970	163,000	15
Y	176	7633	63,000	25
Z	150	7708	111,000	34

**Not routinely recorded, therefore an estimate only.*

†22 animals were initially enrolled onto the study, a further 22 were enrolled and monitored for clinical mastitis only (Section 2.3.2.).

2.3.2. Enrolment Data

1056 cows were dried off between 30th of September 1999 and the 7th of April 2000 on the 16 farms. Of these, 505 (47.8%) fulfilled the enrolment criteria; 253 received antibiotic DCT and 252 received the teat sealer. The number of animals enrolled on each farm ranged from ten to 80 (Figure 2.1). The proportion of animals fulfilling the enrolment criteria on each farm ranged from 23.8 to 71.3% (Figure 2.2).

A further 22 animals (11 in each group) were enrolled between 8th April and 2nd June 2000 on farm B. Only data on clinical mastitis incidence in the first 100 days of lactation were collected from these 22 animals (these animals are not shown in Figures 2.1 and 2.2).

Figure 2.1: Number of Cows Enrolled on each Farm

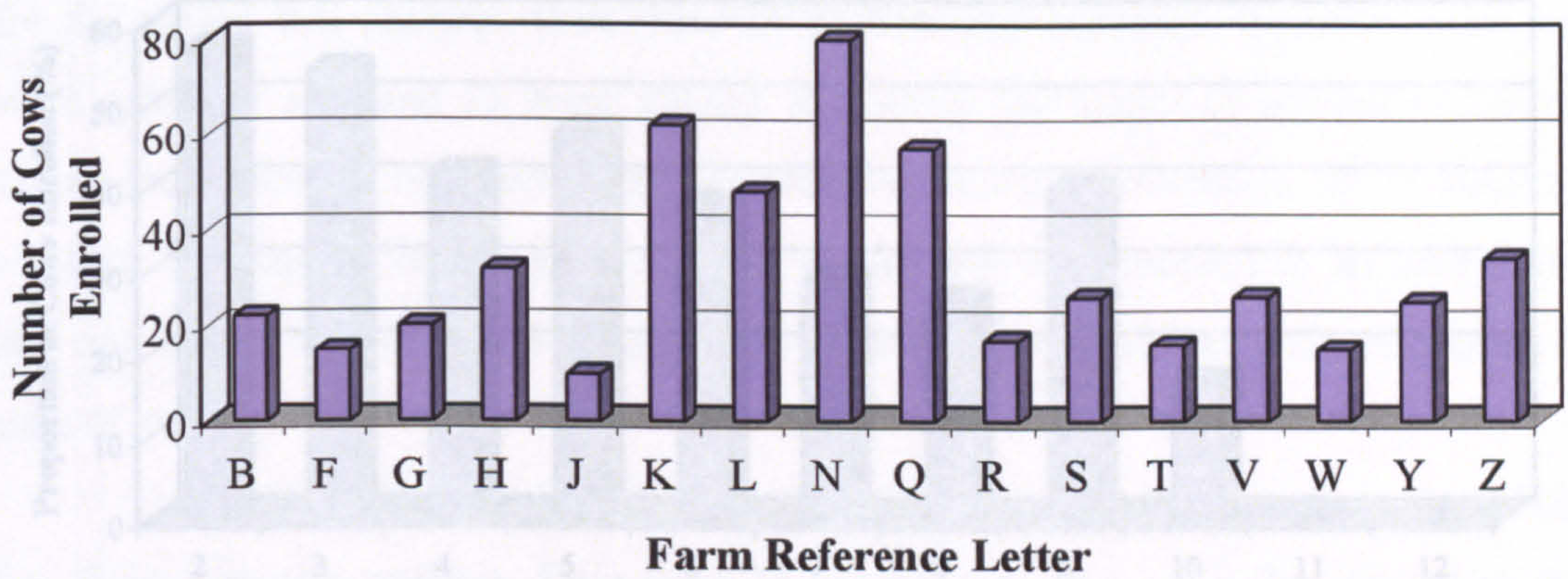
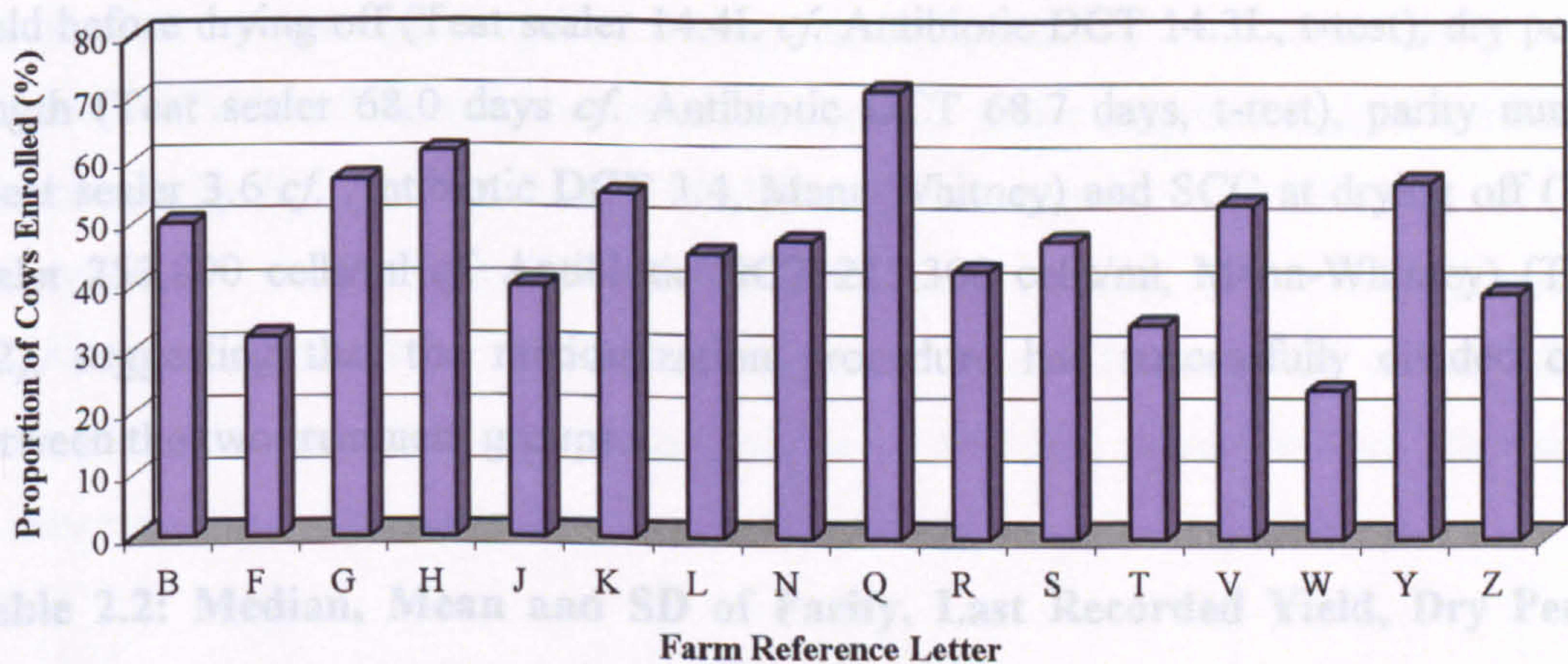
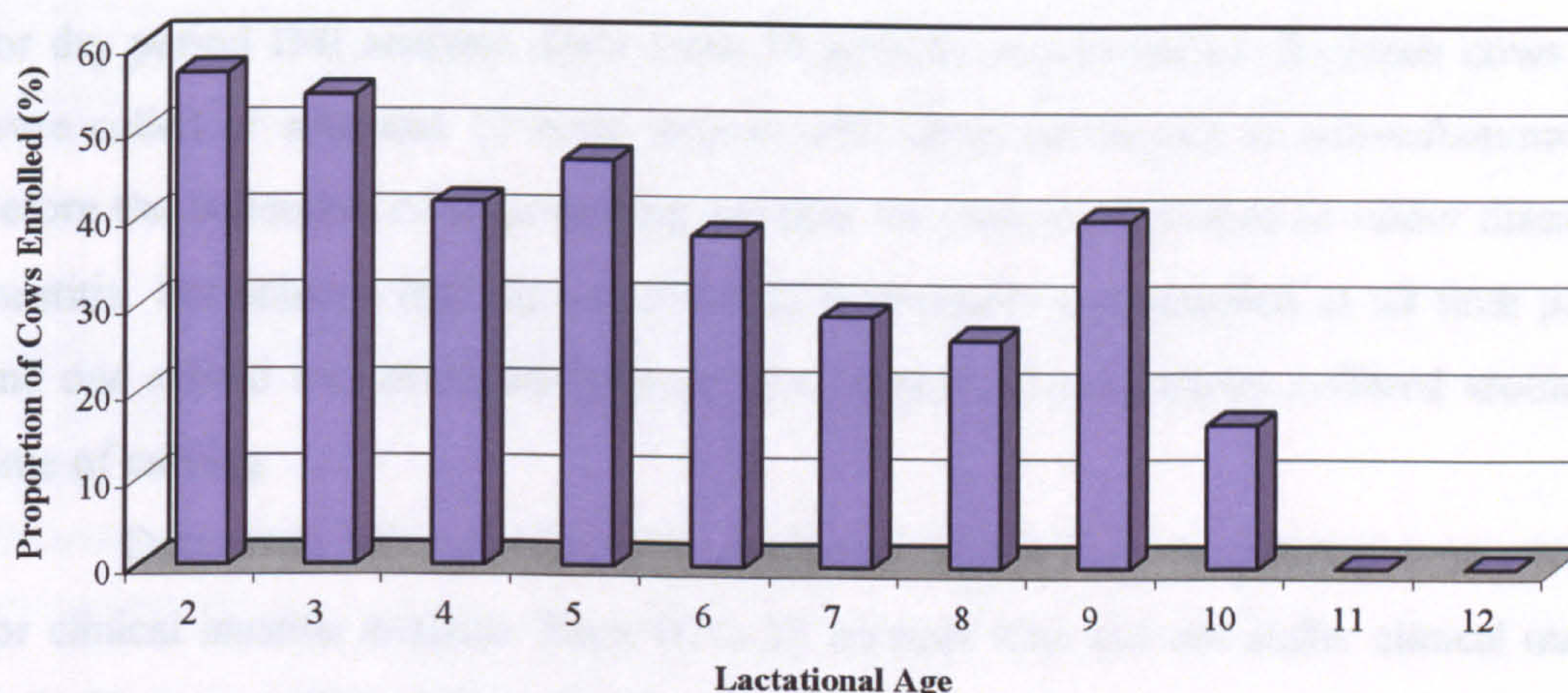


Figure 2.2: Proportion of Cows Enrolled (Fulfilling Enrolment Criteria) on each Farm



The proportion of animals meeting the enrolment criteria decreased as lactational age increased (Figure 2.3), predominantly because these animals tended to have at least one SCC above 200,000 cells/ml during lactation.

Figure 2.3: Proportion of Cows Enrolled by Lactational Age



2.3.3. Randomization Data

There was no significant difference between the treatment groups in the last recorded yield before drying off (Teat sealer 14.4L *cf.* Antibiotic DCT 14.3L, t-test), dry period length (Teat sealer 68.0 days *cf.* Antibiotic DCT 68.7 days, t-test), parity number (Teat sealer 3.6 *cf.* Antibiotic DCT 3.4, Mann-Whitney) and SCC at drying off (Teat sealer 253,800 cells/ml *cf.* Antibiotic DCT 225,300 cells/ml, Mann-Whitney) (Table 2.2), suggesting that the randomization procedure had successfully divided cows between the two treatment groups.

Table 2.2: Median, Mean and SD of Parity, Last Recorded Yield, Dry Period Length and Drying Off SCC by Treatment Group

	Teat Sealer Group				Antibiotic DCT Group			
	n	Median	Mean	SD	n	Median	Mean	SD
Parity	252	3	3.6	1.6	253	3	3.4	1.5
Last Recorded Yield (L)*	238	14.4	14.4	5.02	241	14.0	14.3	4.55
Dry Period Length (d)	241	62	68.0	21.8	242	62	68.7	22.2
Drying off SCC (X 1000cells/ml)†	1002	251	253.8	1250.5	992	242.5	225.3	850.4

**Farm S excluded as data not recorded.*

†Quarter geometric mean somatic cell count of samples taken at drying off (>200,000 cells/ml, likely result of concentration effect caused by low milk yield prior to drying off, collection of strippings after milking and effect of geometric mean of quarter data not a composite quarter sample)

2.3.4. Data Available for Analysis

Data from 467 (232 teat sealer & 235 antibiotic DCT treated) animals was available for dry period IMI analysis. Data from 38 animals was excluded. Eighteen cows died, were culled or sold and 13 were treated with either antibiotics or anti-inflammatories before the collection of all screening samples for reasons unrelated to udder disease or mastitis. Six animals had incomplete data (principally not sampled at all time points) and one animal was excluded because of very serious teat injuries suffered around the time of calving.

Data from 502 animals (251 animals in both treatment groups) were available for clinical mastitis analysis. Data from 25 animals who did not suffer clinical mastitis and who were culled, sold or died was excluded.

2.3.5. Univariate Analysis

2.3.5.1. New IMI Acquired During the Dry Period

The number of new IMI acquired during the dry period by each treatment group is outlined in Table 2.3. Cows that received the teat sealer acquired significantly fewer IMI caused by *E. coli* ($P < 0.001$), all *Enterobacteriaceae* ($P < 0.001$) and all major pathogens ($P < 0.01$) at the quarter level and *E. coli* ($P < 0.001$) and all *Enterobacteriaceae* ($P < 0.001$) at the cow level. There were no significant differences between the groups in the number of new IMI caused by any other major or minor mastitis pathogen.

2.3.5.2. Clinical Mastitis

Two cases of clinical mastitis occurred in cows that received antibiotic DCT during the dry period, compared with no cases in animals that received the teat sealer; the difference was not significant. The number of cases and causes of clinical mastitis during the first 100 days of lactation is outlined in Table 2.4. There was no difference between the groups in the number of cases by quarter (Teat sealer 30/1004 *cf.* Antibiotic DCT 35/1004) or cow (Teat sealer 25/251 *cf.* Antibiotic DCT 31/251). There was no difference between the groups in severity of clinical mastitis (Mann-Whitney); Teat sealer: grade 1 - 33.3%, grade 2 - 54.2%, grade 3 - 12.5%, Antibiotic DCT: grade 1 - 59.3%, grade 2 – 18.8%, grade 3 – 21.9%.

Table 2.3: Number of Quarter and Cow IMI Acquired During the Dry Period, by Causative Organism

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	New quarter IMI during dry period (n=928)	New cow IMI during dry period (n=232)	New quarter IMI during dry period (n=940)	New cow IMI during dry period (n=235)
Coagulase +ve Staphylococci	10	9	7	6
S. dysgalactiae	2	2	0	0
S. uberis	11	11	12	11
Streptococcus spp. (Other)	11	11	14	13
Enterococcus spp.	20	18	35	31
E. coli	13[c]	11[c]	42[d]	35[d]
Klebsiella spp.	0	0	1	1
Serratia spp.	0	0	2	2
Pantoea spp.	1	1	0	0
Citrobacter spp.	1	1	0	0
Morganella spp.	0	0	1	1
Proteus spp.	2	2	7	7
Enterobacter spp.	0	0	1	1
Hafnia spp.	0	0	1	1
All *Enterobacteriaceae* spp.	17[c]	15[c]	55[d]	48[d]
Ochrobacter spp.	1	1	1	1
Chryseomonas spp.	1	1	0	0
Aerococcus spp.	0	0	2	2
Acinetobacter spp.	14	13	14	12
Non fermenters	11	10	11	10
Bacillus spp.	8	7	5	4
Other non speciated organisms	0	0	1	1
Aspergillus spp.	1	1	2	2
Yeast spp.	0	0	1	1
Mucur spp.	0	0	4	4
All Yeast & Fungi	1	1	7	7
All Major Pathogens	103[a]	81	145[b]	100
Micrococcus spp.	19	14	13	12
Coagulase –ve Staphylococci	162	108	184	126
Corynebacterium spp.	48	39	39	31
All Minor Pathogens	218	135	224	142

[a,b] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.01$)*
[c,d] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)*

Quarters which acquired a new dry period IMI were significantly more likely to suffer clinical mastitis during the first 100 days of the next lactation compared to those that did not (18/248 *cf.* 36/1679, $P < 0.001$). This was true in both antibiotic (11/145 *cf.* 18/818, $P < 0.01$) and teat sealer (7/103 *cf.* 18/861, $P = 0.01$) treated quarters.

In the antibiotic treated group, seven quarters that acquired a new IMI during the dry period suffered a case of clinical mastitis (within 100 days of calving) caused by the same organism, compared to two quarters in the teat sealer group. The difference between the two groups was not significant.

Table 2.4: Number of Clinical Mastitis Cases in the First 100 days of Lactation, by Causative Organism

	Teat sealer Group		Antibiotic DCT Group	
Diagnosis	Quarter cases (n=1004)	Cow cases (n=251)	Quarter cases (n=1004)	Cow cases (n=251)
E. coli	7	6	7	7
S. uberis	8	5	3	3
Coagulase +ve Staphylococci	0	0	1	1
S. dysgalactiae	1	1	2	2
Enterococcus spp.	1	1	1	1
Acinetobacter spp.	1	1	0	0
Coagulase –ve Staphylococci	2	2	2	2
Corynebacterium spp.	1	1	0	0
Mixed growth	1	1	4	4
No growth	3	3	8	6
No sample	5	4	7	6
Total	30	25	35	31

2.3.5.3. *IMI Present at Drying Off*

60 out of 1868 quarters (3.2%) were infected with one or more major pathogens at drying off, 27 (2.9%) in teat sealer and 33 (3.5%) in antibiotic DCT animals (Table 2.5). Of these quarter infections, ten were identified at calving in each group. The difference in cure rate was not significant. 15 of the 20 infected quarters that remained at calving occurred on one farm (farm K).

645 out of 1868 quarters (34.5%) were infected with one or more minor pathogens at drying off; 310 in teat sealer and 335 in antibiotic DCT animals (Table 2.5). The quarter cure rate for *Corynebacterium* spp. and all minor pathogens was significantly greater in cows that received antibiotic DCT ($P < 0.001$).

Table 2.5: Number of IMI Present at Drying Off and Number and Percentage Cured During Dry Period

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	Quarter IMI present at drying off (n=928)	Quarter IMI cured during dry period (%)	Quarter IMI present at drying off (n=940)	Quarter IMI cured during dry period (%)
Coagulase +ve Staphylococci	8	1 (12.5)	13	5 (38.5)
S. uberis	0	0 (0)	2	2 (100)
E. coli	0	0 (0)	3	3 (100)
Streptococcus spp. (Other)	3	1 (33.3)	2	0 (0)
Enterococcus spp.	6	6 (100)	6	4 (66.7)
Klebsiella spp.	1	1 (100)	0	0 (0)
Pantoea spp.	0	0 (0)	1	1 (100)
Pseudomonas spp.	1	1 (100)	0	0 (0)
Acinetobacter spp.	2	2 (100)	1	1 (100)
Non fermenters	2	1 (50.0)	0	0 (0)
Aspergillus spp.	1	1 (100)	1	1 (100)
Yeast spp.	1	1 (100)	1	1 (100)
Bacillus spp.	5	5 (100)	3	3 (100)
All Major Pathogens	27	17 (63.0)	33	23 (69.7)
Micrococcus spp.	8	8 (100)	12	12 (100)
Coagulase -ve Staphylococci	79	54 (68.4)	118	76 (64.4)
Corynebacterium spp.	237	135^{a} (57.0)	219	205^{b} (93.6)
All Minor Pathogens	310	183^{a} (59.0)	335	279^{b} (83.3)

[a,b] Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)

2.3.6. Logistic Regression Analysis

2.3.6.1. New IMI Acquired During the Dry Period

Cows that received the teat sealer were significantly less likely to acquire new quarter IMI caused by a major pathogen (Odds ratio = 0.66, 95% CI = 0.47 to 0.93, $P = 0.02$, Table 2.6), all *Enterobacteriaceae* (Odds ratio = 0.30, 95% CI = 0.16 to 0.55, $P < 0.001$, Table 2.6) and *E. coli* (Odds ratio = 0.29, 95% CI = 0.14 to 0.63, $P = 0.002$,

Table 2.6) during the dry peirod. There was no significant difference between the groups in the number of new IMI caused by minor pathogens.

2.3.6.2. IMI Cure During the Dry Period

There was no significant difference between the groups in the proportion of IMI caused by a major pathogen that were cured during the dry period. Cows that received teat sealer were significantly less likely to cure IMI caused by a minor pathogen (Odds ratio = 0.24, 95% CI = 0.15 to 0.40, $P < 0.001$, Table 2.6) and *Corynebacterium* spp. (Odds ratio = 0.06, 95% CI = 0.02 to 0.13, $P < 0.001$, Table 2.6) during the dry period.

Table 2.6: Results of Five Logistic Regression Models Used to Assess the Efficacy of the Teat Sealer during the Dry Period

Variable	Coefficient	SE	Odds Ratio	95% CI	*P* value
Model 1: Outcome variable – New IMI caused by a major pathogen during dry period					
CONSTANT	-1.99	0.14			
EXPOSURE VARIABLE					
DRY COW PRODUCT					
Antibiotic DCT (Ref)	-	-	-	-	
Teat sealer	-0.42	0.17	0.66	0.47-0.93	0.02
% SCL*	0.96	0.14			
Model 2: Outcome variable – New IMI caused by a *Enterobacteriaceae* species during the dry period					
CONSTANT	-4.10	0.53			
CONFOUNDERS					
LAST RECORDED YIELD					
Every ↑ in 1L	0.06	0.03	1.06	1.00-1.12	0.036
EXPOSURE VARIABLE					
DRY COW PRODUCT					
Antibiotic DCT (Ref)	-	-	-	-	
Teat sealer	-1.21	0.31	0.30	0.16-0.55	< 0.001
% SCL	0.98	0.31			
Model 3: Outcome variable - New IMI caused by *E. coli* during the dry period					
CONSTANT	-4.05	0.42			
EXPOSURE VARIABLE					
DRY COW PRODUCT					
Antibiotic DCT (Ref)	-	-	-	-	
Teat sealer	-1.22	0.38	0.29	0.14-0.63	0.002
% SCL	1.52	0.34			

Model 4: Outcome variable – A minor pathogen IMI cured during the dry period					
CONSTANT	1.91	0.22			
EXPOSURE VARIABLE					
DRY COW PRODUCT					
Antibiotic DCT (Ref)	-	-	-	-	
Teat sealer	-1.41	0.26	0.24	0.15-0.40	< 0.001
% SCL	1.07	0.24			
Model 5: Outcome variable – *Corynebacterium* spp. IMI cured during the dry period					
CONSTANT	4.07	0.63			
CONFOUNDERS					
MONTH OF CALVING					
1	0.00	1.00	-	-	
2	-0.14	0.66	0.87	0.24-3.17	0.827
3	-1.61	0.61	0.20	0.06-0.66	0.008
4	-1.72	0.57	0.18	0.06-0.54	0.003
5-7	-1.57	0.69	0.21	0.05-0.80	0.022
11	0.19	0.90	1.20	0.21-7.07	0.837
12	-0.53	0.53	0.59	0.21-1.65	0.314
EXPOSURE VARIABLE					
DRY COW PRODUCT					
Antibiotic DCT (Ref)	-	-	-	-	
Teat sealer	-2.89	0.42	0.06	0.02-0.13	< 0.001
% SCL	0.90	0.28			

** % SCL is a scalar term for the random effect of cow (Egret Manual Version 2.0.3, Cytel Statistical Software Corp, USA)*

2.4. DISCUSSION

This is the first controlled study to demonstrate the efficacy of an internal bismuth subnitrate teat sealer in protecting quarters against new dry period IMI caused by major mastitis pathogens under UK field conditions, compared to antibiotic DCT. Previously the efficacy of the teat sealer has been demonstrated under field conditions in New Zealand (Woolford *et al.* 1998). In the New Zealand study, quarters which received the teat sealer acquired significantly fewer new dry period IMI compared to negative control quarters and an equivalent number compared to quarters which received antibiotic DCT or antibiotic DCT and teat sealer together. The effect was particularly marked against streptococci, especially *S. uberis,* the predominant clinical mastitis pathogen in New Zealand. In comparison with this study, the difference in the

number of new dry period IMI in quarters that received the teal sealer compared to those that received antibiotic DCT was not significant. A recent negative control study in the UK has demonstrated that the teat sealer was significantly better than no treatment at preventing new dry period IMI, clinical mastitis during the dry period and clinical mastitis during the first 100 days of the next lactation (Berry and Hillerton 2002).

The reduction in new dry period IMI in this study was particularly marked for members of the *Enterobacteriaceae* genus. *E. coli* and closely related organisms are opportunistic invaders of the mammary gland following migration into the teat cistern via the sphincter. The non-lactating gland is particularly prone to IMI. In a recent UK study, 12.8% of quarters acquired new enterobacterial IMI during the dry period (Bradley and Green 2000). The early and late dry periods are the times of greatest risk (Smith *et al.* 1985). The internal teat sealer used in this study sinks to the base of the teat cistern and covers the teat sphincter. Radiographical examination has demonstrated the presence of the sealer at the base of the teat cistern 100 days after infusion (Woolford *et al.* 1998). In agreement with previous work conducted originally in Ireland and lately in New Zealand, the results of this UK study indicate that the sealer in its current formulation effectively prevents the entry of mastitis pathogens, particularly environmental organisms, during the dry period.

Whilst the physical barrier effect that the teat sealer produces is likely to be a significant part of the protective effect it affords there are other possible explanations. The dry period cure rate of *Corynebacterium* spp. was significantly lower in quarters receiving the teat sealer (57.0%) compared with quarters receiving the antibiotic tube (93.6%). It has been demonstrated by some authors that quarters infected with *C. bovis* are significantly less likely to become infected with major pathogens (Pociecha 1989; Lam *et al.* 1997; Schukken *et al.* 1999). The role of *C. bovis* in increasing or decreasing the susceptibility of quarters to subsequent infection with other mastitis pathogens undoubtedly remains a complex issue. It remains a possible explanation for the lower proportion of major pathogen IMI acquired by cows that received the teat sealer and will be discussed in more detail in the later parts of this thesis.

IMI acquired during the dry period can cause clinical mastitis during the following lactation (Smith *et al.* 1985; Bradley and Green 2000). In this study, however, there was no significant difference between the groups in the number of cases of clinical

mastitis during the first 100 days of lactation despite the significantly greater number of new IMI acquired during the dry period by cows that received antibiotic DCT. Woolford *et al* (1998) also demonstrated a non significant decrease in the number of cases of clinical mastitis in cows that received the teat sealer compared to those that received antibiotic DCT. The total number of cases of clinical mastitis in this UK study was low; only 3.4% of quarters and 11.7% of cows were affected, compared with 6.5% of quarters and 25.1% of cows suffering clinical mastitis over an identical time period in another recent UK study (Bradley and Green 2001). This may be indicative of the selection criteria used to select cows for this study (*i.e.* cows which did not suffer clinical mastitis during the previous lactation) or lack of exposure to the pathogen because of the farms or season studied.

In this study, quarters that acquired a new dry period IMI were at significantly greater risk of suffering a case of clinical mastitis during the first 100 days of the next lactation. Quarters in the antibiotic treated group acquired significantly more new dry period IMI. A significant increase in the number of cases of clinical mastitis in the cows which received antibiotic DCT may have been apparent if the group sizes had been larger.

Selecting cows suitable for non-antibiotic dry cow therapy (such as teat sealers) is dependent on reliably identifying uninfected quarters at drying off. The cost and practicalities of quarter bacteriology make it an unrealistic method on commercial UK dairy units. Historical clinical mastitis and SCC data are available on many farms and may provide a practical alternative method to identify eligible cows. A cow level threshold of 200,000 cells/ml was selected as it is commonly used as a cut off point above which one or more quarters are likely to be infected (Dohoo 2001). The sensitivity and specificity of this threshold have previously been calculated as 83.4 and 58.9% for all major pathogens (Dohoo and Leslie 1991) and 89.0 and 75.0% for all pathogens (McDermott *et al.* 1982). More recently, the ability of the last SCC above 200,000 cells/ml, any of the last three SCC above 200,000 cells/ml and a geometric mean of the last three SCC above 200,000 cells/ml to identify infected quarters at drying off, have been assessed (Bradley *et al.* 2002). To detect quarters infected with any major pathogen, any of the last three counts above 200,000 cells/ml had the highest sensitivity (67%). These criteria also had the highest sensitivity when they were used to detect quarters infected with Gram-positive organisms only (80%).

Gram-positive infections present at drying off are the most clinically relevant because the gland self cures *E. coli* infection present at drying off or acquired during the early dry period (McDonald and Anderson 1981).

The use of historical SCC and clinical mastitis data will inevitably lead to the misclassification of both unifected (not infected, but excluded by the enrolment criteria) and infected cows (infected, but selected by the enrolment criteria). In the study described here, the use of a cow SCC threshold of 200,000 cells/ml led to 11.3% of cows (53 in 467) and 3.2% of quarters (60 in 1868) being incorrectly identified as uninfected with a major pathogen. It is not possible, using the data collected, to calculate the number of uninfected cows that were excluded inappropriately. However, it is interesting to note that there was no significant difference in the apparent quarter cure rate between the two treatment groups (63% teat sealer *cf.* 70% antibiotic tube). A significant difference may have become apparent if the group sized in this study had been larger.

Establishing any SCC threshold as a cut off is an arbitrary decision, irrespective of the level at which it is set and will always lead to some infected quarters being "missed". If historical clinical mastitis and SCC data alone are used to select cows suitable for non-antibiotic dry cow therapy, lowering the SCC threshold to reduce the number of false negative cows included will increase the number of false positive cows excluded to the point that the total number of animals eligible may be unacceptably low in commercial situations. Using the data generated during this study it was necessary to reduce the SCC threshold to 25,000 cells/ml (*i.e.* all SCC less than 25,000 cells/ml during the previous lactation) before all infected cows were identified (Huxley *et al.* 2001). At this SCC threshold the number of cows meeting the selection criteria would be negligible. Sealing a small number of infected quarters is acceptable providing the potential benefits (*e.g.* reduced numbers of new dry period IMI) outweighs either the impact of missing IMI (*e.g.* clinical dry period IMI or the persistence of contagious organism through the dry period), or the cost of identifying uninfected cows with a higher degree of precision (*e.g.* quarter sample bacteriology). In this study none of the quarters infected at drying off in the teat sealer group suffered clinical mastitis during the dry period, although the number infected with major pathogens was small (27). More research is needed to identify the most suitable regimes and inclusion values (*e.g.* <200,000 cells/ml) for using historical SCC and clinical mastitis data for identifying uninfected cows at the end of lactation.

If the use of non antibiotic dry cow therapies becomes widespread the ability to diagnose quarter infection status by methods other than bacteriology (which remains expensive and time consuming) or analysis of historical data (as used in this study) will become increasingly important. California mastitis test results (Rindsig *et al.* 1978; Poutrel and Rainard 1981) and N-acetyl-beta-D-glucosaminidase levels (Hassan *et al.* 1999) at drying off have previously been used to diagnose cows suitable for selective dry cow therapy regimes. Milk conductivity (Chamings *et al.* 1984; Hillerton and Walton 1991) and more recently acute phase proteins (Gronlund *et al.* 2001) have also been suggested as detection methods for subclinical mastitis. The relative merit of these methods is dependent on their abilities to detect subclinical major pathogen IMI present at drying off with a high degree of sensitivity and specificity. This remains an area in need of further research.

New dry period IMI were diagnosed with a single bacteriological isolation of a pathogen at calving in quarters uninfected at drying off. The author accepts that this may lead to an increase in the proportion of both false positive (transient IMI) and false negative quarters in both groups, compared with methods based on re-isolation of causal pathogens in second or third samples. However, the increase in accuracy and repeatability between these methods can be as little as five percent (Griffin *et al.* 1987) providing precautions such as careful aseptic sampling technique, use of accredited laboratories and "blinding" of microbiological operators are taken to minimise problems. These precautions were employed during this study.

Quarter SCC thresholds can be used in conjunction with single bacteriological isolation of a pathogen in an attempt to decrease the number of false positive and negative diagnoses; levels of 125, 250, 500 or 1000 $x10^3$ cells/ml have been suggested (Griffin *et al.* 1987). Adding any of these SCC thresholds using the quarter level SCC data from the drying off and calving samples to the bacteriological data produced did not alter the outcome of the study (Tables AII.I, AII.II, AII.III, AII.IV, Appendix II). The difference in the number of *E. coli*, all *Enterobacteriaceae* and all major pathogen IMI acquired (at the quarter level) during the dry period was significantly lower in the group that received the teat sealer at all SCC thresholds. The only exception was when the SCC was 1000,000 cells/ml when the reduction in all major pathogen IMI was only significant at the $P < 0.1$ level.

The author also accepts that assessing bacteriological cure rate during the dry period on single isolation after the dry period, especially for organisms such as *S. aureus*, which are intermittently shed from infected glands, will inevitably lead to an overestimation of the efficacy of a product. This study was designed to investigate the rate of new dry period IMI, not dry period cure rates. Cure rate results from this study should be interpreted with care as they may not truly represent the actually efficacy of either product.

Across the 16 herds the proportion of cows eligible for recruitment to the study ranged from 24 to 71 percent with a mean value of 47.8%. Herd BMSCC, clinical mastitis incidence and particularly the proportion of the herd affected with clinical mastitis had the most bearing on numbers enrolled. Dry period length (less than 51 days), treatment within the previous 30 days and minor teat abnormalities did however exclude significant numbers of cows on some farms. Calving seasonality was an issue on farms J, T and W because the recruitment period was late in the season for these herds, biasing the sample towards older (and therefore higher SCC) cows, resulting in a lower recruitment proportion in these herds.

It can be expected that the enrolment rate in herds with higher BMSCC will be lower. In these herds contagious organisms are more prevalent and therefore treatment of infected quarters at drying off is a priority. Teat sealants may have a role for the protection of uninfected cows on these farms, however the proportion eligible is likely to be low. If the sensitivity and specificity of the enrolment criteria are poor there is an increased risk that animals infected with contagious organisms (particularly *S. aureus*) will be left untreated. It must be remembered that non-antibiotic dry cow therapies (such as the teat sealer used in this study) are not designed to cure existing IMI present at drying off. For animals not deemed eligible to receive non-antibiotic therapies, antibiotic dry cow treatment will remain vital. Failure to treat these animals effectively could lead to the build up of a reservoir of subclinical IMI within herds, which will increase BMSCC and the incidence of clinical mastitis.

Logistic regression analysis demonstrated that last recorded milk yield prior to drying off was significantly related to the risk of acquiring a new enterobacterial IMI during the dry period. For every one litre increase in final yield cows were 1.06 times more likely to acquire an enterobacterial IMI. This data suggests that management at the

end of lactation should include strategies to minimise milk yield prior to drying off (*e.g.* reducing the energy density of diets *i.e.* based on poor quality roughage, reducing the frequency of milking prior to drying off, reducing the stimulation of milk production *i.e.* minimise exposure to calves and milking parlour), to reduce this risk. However the model used here did not contain total lactation yield as a potential confounding factor. The possibility remains that the increased risk of acquiring a new enterobacterial IMI is a function of high yielding cattle rather than of yield prior to drying off. Further work is needed to investigate this potentially very interesting and clinically plausible relationship.

The introduction of an internal teat sealer without antibacterial properties into the mammary gland represents a potential risk to the quarters treated. Pathogens present around the teat sphincter or in the environment could be inoculated into the quarter or the seal material may act as a nidus for IMI if the product is contaminated during the infusion process. The teat disinfection process employed during this study prior to product infusion was fastidious. If this product becomes available in the UK, users in the field need to be made aware of the potential dangers and adequately trained to maintain aseptic infusion techniques of the highest standards. These precautions will minimise the potential risks of inoculating pathogenic organisms into the quarter, at the time of product administration.

In an attempt to prevent anti-microbial resistance in human pathogens the antibiotics avoparcin, tylosin phosphate, spiramycin, virginiamycin and bacitracin zinc have been banned as growth promoters within the EU. It is planned that the remaining four (monensin sodium, salinomycin sodium, avilamycin and flavophospholipol) will be banned by January 1st 2006 (Anon 2002). The continuing drive to reduce the use of routine prophylactic antibiotic treatments in animals will increase the pressure to prescribe DCT rationally, justifiably and to the individual not the herd. The results of this study indicate that the continued blanket use of antibiotic dry cow therapy in low SCC cows needs to be re-evaluated. It is likely that the rationale for the use of high doses of long acting antibiotics in cows uninfected with major pathogens at drying off will become increasingly untenable if effective alternatives for the prevention of new IMI during the dry period are available. The internal teat sealer used in this study

could be one such viable alternative. Further work to support the usage of internal teat sealers is needed in this novel and evolving area of DCT.

CHAPTER 3: SPECIATION OF LIPOPHILIC *CORYNEBACTERIUM* SPECIES OF MILK ORIGIN BY ENDONUCLEASE RESTRICTION ANALYSIS OF THE 16S rRNA GENE SEQUENCE.

3.1. INTRODUCTION

Use of a dry period internal teat sealer significantly reduced the number of IMI acquired during the dry period compared to the UK's market leading antibiotic dry cow therapy (Chapter 2). It has been demonstrated by some authors that quarters infected with *C. bovis* are less likely to become infected with major pathogens compared to uninfected quarters (Black *et al.* 1972; Lam *et al.* 1997; Schukken *et al.* 1999). The significantly lower *C. bovis* cure rate in animals that received the teat sealer is one possible explanation for the decrease in the number of major pathogen IMI acquired during the dry period in this group.

In order to investigate any potential protective effect afforded by *C. bovis*, it is first necessary to differentiate *C. bovis* from other coryneforms present in milk. *Corynebacterium* species can be divided into lipophilic and non-lipophilic sub-groups; the growth of members of the lipophilic subgroup is enhanced by the addition of free fatty acid *e.g.* one percent Tween 80 to the growth medium (Funke *et al.* 1997). *C. bovis* is a member of the much smaller lipophilic grouping (Funke *et al.* 1997). The non-lipophilic *Corynebacterium* species, *C. amycolatum*, *C. ulcerans*, *C. pseudotuberculosis*, *C. minutissimum* (Hommez *et al.* 1999), *C. ammoniagenes*, *C. vitarumen* and *C. pilosum* (Watts *et al.* 2000) have recently been identified in bovine milk. To date, *C. bovis* is the only lipophilic *Corynebacterium* species that has been isolated in milk of bovine origin, although a new lipophilic species (*C. mastitidis*) has recently been isolated from sheep with subclinical mastitis (Fernandez-Garayzabal *et al.* 1997).

Many mastitis laboratories presumptively diagnose *C. bovis* based on the presence of tiny (less than one millimetre) off-white non-haemolytic colonies after 48-72 hours growth on blood agar cultured at 37°C (Watts *et al.* 2000). Some may strengthen their diagnosis based on Gram stain (Gram-positive), morphology (coryneform), catalase test (positive) and demonstration of enhanced growth with the

addition of Tween 80 to the media. None of these tests will, however, provide a definitive diagnosis of *C. bovis*. For routine bacteriological purposes, the misclassification of some isolates as *C. bovis* is of little importance because *C. bovis* is of limited clinical significance. However, for studies and trials that ascribe either pathogenicity or biological significance to *C. bovis* it is necessary to confirm the identity with other more definitive tests.

In the past confirmation of identity to a species level has been based on the results of biochemical reactions and fermentation profiles (Funke *et al.* 1997), compared with the reactions of type strains. Commercial kits based on this information, composed of a series of biochemical reactions and a database for predicting the most likely identity of the isolate in question, are available *e.g.* API Coryne (bioMérieux sa, Lyon, France). However, biochemical reactions can vary even between members of the same species (Funke *et al.* 1997); identities based on their results are very often a "most likely" rather than a "definitive" diagnosis.

16S rRNA gene sequencing followed by phylogenetic analysis is now recognised as the "reference" technique for differentiating closely related species. It is particularly useful for Corynebacteria because 16S rRNA gene sequence divergence rates are relatively high (Pascual *et al.* 1995; Ruimy *et al.* 1995). The comparison of unknown 16S rRNA sequences with the sequence of the type strain has become the reference method of identification for taxonomic studies (Watts *et al.* 2000). However gene sequencing is still relatively expensive and out-with the capabilities of many laboratories, which makes it an unsuitable technique for large scale studies such as the one described in this thesis.

Restriction endonucleases are a group of enzymes, purified from bacteria, which "cleave" double stranded DNA leaving a 3' hydoxyl group on one side and a 5' phosphate group on the other. Each endonuclease recognises and binds to a short DNA "target" sequence (usually but not exclusively between four and eight base pairs in length) specific to that enzyme and cleaves the DNA either within the target sequence or within a defined distance of it (up to 20 base pairs away). For any given endonuclease, a DNA sequence is restricted at every point in its length at which that specific target sequence occurs. If a piece of DNA does not contain the target sequence, it will not be restricted. The result is a "pattern" of smaller DNA pieces (varying in number and size) specific to the original DNA sequence and the restriction enzyme used. The restriction pattern of DNA fragments can be separated and

visualised by techniques such as agarose gel electrophoresis and ultraviolet trans-illumination after staining with ethidium bromide. Two identical DNA sequences will produce identical restriction patterns after digestion with the same enzyme. Two DNA sequences with minor differences will also restrict in an identical manner providing any alterations between the sequences do not affect the target sequence for the endonuclease employed. As sequences diverge in similarity from each other, the chances that both contain the same number of target sequences at the same position becomes more remote and therefore differences in restriction pattern will become evident.

The extensive use of 16S rRNA gene sequencing as a tool for differentiating closely related species has facilitated the use of endonuclease restriction analysis as a diagnostic tool because the entire 16S sequence of the type strain for nearly all currently recognised species have been published (www.ncbi.nlm.nih.gov). For any species it becomes possible to analyse the available sequence data and predict the number and sizes of DNA fragments that would be produced after restriction with any restriction endonuclease. By database analysis and comparison of the restriction patterns produced with a host of different endonucleases, it is possible to identify enzymes that will differentiate even closely related species based on small variations in the 16S rRNA gene sequence.

The aim of this part of the study was to develop a method of speciating and differentiating *C. bovis* from other lipophilic coryneform bacteria of milk origin based on endonuclease restriction analysis of the 16S rRNA gene sequence.

3.2. Materials and Methods

3.2.1. Bacterial Isolates

During the teat sealer study the author collected a third set of quarter samples from all study animals in the second week post calving (*i.e.* 7-14 post calving) during the weekly farm visit. This set of samples were denoted "Post Calving Samples". The samples were collected to increase the number of *C. bovis* isolates available for analysis in the later parts of this thesis in case the number cultured from calving samples was low. These samples were collected, transported and handled in an

identical manner to those collected at drying off and calving. Samples were submitted for bacteriological and SCC analysis as previously described.

All coryneform organisms isolated from drying off, calving and post calving samples were utilized during the remaining parts of this study.

3.2.2. Characterisation of Coryneform Isolates as Lipophilic or Non-Lipophilic

Coryneform isolates were recovered from storage at –80°C by placing one storage bead in ten millilitres of brain heart broth (Merck, Darmstadt, Germany) supplemented with one percent v/v Polyoxyethylene Sorbitans Mono-oleate (Tween 80, Sigma, St Louis, USA), in a sterile universal container. Broth was incubated aerobically at 37°C and examined every 24 hours for the presence of visible growth, for up to 120 hours. Cultures containing visible growth were streaked out onto brain heart agar (Merck, Darmstadt, Germany) with and without the presence of one percent Tween 80 v/v to differentiate lipophilic and non-lipophilic isolates (Riegel *et al.* 1995). Plates were compared after aerobic incubation at 37°C for 48 and 72 hours.

Broth cultures containing more than one bacterial isolate were discarded and recultured from bead stocks until a pure culture was obtained. Lipophilic species were considered those that demonstrated no or minimal growth on agar without Tween 80 and increased or luxuriant growth on agar containing Tween 80.

3.2.3. Compilation of a 16S rRNA Gene Sequence Database

A 16S rRNA gene sequence database was created from published data for all lipophilic *Corynebacterium* species. Sequences were imported from "GenBank", held by the "National Centre for Biotechnology Information" (www.ncbi.nlm.nih.gov), into sequence manipulation software (Omiga 2.0, Oxford Molecular Ltd). At least one 16S rRNA gene sequence was available for the ten validated lipophilic species (*C. accolens*, *C. afermentans* subsp. *lipophilum*, *C. bovis*, group F-1, group G-2, *C. jeikeium*, *C. lipophiloflavum*, *C. macginleyi*, *C. mastitidis* and *C. urealyticum*) and the three species whose presence within the genus has been proposed but never validated (*"C. genitalium"*, *"C. pseudogenitalium"* and *"C. tuberculostericum"*).

3.2.4. Universal Primer Design

Sequences downloaded into the database were aligned using the sequence manipulation software. Universal forward and reversed primers were designed and produced (Invitrogen Custom Primers, Invitrogen Life Technologies) outside the hyper-variable regions and denoted "coryneallfor" and "coryneallrev" (Figure 3.1.). Primers were designed to have a G plus C content of 50 to 55 percent, similar melting temperatures (approximately 60°C) and at least one G or C at the 3' end. Homopolymeric regions, sequence repeats and self complimentary areas were avoided.

Sequences within the database were "trimmed" at the 5' and 3' ends, so they all began and ended with the two primer sequences. Trimmed sequences ranged in length from 1181 to 1241bp.

Figure 3.1: Oligonucleotide Primers, "Coryneallfor" and "Coryneallrev"

Forward Primer

"Coryneallfor" 5' – GCG AAC GGG TGA GTA ACA CG – 3'

Reverse Primer

"Coryneallrev" 5' – TCT GCG ATT ACT AGC GAC TCC G – 3'

3.2.5. Prediction of Restriction Endonuclease Digest Patterns

Using the database, restriction endonuclease digest patterns (number and size of fragments) were predicted for the following common enzymes: *Alu* I, *Bam* HI, *Bg* II, *Bg* III, *Eco* RI, *Eco* RV, *Hae* III, *Hind* III, *Hinf* I, *Kpn* I, *Mlu* I, *Msp* I, *Nco* I, *Not* I, *Nru* I, *Pst* I, *Pvu* II, *Rsa* I, *Sal* I, *Sfi* I, *Sma* I, *Sph* I, *Taq* I, *Xba* I and *Xho* I.

Enzymes were excluded from further analysis if they cut the sequence into many small fragments or if all/most species within the database were cut the same number of times and/or produced fragments of the same size. *Sma* I, *Pst* I, *Hind* III, *Mlu* I, *Pvu* II, *Rsa* I and *Nru* I were identified as potentially the most useful endonucleases. For these seven enzymes the number and size of fragments were compared for all lipophilic species. Based on the number and size of fragments, it was predicted that endonuclease restriction with *Sma* I and *Hind* III could be used to differentiate *C. bovis* from all other lipophilic species. Other lipophilic species could

be differentiated from each other using *Sma* I and *Hind* III plus *Pst* I, *Rsa* I, *Pvu* II and *Mlu* I (Table 3.1.). The database-predicted restriction patterns for two of the species not currently validated (*C. "genitalium"* and *C. "pseudogenitalium"*) were very variable indicating these sequences may well represent more than one species.

3.2.6. Extraction of Genomic DNA

DNA extraction was carried out based on a method described by Vaneechoutte *et al* (1995). After 48 hours incubation, two colonies were "picked" from a purity plate, using a sterile disposable plastic pick. Cellular mass was placed in 0.5ml of double distilled water in a 1.5ml sterile epindorff tube and emulsified by vortexing for 30 seconds. Epindorffs were placed in a boiling water bath for ten minutes to disrupt cellular membranes and release genomic DNA. Tubes were allowed to cool, agitated by vortexing and centrifuged briefly to pellet cellular debris.

3.2.7. Polymerase Chain Reaction

DNA was amplified by the polymerase chain reaction (PCR) using *Taq* DNA polymerase in a PCR premix solution (*Taq* PCR Mastermix, Qiagen). The premix solution provided a final concentration of 1.5μM magnesium chloride and 200μM of each dNTP. Amplification of the 16S rRNA gene sequence was performed using the primer pair "Coryneallfor" and "Coryneallrev" (Figure 3.1.) by the following methodology.

1. 2.5μl of boiled product was added to 12.5μl of PCR Mastermix (Qiagen), 7.5μl ultra-pure water (prepared in an Elgastat UHQPS to 18MΩ/cm purity) and 2.5μl of a working oligonucleotide solution (0.02μg primer per microlitre, to provide a working primer concentration in the reaction of 0.3 molar)
2. The PCR was performed in a Techne Genius FGEN02TD thermocycler with a heated lid using the following temperature regime:
    a) 94°C for 2 min – (Initial "melt")
    b) 94°C for 1min – (Melting)
    c) 56°C for 1 min – (Annealing)
    d) 72°C for 2.5 min – (Extension)
    (b – d were repeated for 35 cycles)

Table 3.1: Predicted Number of Restriction Sites and Size of Fragments Produced (Italics), in Base Pairs, After Endonuclease Restriction of Lipophillic *Corynebacterium* Species 16S rRNA Gene Sequences with *Hind* III, *Sma* I, *Pst* I, *Mlu* I, *Pvu* II and *Rsa* I

	bovis AJ222965*	*bovis* X84444*	*bovis* AF311389*	*accolens* X80500	*afermentans* X81874	*afermentans* X82054	*afermentans* X82055	F-1 X81904	F-1 X81905	G-2 X80498	"*genitalium*" X84253	"*genitalium*" U87817	"*genitalium*" U87818	"*genitalium*" U87819	"*genitalium*" U87820	"*genitalium*" U87821	"*genitalium*" U87824	*jeikeium* X84250	*jeikeium* X82062	*jeikeium* U87815	*jeikeium* U87816	*jeikeium* U87823	*lipophiloflavum* Y09045	*macginleyi* X80499	*mastitidis* Y09806	"*pseudogenitalium*" X81872	"*pseudogenitalium*" U87822	*urealyticum* X81913	*urealyticum* X84439	"*tuberculostericum*" X84247
Hind III	1 *346* *888*	1 *346* *886*	1 *345* *889*	2 *48* *299* *888*	0	0	0	2 *48* *45* *1132*	2 *48* *58* *1132*	1 *347* *888*	0	0	0	0	0	0	1 *331* *867*	0	0	0	0	0	2 *48* *58* *1131*	2 *48* *299* *888*	2 *48* *58* *1131*	3 *48* *58* *241* *889*	1 *328* *880*	0	0	1 *347* *888*
Sma I	1 *488* *746*	1 *488* *746*	1 *487* *747*	0	0	0	0	0	0	0	0	0	1 *464* *734*	0	0	0	0	1 *494* *747*	1 *493* *747*	1 *485* *737*	1 *472* *732*	1 *483* *740*	0	0	0	0	0	0	0	0
Pst I	0	0	0	1 *342* *893*	1 *512* *724*	1 *512* *724*	1 *512* *724*	1 *512* *726*	1 *513* *726*	0	1 *341* *891*	0	1 *338* *860*	2 *343* *377* *488*	1 *333* *871*	1 *334* *864*	1 *338* *860*	1 *343* *893*	1 *343* *897*	1 *338* *884*	1 *336* *868*	1 *341* *882*	0	1 *343* *892*	0	1 *512* *724*	0	1 *344* *892*	1 *344* *892*	0
Mlu I	0	0	0	0	1 *107* *1129*	1 *107* *1129*	1 *107* *1129*	0	0	0	0	0	1 *482* *716*	1 *108* *1100*	0	0	0	0	0	0	0	0	1 *108* *1129*	1 *106* *1129*	0	0	0	0	0	0
Pvu II	0	0	0	0	0	0	0	0	0	0	0	1 *261* *920*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 *271* *937*	0	0	0
Rsa I	4 *110* *160* *201* *356* *407*	4 *110* *160* *201* *356* *407*	4 *110* *160* *200* *357* *407*	5 *110* *151* *161* *202* *203* *408*	5 *111* *151* *162* *201* *204* *407*	5 *111* *151* *162* *201* *204* *407*	5 *111* *151* *162* *201* *204* *407*	6 *37* *108* *111* *161* *165* *248* *408*	6 *37* *108* *111* *162* *165* *248* *408*	5 *110* *151* *172* *203* *236* *363*	5 *110* *150* *160* *202* *203* *407*	6 *19* *109* *133* *146* *172* *198* *404*	5 *110* *144* *147* *198* *200* *399*	5 *112* *142* *148* *201* *202* *403*	6 *36* *110* *113* *141* *195* *200* *409*	5 *107* *145* *146* *199* *199* *402*	4 *19* *146* *291* *346* *396*	5 *110* *150* *165* *202* *205* *409*	5 *110* *150* *164* *202* *205* *409*	5 *110* *145* *159* *200* *202* *406*	4 *143* *148* *200* *309* *404*	7 *7* *44* *103* *110* *157* *200* *203* *399*	6 *37* *112* *150* *161* *165* *205* *407*	5 *110* *152* *160* *202* *203* *408*	4 *111* *150* *202* *205* *569*	5 *37* *111* *161* *165* *355* *407*	7 *26* *37* *109* *111* *145* *200* *237* *343*	3 *151* *315* *364* *406*	3 *151* *315* *364* *406*	6 *37* *110* *151* *172* *203* *236* *326*

**The sequence data from 49 C. bovis isolates was available for analysis, 3 representative isolates are presented here, fragment patterns for the other 46 isolates are presented in Table AIII.I (Appendix III)*

e) 72°C for 10 min – (Final extension)

Negative controls were generated by replacing extracted DNA solution with 2.5μl of double distilled water.

3.2.8. PCR Product Purification

The resulting PCR products were purified using a commercially available filter kit (Microcon PCR Centrifugal Filter Devices, Millipore Corporation, Bedford, MA, USA), according to the manufactures instructions. PCR product was recovered in 20μl ultra-pure water and frozen at –20°C until required.

3.2.9. Gel Electrophoresis

Purified PCR product was visualized by agarose gel electrophoresis. One percent (w/v) agarose gels were prepared by melting agarose (Agarose, Transgenomic, Crewe, UK) in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.5) by heating in a microwave oven. Liquid gels were allowed to cool (<60°C) before casting in gel apparatus (Jencons HU13). Prior to loading, DNA samples were diluted in ten times loading buffer (50% v/v glycerol, one percent w/v xylene cyanole FF, one percent w/v orange G in ultra pure water). Electrophoresis was carried out in 1x TAE buffer at between four and six volts per centimetre. Gels were stained with ethidium bromide, visualized by ultraviolet transillumination and photographed using a gel documentation system (UVP, Duel Intensity Tranilluminator GDS 7500, Cambridge, UK). Gel images were captured and manipulated using GRAB IT 2.59 (Synoptics Ltd).

3.2.10. Restriction Endonuclease Cleavage

Purified PCR product was cleaved using *Hind* III and *Sma* I restriction endonuclease enzymes. Digests were performed in ten microlitre reactions in the buffer supplied by the manufacturer (*Hind* III (Buffer SB) and *Sma* I (Buffer SA), Sigma, Saint Louis, USA), with 2.5 units of enzyme (*Hind* III and *Sma* I restriction endonucleases, Sigma, Saint Louis, USA) and approximately 500ng of DNA. Reactions were incubated for at least six hours and usually over night at 37°C (*Hind* III) and 25°C (*Sma* I). Cleavage

products were separated, visualized and photographed in one percent agarose gels as previously described.

The number and size of cleavage products were estimated by comparison to a DNA molecular weigh marker ladder (GeneRuler 1Kb DNA Ladder, MBI Fermentas). Samples that did not restrict in the database pattern predicted for *C. bovis* were further cleaved with *Mlu* I, *Pvu* II, *Pst* I and *Rsa* I restriction endonucleases employing a protocol similar to that previously described for *Hind* III and *Sma* I using buffer and reaction conditions recommended by the manufacturer (Sigma, Saint Louis, Missouri, USA).

Results of restriction were electrophoresed in one percent (*Mlu* I, *Pvu* II, *Pst* I) and three precent (*Rsa* I) gels (to allow increased separation and differentiation of small DNA fragments produced by restriction with *Rsa* I). Restriction patterns were compared to database predictions to allow species identification.

The first lane of every gel was loaded with a negative control restriction reactions containing all components of the reaction except PCR product. In cases where test PCR product was not cleaved by the endonuclease enzyme in question the digests were repeated including positive control reactions to demonstrate that the enzyme was functioning. The two positive control reactions contained either a PCR product or a plasmid that the enzyme had previously been shown to restrict. The first positive control contained all components of the reaction except the endonuclease; the second contained all components including the endonuclease enzyme. Where their presence was necessary positive control reactions were loaded into lanes 2 and 3 of the gel.

3.2.11. Reference Strains

Six *C. bovis* reference strains were obtained from a culture collection held by the Veterinary Laboratories Agency, England, and processed in the manner outlined above, to act as control isolates for the procedure.

3.3. RESULTS

3.3.1. Screening Sample Isolates

Nine hundred and thirty nine coryneform isolates were identified in screening samples during the teat sealer study. Of these, six were not available for further analysis. Of the 933 isolates examined 762 (81.7%) were lipophilic and 171 (18.3%) were non-lipophilic (Figure 3.2.).

Of the 762 lipophilic isolates, 741 (97.2%) restricted in the manner predicted for *C. bovis, i.e. Hind* III and *Sma* I cut the PCR product once producing fragments of approximately 350 and 890bp (Representative examples are given in Figure 3.3) and 490 and 750bp (Representative examples are given in Figure 3.4) respectively. Twenty one (2.8%) lipophilic isolates did not restrict in the pattern predicted for *C. bovis.*

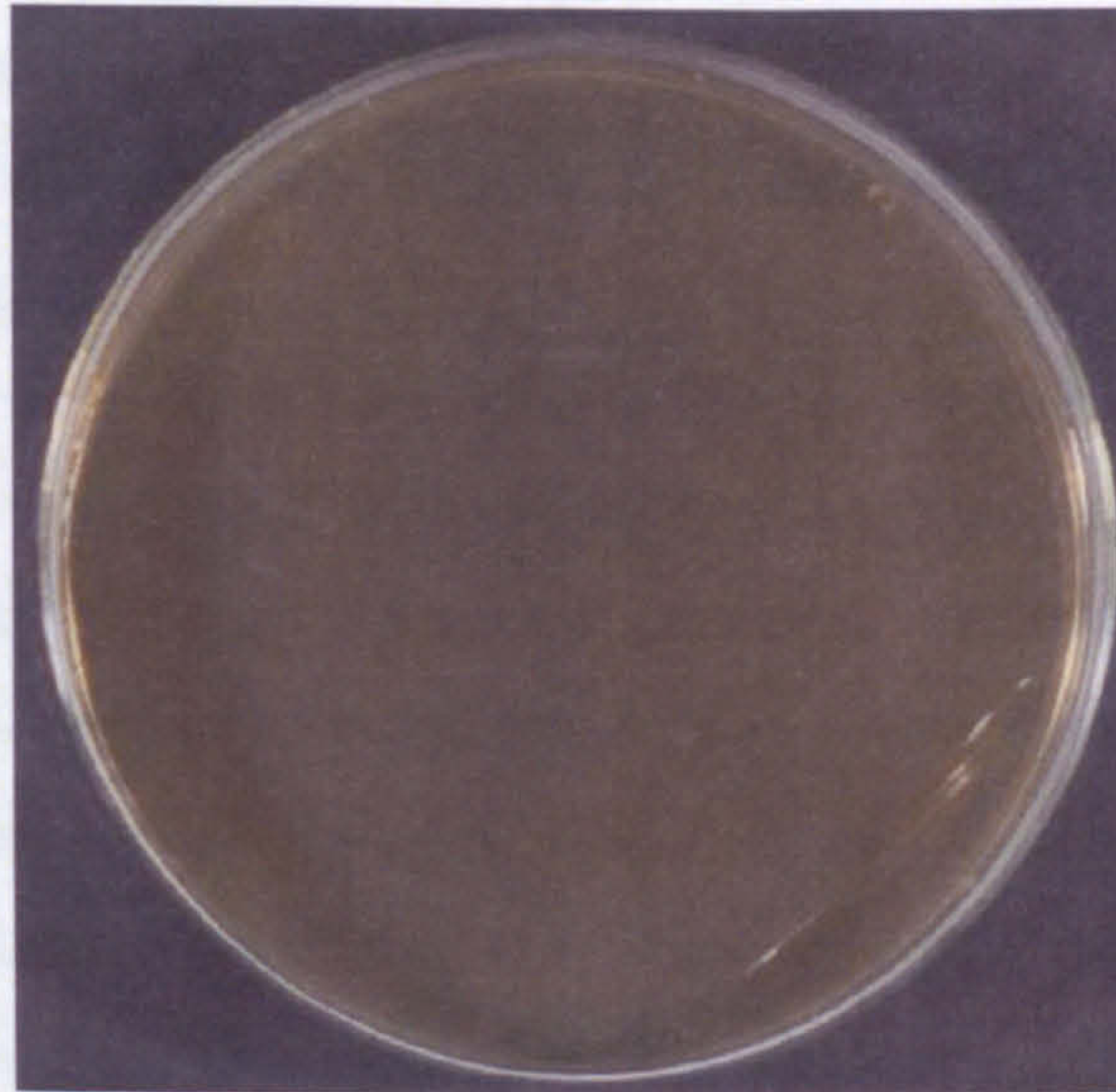
3.3.2. Atypical Lipophilic Isolates

Twenty three isolates (21 screening and two clinical mastitis) did not restrict in the pattern predicted for *C. bovis.* Of these, 20 were not cut by *Hind* III (Representative examples are given in Figure 3.5) but were cut in an identical fashion to *C. bovis* isolates with *Sma* I (Representative examples are given in Figure 3.6), two were not cut by either enzyme (Representative examples are given in Figures 3.7 & 3.8) and one was not cut by *Sma* I but was cut in an identical fashion to *C. bovis* isolates with *Hind* III (Representative examples are given in Figures 3.9 & 3.10).

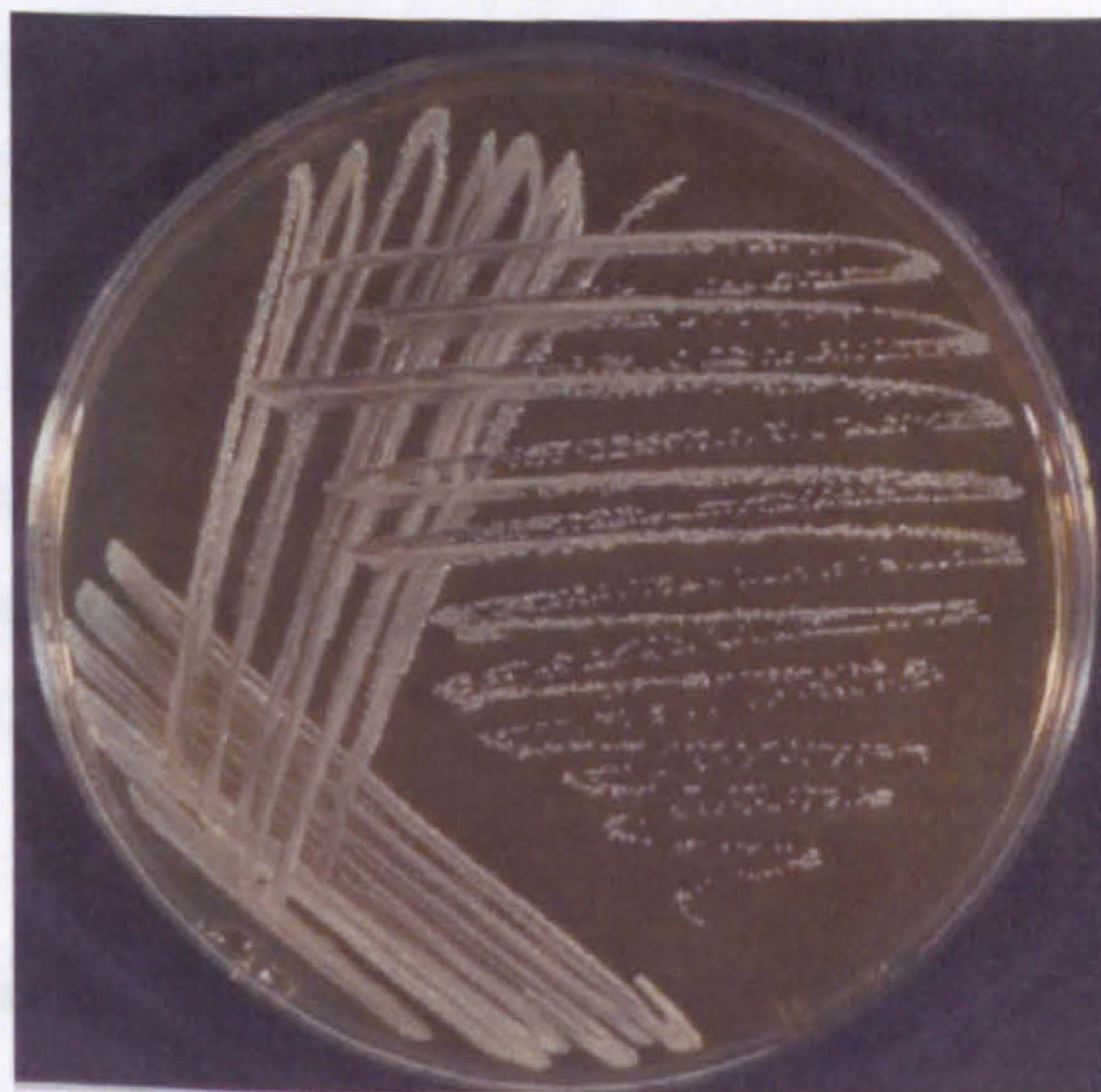
3.3.2.1. Hind III Negative, Sma I Positive Isolates (20 Isolates)

After *Hind* III and *Sma* I restriction, database predictions indicated that these isolates were either *C. jeikeium* or *"C. genitalium"* (Accession number U87818) (Table 3.1). These isolates were further restricted with *Pst* I and *Mlu* I to confirm their identity. *Pst* I and *Mlu* I did not cleave any of these three isolates (Representative examples are given in Figures 3.11 & 3.12) and they did not restrict in the pattern predicted for *C. jeikeium, "C. genitalium"* (Accession number U87818) or any other lipophilic species present within the data base.

Figure 3.2: Differentiation of Lipophilic From Non-Lipophilic *Corynebacterium* Species Based on Their Differential Growth Characteristics on Brain Heart Agar With and Without the Addition of Tween 80



a. *C. bovis* growing on brain heart agar without Tween 80 after 48 hours incubation at 37°C (Lipophilic species demonstrate very poor or negligible growth on media which do not contain free fatty acid).



b. *C. bovis* growing on brain heart agar with one percent v/v Tween 80 after 48 hours incubation at 37°C.

3.3.2.2. Hind III Negative, Sma I Negative Isolates (Nos. 1120 & 5525)

After *Hind* III and *Sma* I restriction, database predictions indicated that these isolates were *C. afermentans*, *"C. genitalium"* (Accession numbers X84253, U87817, U87819, U87820, U87821) or *C. urealyticum* (Table 3.1). These isolates were further restricted with *Pst* I and *Rsa* I.

Isolate 5525 was cut once with *Pst* I, producing fragments of approximately 350 and 875bp (Figure 3.13) and four times with *Rsa* I producing fragments of approximately 110, 160, 205, 380 and 405bp in length (Figure 3.14).

Isolate 1120 was not cut by *Pst* I (Figure 3.13) but restricted multiple times with *Rsa* I producing fragments of approximately 110, 160, 200 and 405 bp in length (Figure 3.14). The fragment sizes did not add up to the approximate 1250bp PCR product, however the 160bp band is broad and is possibly composed of three fragments of approximately equal size (Compared to 5525, the 380bp fragment was not present, therefore a possible extra restriction site may exist close to the mid point of this fragment).

The restriction profiles for 1120 and 5525 did not fit any database predictions for currently recognised lipophilic *Corynebacterium* species.

3.3.2.3. Hind III Positive, Sma I Negative Isolate (No. 5724)

After *Hind* III and *Sma* I restriction, database predictions indicated that this isolate was type G-2, *"C. genitalium"* (U87824), *"C. pseudogenitalium"* (U87822) or *"C. tuberculostericum"* (Table 3.1).

Isolate 5724 was further restricted with *Pvu* II and *Pst* I; it was not cut with *Pvu* II (Figure 3.16) but restricted once with *Pst* I (Figure 3.15) producing fragment sizes of approximately 350 and 850bp in length. The restriction patterns of 5724 produced an overall profile consistent with data base predictions for *"C. genitalium"* accession number U87824.

3.3.3. Reference Strains

All six isolates obtained from the Veterinary Laboratory Agencies culture collection restricted in the manner predicted for *C. bovis* confirming the validity of the identification system on reference cultures.

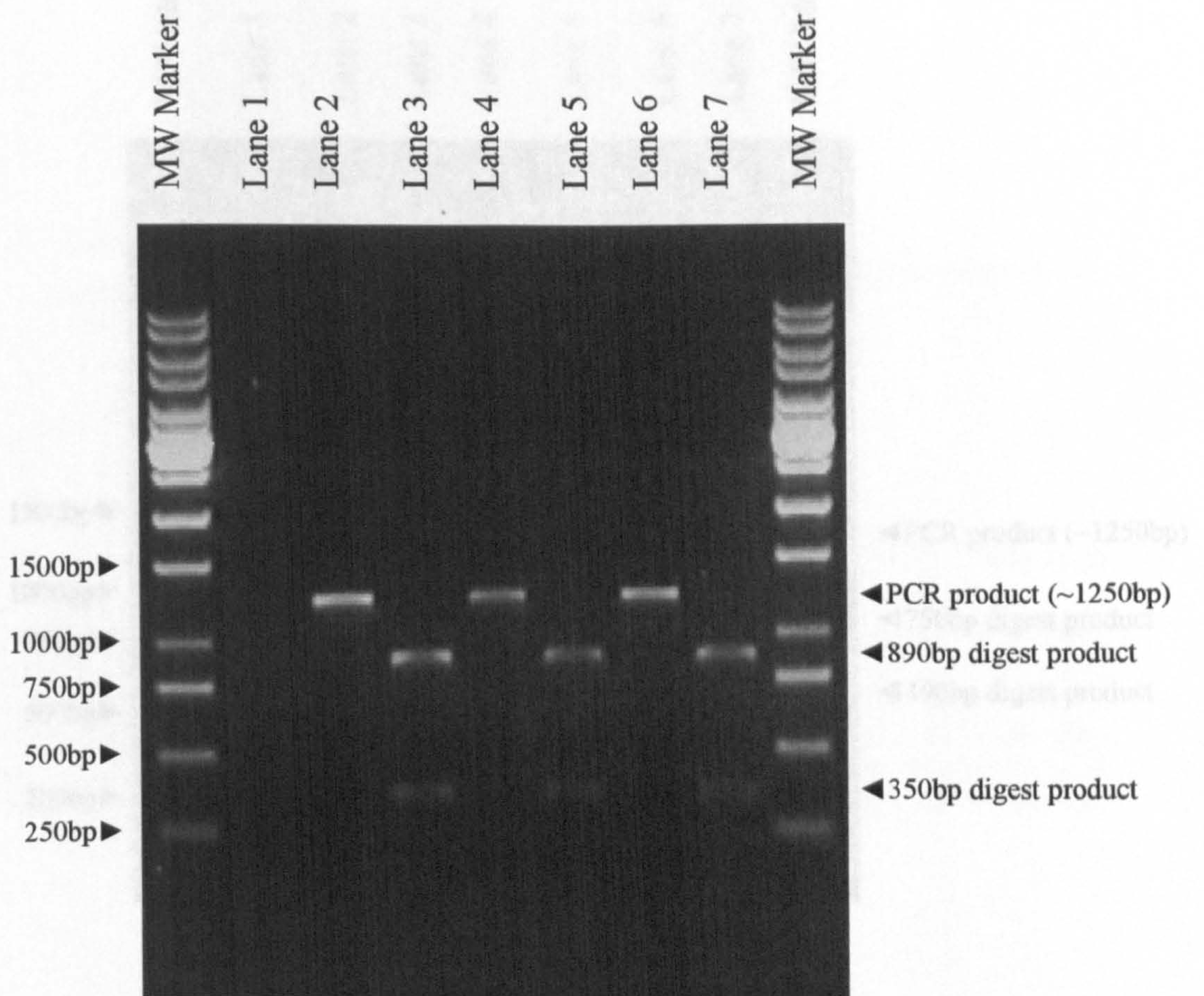


Figure 3.3: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of *C. bovis* After Endonuclease Restriction With *Hind* III

Hind III digestion of the 16S rRNA PCR product of *C. bovis* produced two digest products (MW ~ 350bp and 890bp), visualisation here on a one percent agarose gel. Lane 1: Negative control (*Hind* III digest reaction with no DNA), Lane 2: Isolate 47 (-ve control, no *Hind* III), Lane 3: Isolate 47 after *Hind* III digestion, Lane 4: Isolate 462 (-ve control, no *Hind* III), Lane 5: Isolate 462 after *Hind* III digestion, Lane 6: Isolate 3933 (-ve control, no *Hind* III), Lane 7: Isolate 3933 after *Hind* III digestion.

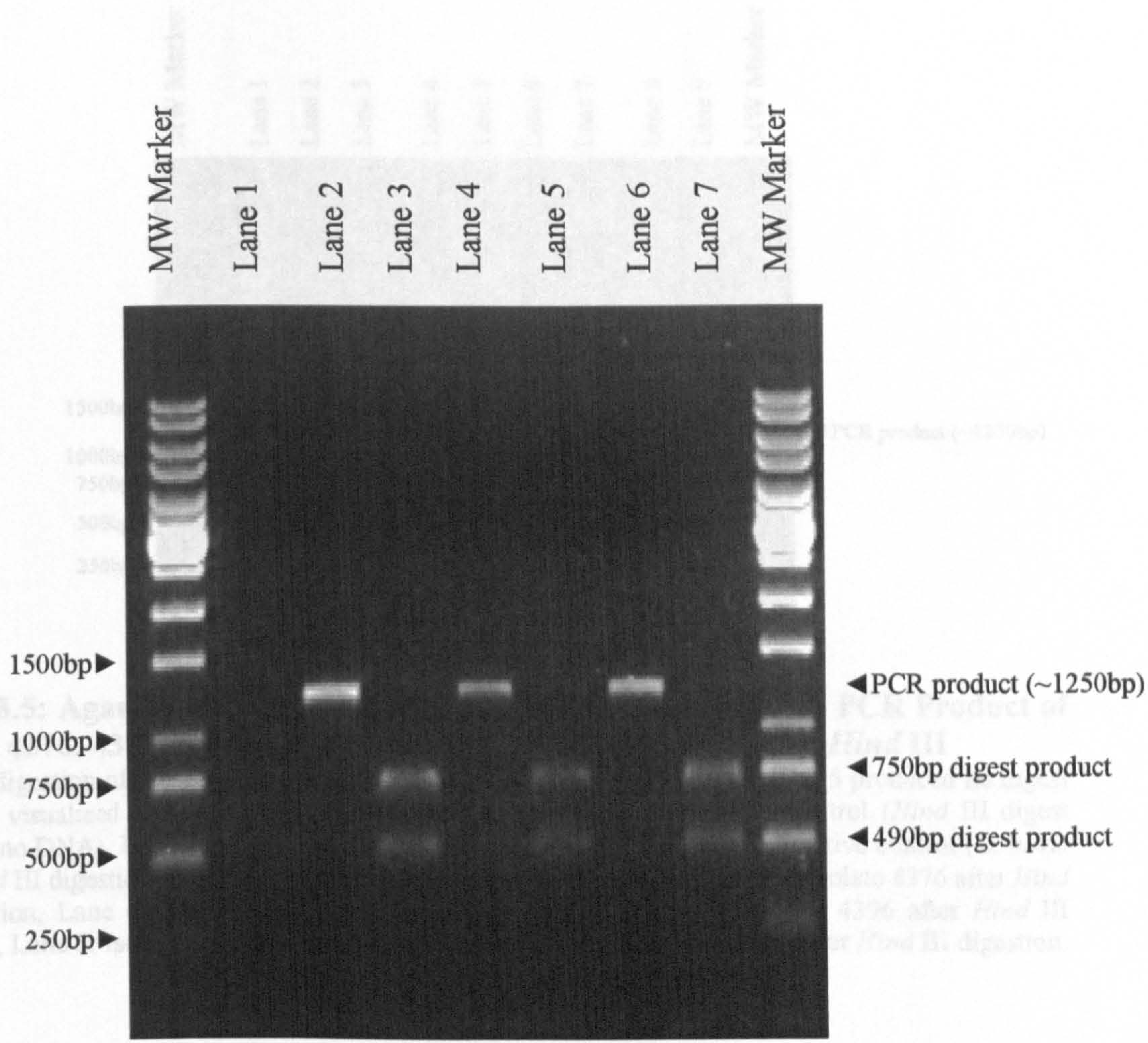


Figure 3.4: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of *C. bovis* After Endonuclease Restriction With *Sma* I

Sma I digestion of the 16S rRNA PCR product of *C. bovis* produced two digest products (MW ~ 490bp and 750bp), visualisation here on a one percent agarose gel. Lane 1: Negative control (*Sma* I digest reaction with no DNA), Lane 2: Isolate 47 (-ve control, no *Sma* I), Lane 3: Isolate 47 after *Sma* I digestion, Lane 4: Isolate 462 (-ve control, no *Sma* I), Lane 5: Isolate 462 after *Sma* I digestion, Lane 6: Isolate 3933 (-ve control, no *Sma* I), Lane 7: Isolate 3933 after *Sma* I digestion.

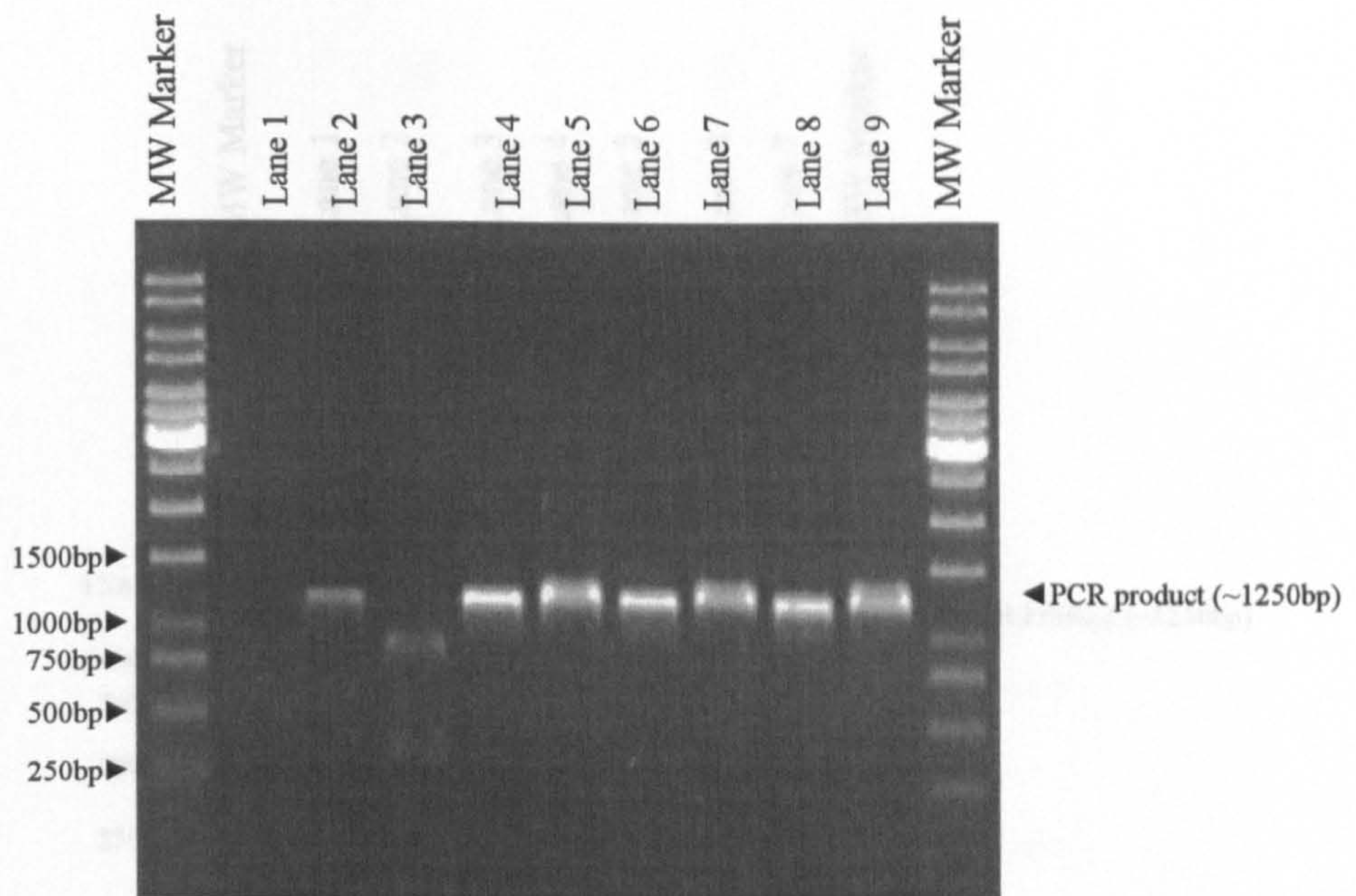


Figure 3.5: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 4376, 4396 & 4465 After Endonuclease Restriction With *Hind* III

Hind III digestion of the 16S rRNA PCR product of isolates 4376, 4396 and 4465 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Hind* III digest reaction, no DNA), Lane 2: Positive control (*C. bovis*, no *Hind* III), Lane 3: Positive control (*C. bovis* after *Hind* III digestion), Lane 4: Isolate 4376 (-ve control, no *Hind* III), Lane 5: Isolate 4376 after *Hind* III digestion, Lane 6: Isolate 4396 (-ve control, no *Hind* III), Lane 7: Isolate 4396 after *Hind* III digestion, Lane 8: Isolate 4465 (-ve control, no *Hind* III), Lane 9: Isolate 4465 after *Hind* III digestion.

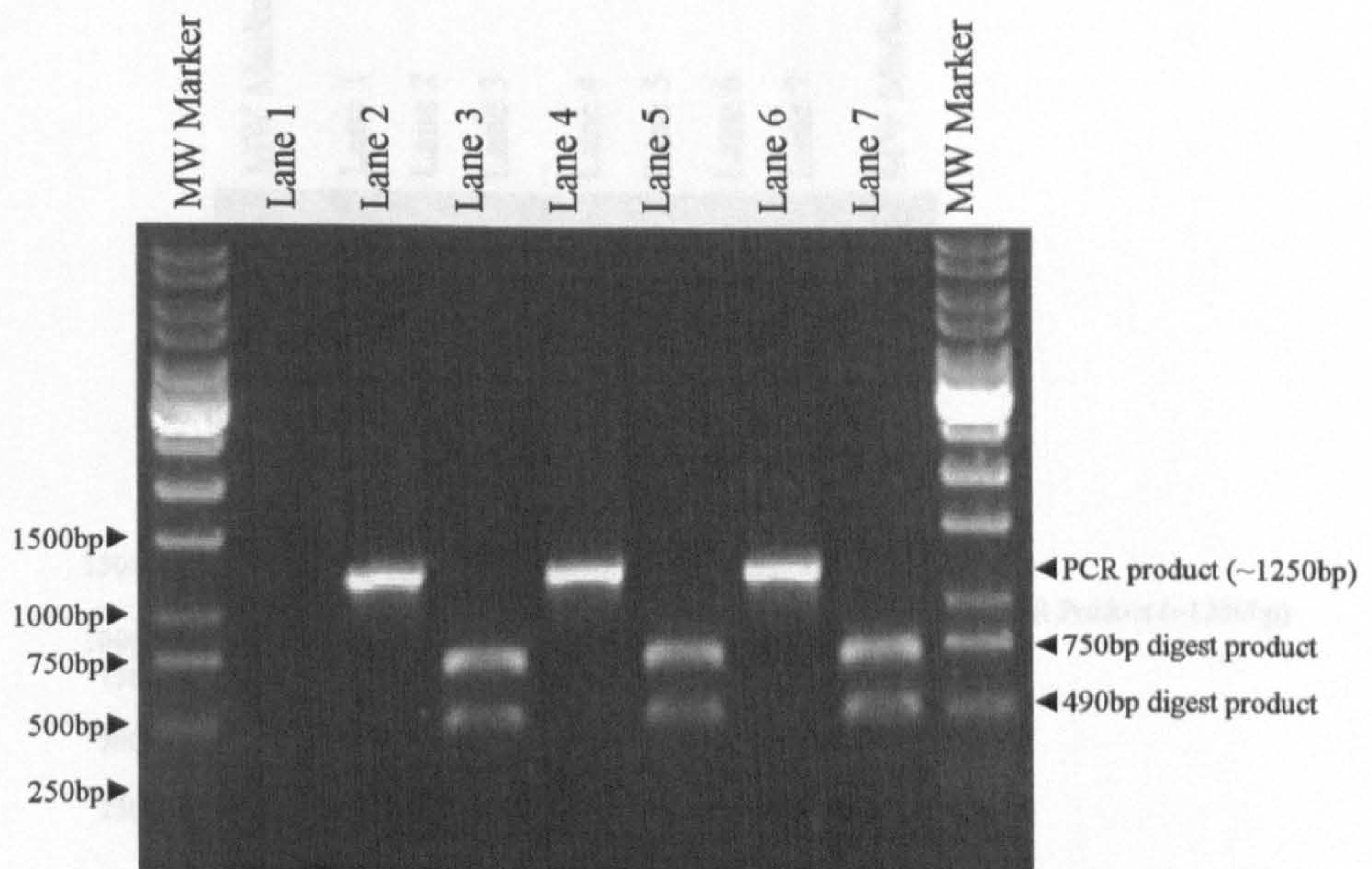


Figure 3.6: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 4376, 4396 & 4465 After Endonuclease Restriction With *Sma* I

Sma I digestion of the 16S rRNA PCR product of isolates 4376, 4396 & 4465 produced two digest products (MW ~ 490bp and 750bp), visualised here on a one percent agarose gel. Lane 1: Negative control (*Sma* I digest reaction, no DNA), Lane 2: Positive control (*C. bovis*, no *Sma* I), Lane 3: Positive control (*C. bovis* after *Sma* I digestion), Lane 4: Isolate 1120 (-ve control, no *Sma* I), Lane 5: Isolate 1120 after *Sma* I digestion, Lane 6: Isolate 5525 (-ve control, no *Sma* I), Lane 7: Isolate 5525 after *Sma* I digestion.

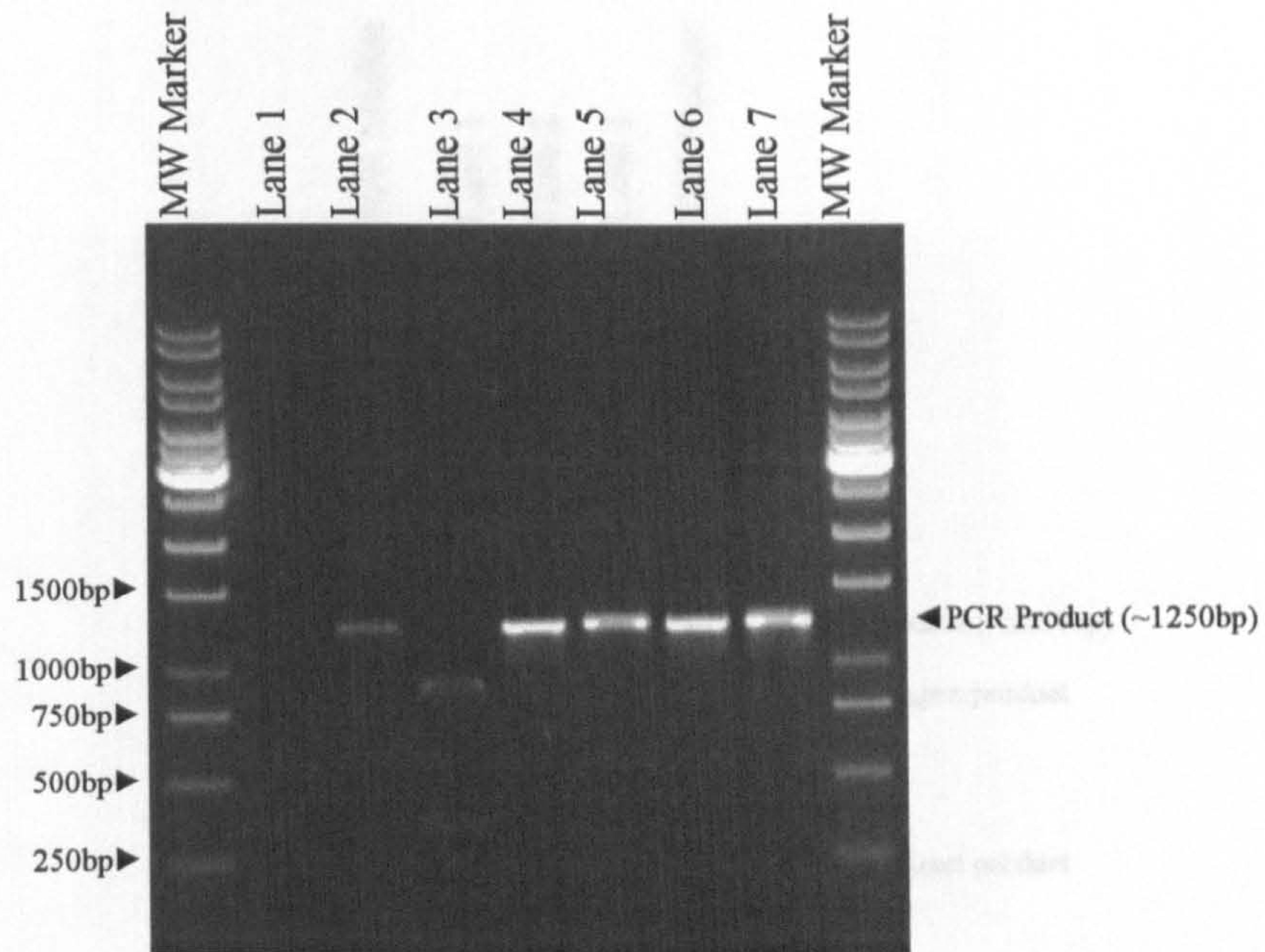


Figure 3.7: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 1120 & 5525 After Endonuclease Restriction With *Hind* III

Hind III digestion of the 16S rRNA PCR product of isolates 1120 & 5525 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Hind* III digest reaction with no DNA), Lane 2: Positive control (*C. bovis*, no *Hind* III), Lane 3: Positive control (*C. bovis* after *Hind* III digestion, Lane 4: Isolate 1120 (-ve control, no *Hind* III), Lane 5: Isolate 1120 after *Hind* III digestion, Lane 6: Isolate 5525 (-ve control, no *Hind* III), Lane 7: Isolate 5525 after *Hind* III digestion.

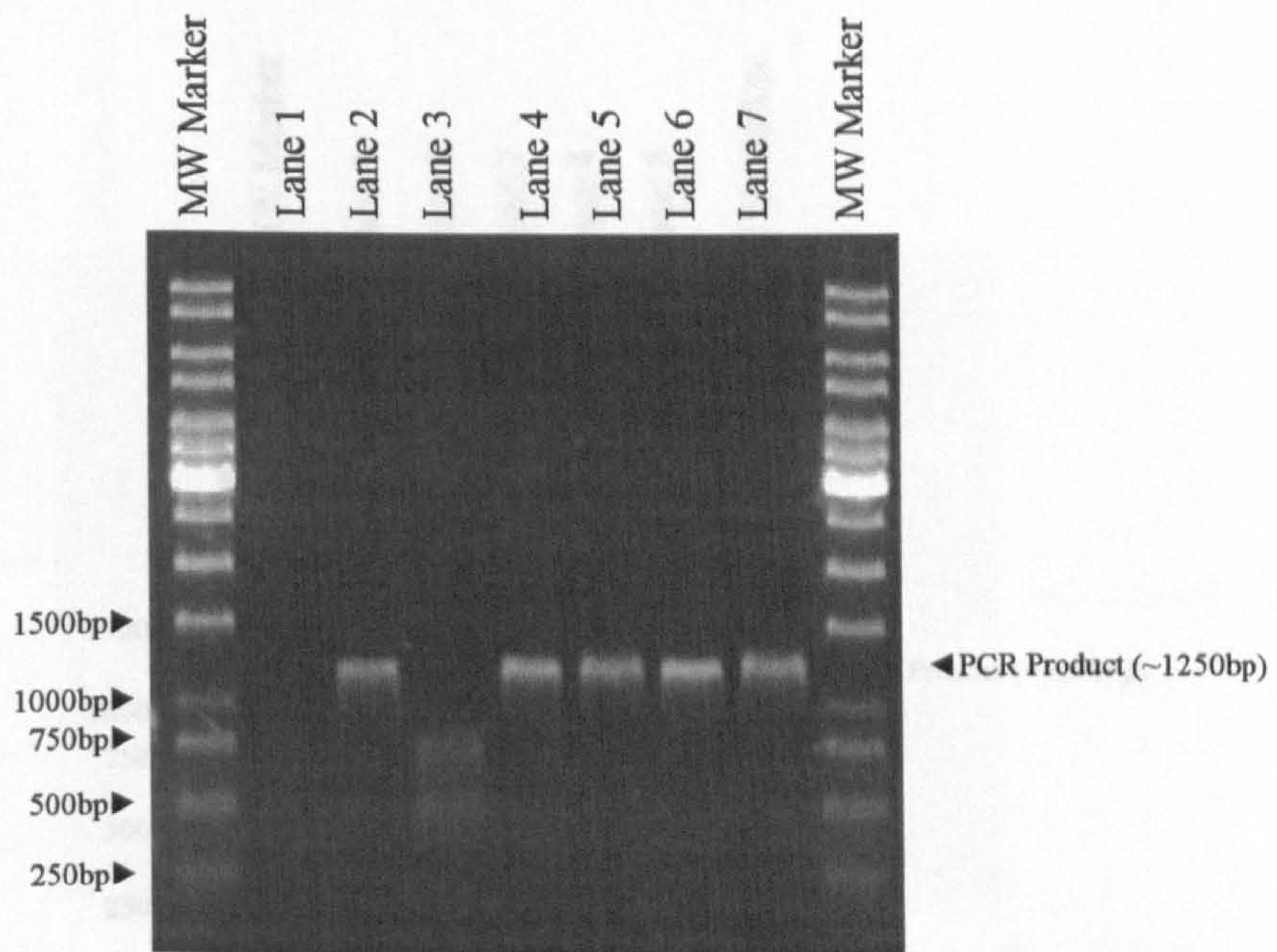


Figure 3.8: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 1120 & 5525 After Endonuclease Restriction With *Sma* I

Sma I digestion of the 16S rRNA PCR product of isolates 1120 & 5525 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Sma* I digest reaction with no DNA), Lane 2: Positive control (*C. bovis*, no *Sma* I), Lane 3: Positive control (*C. bovis* after *Sma* I digestion), Lane 4: Isolate 1120 (-ve control, no *Sma* I), Lane 5: Isolate 1120 after *Sma* I digestion, Lane 6: Isolate 5525 (-ve control, no *Sma* I), Lane 7: Isolate 5525 after *Sma* I digestion.

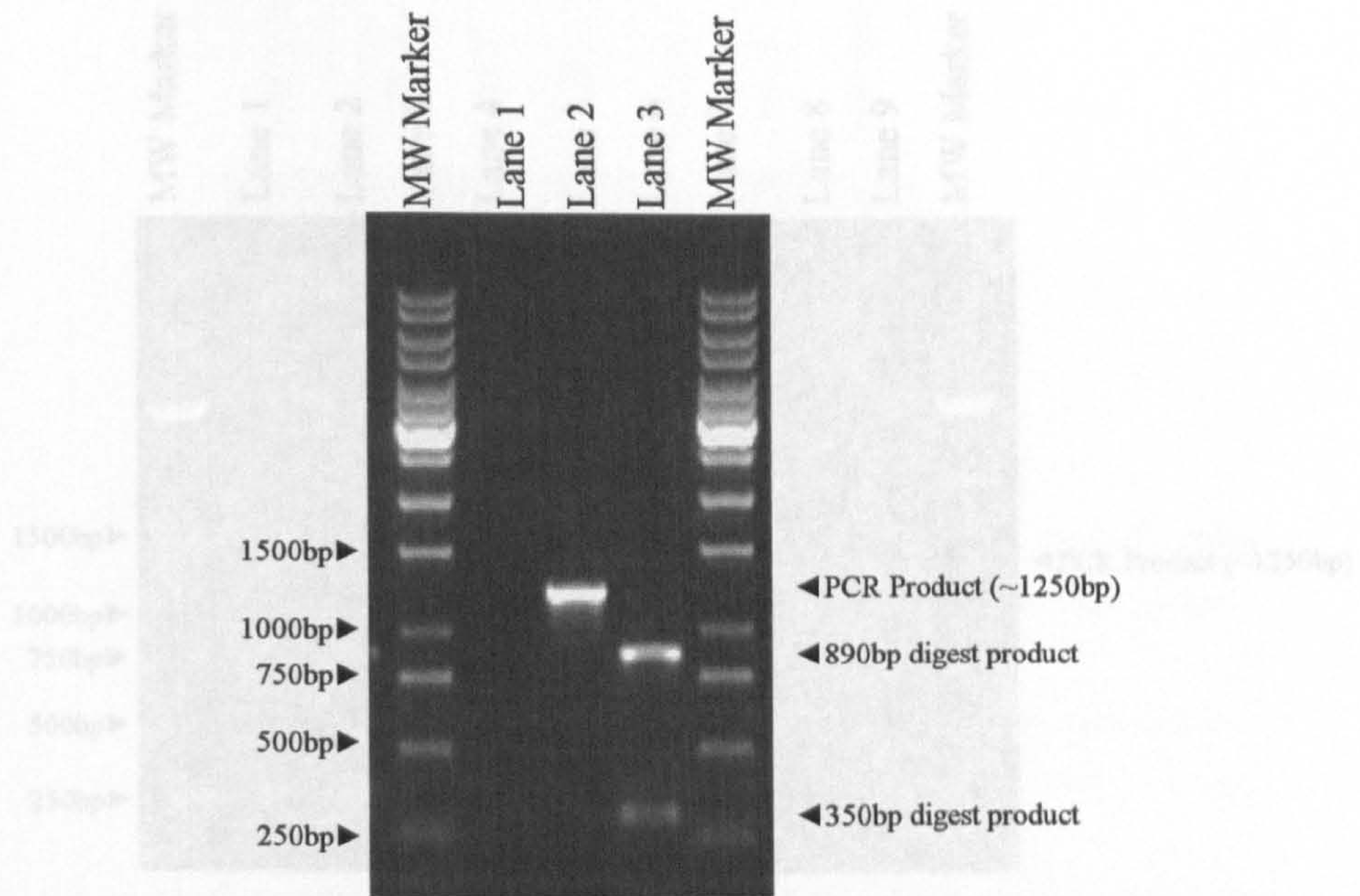


Figure 3.9: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolate 5724 After Endonuclease Restriction With *Hind* III

Hind III digestion of the 16S rRNA PCR product of isolate 5724 produced two digest products (MW ~ 350bp and 890bp), visualised here on a one percent agarose gel. Lane 1: Negative control (*Hind* III digest reaction with no DNA), Lane 2: Isolate 5724 (-ve control, no *Hind* III), Lane 3: Isolate 5724 after *Hind* III digestion.

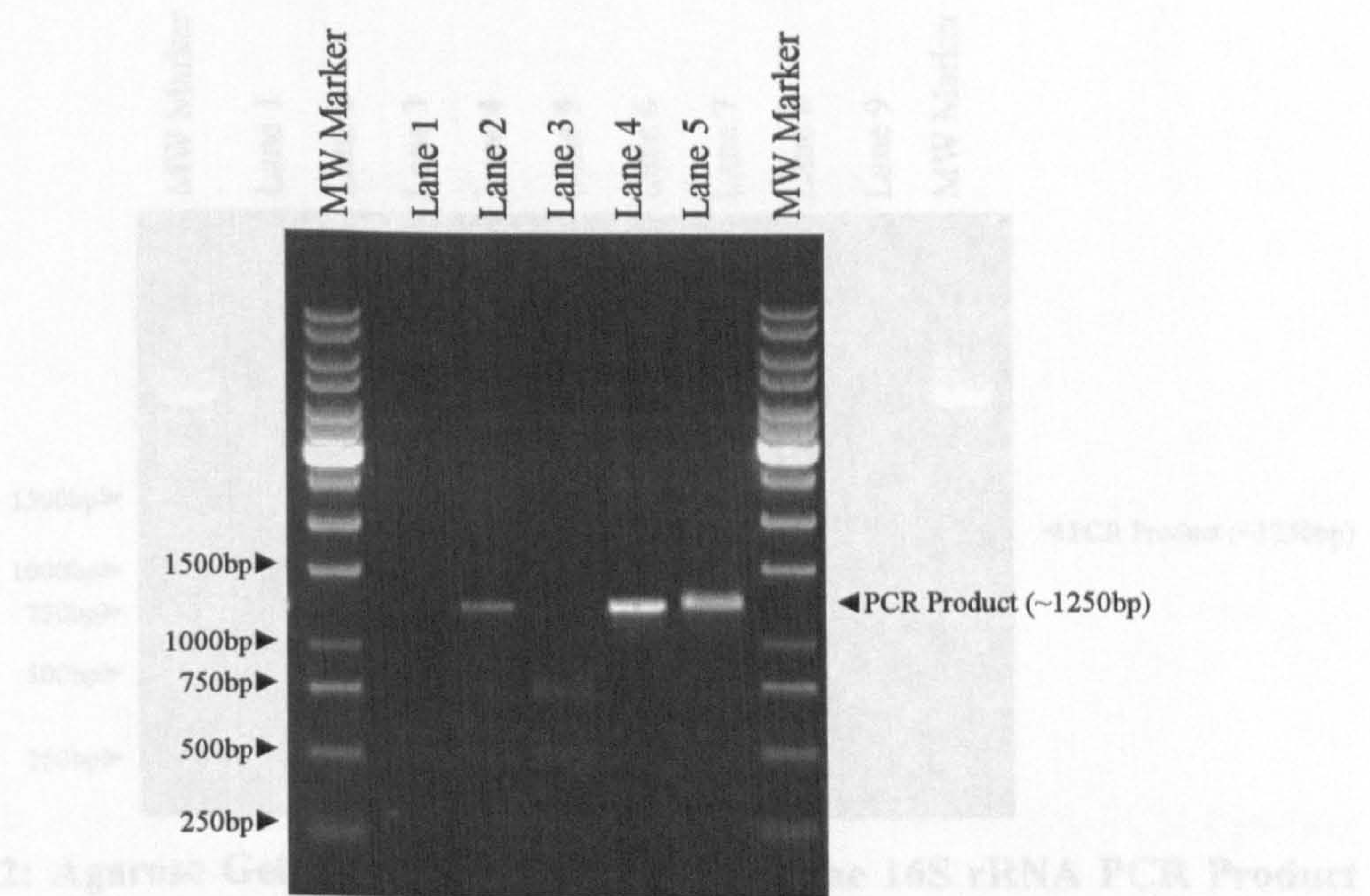


Figure 3.10: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolate 5724 After Endonuclease Restriction With *Sma* I

Sma I digestion of the 16S rRNA PCR product of isolate 5724 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Sma* I digest reaction with no DNA), Lane 2: Positive control (*C. bovis*, no *Sma* I), Lane 3: Positive control (*C. bovis* after *Sma* I digest), Lane 4: 5724 (-ve control, no *Sma* I), Lane 5: 5724 after *Sma* I digestion.

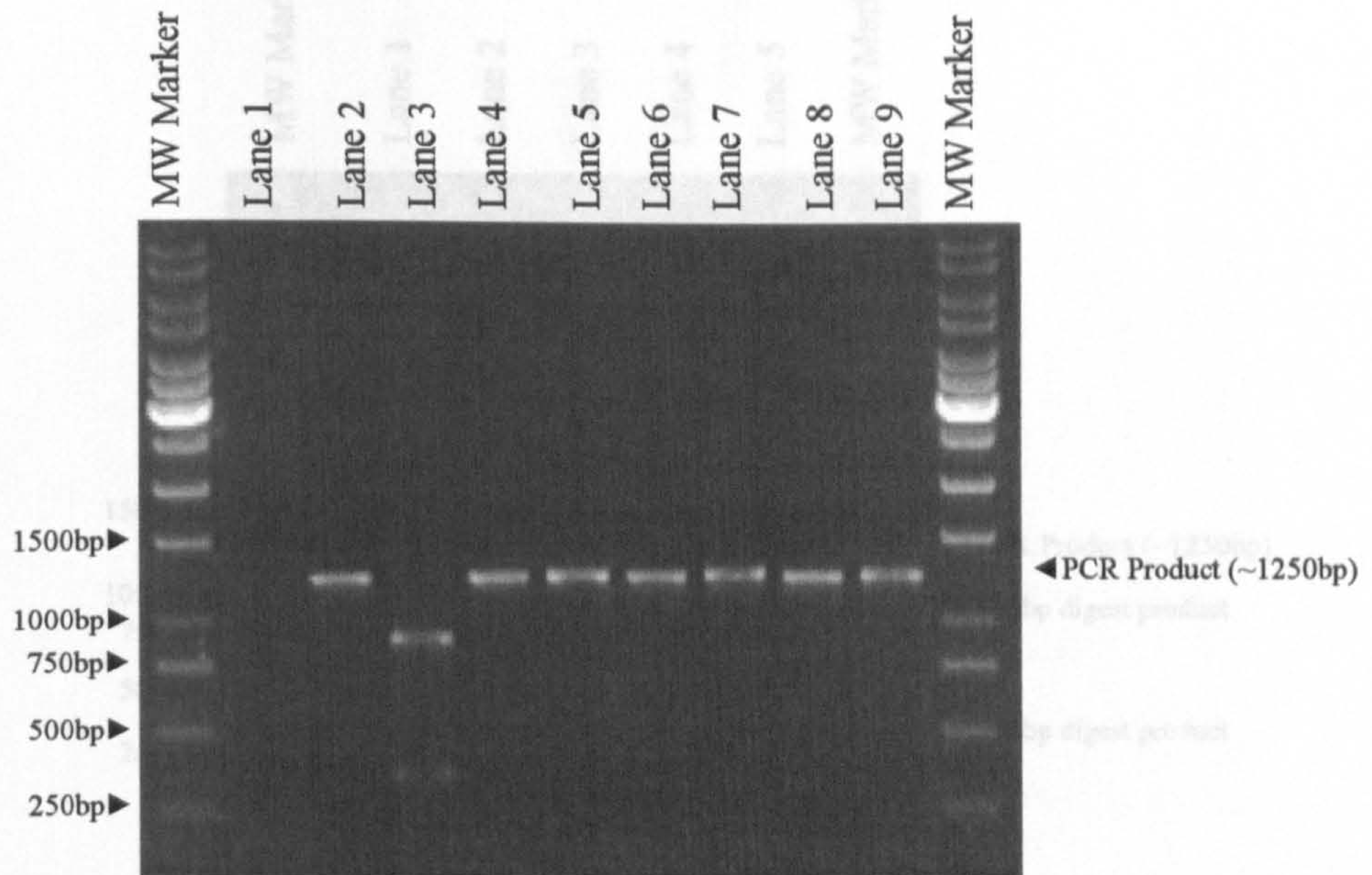


Figure 3.11: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 4376, 4396 & 4465 After Endonuclease Restriction With *Pst* I
Pst I digestion of the 16S rRNA PCR product of isolates 4376, 4396 & 4465 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Pst* I digest reaction with no DNA), Lane 2: Positive control (Isolate 5525, no *Pst* I), Lane 3: Positive control (Isolate 5525 after *Pst* I digestion), Lane 4: Isolate 4376 (-ve control, no *Pst* I), Lane 5: Isolate 4376 after *Pst* I digestion, Lane 6: Isolate 4396 (-ve control, no *Pst* I), Lane 7: Isolate 4396 after *Pst* I digestion, Lane 8: Isolate 4465 (-ve control, no *Pst* I), Lane 9: Isolate 4465 after *Pst* I digestion.

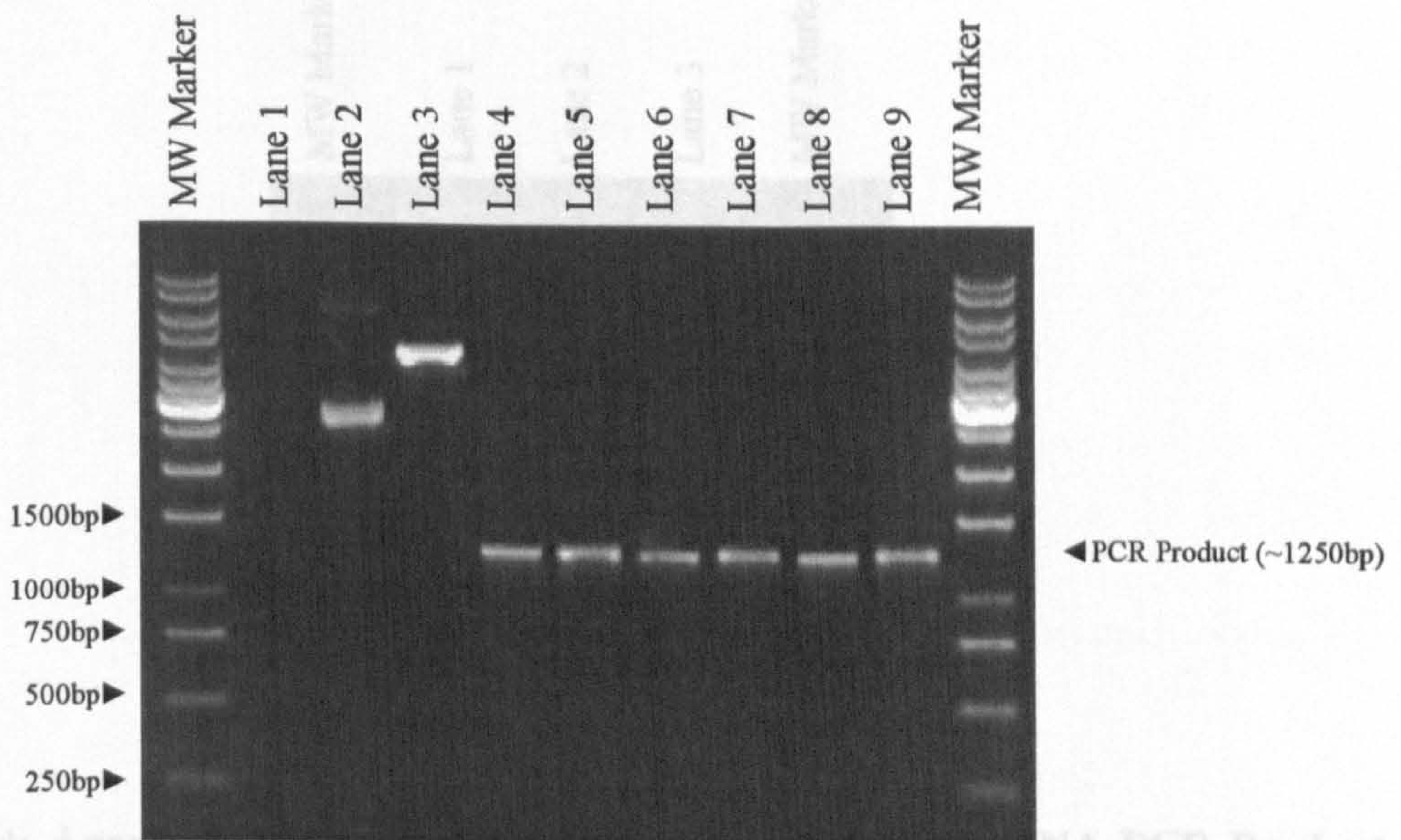


Figure 3.12: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 4376, 4396 & 4465 After Endonuclease Restriction With *Mlu* I
Mlu I digestion of the 16S rRNA PCR product of isolate 4376, 4396 & 4465 produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Mlu* I digest reaction with no DNA), Lane 2: Positive control (pSport I (a plasmid), no *Mlu* I), Lane 3: Positive control (pSport I after *Mlu* I digestion), Lane 4: Isolate 4376 (-ve control, no *Mlu* I), Lane 5: Isolate 4376 after *Mlu* I digestion, Lane 6: Isolate 4396 (-ve control, no *Mlu* I), Lane 7: Isolate 4396 after *Mlu* I digestion, Lane 8: Isolate 4465 (-ve control, no *Mlu* I), Lane 9: Isolate 4465 after *Mlu* I digestion.

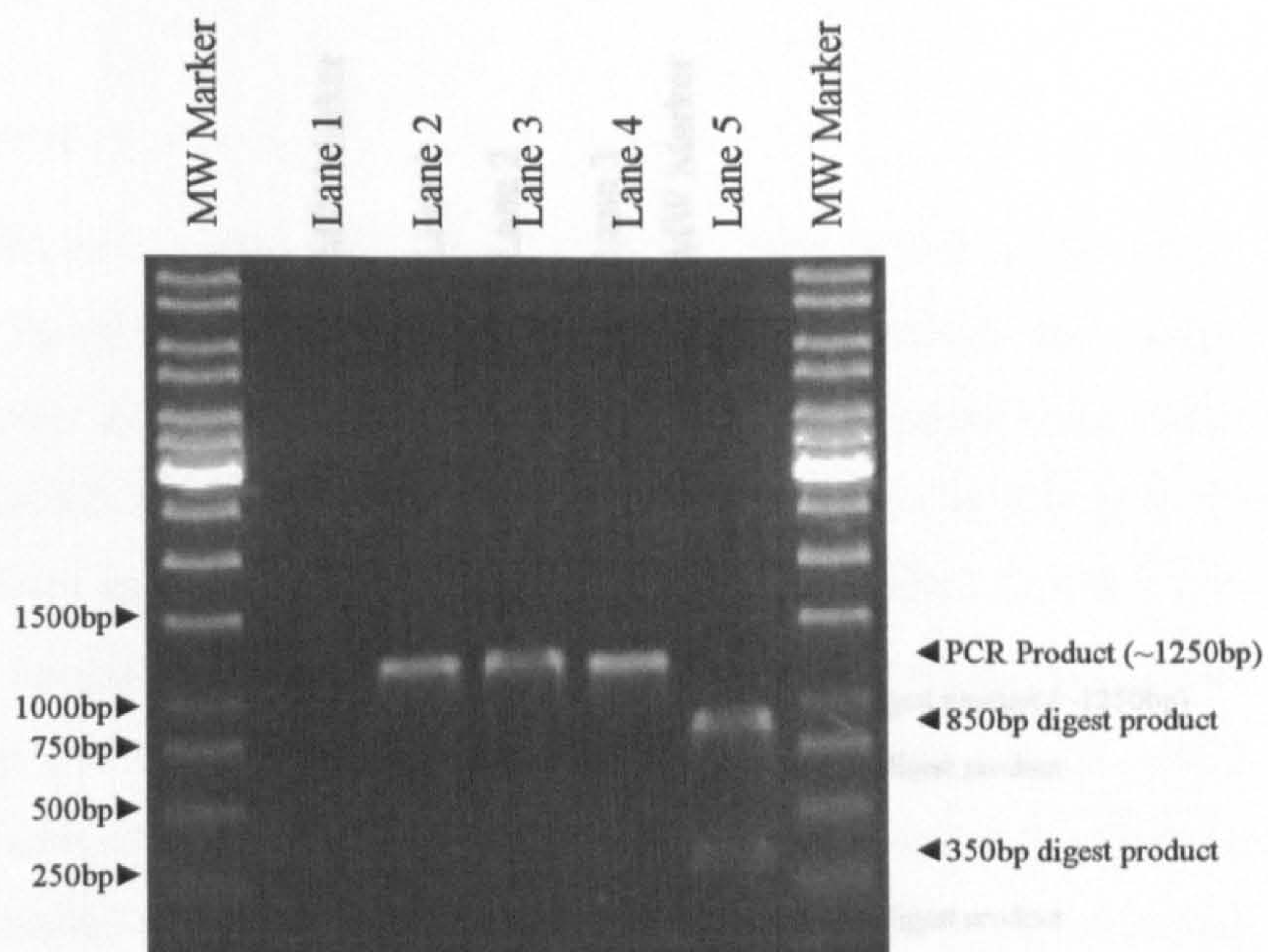


Figure 3.13: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 1120 & 5525 After Endonuclease Restriction With *Pst* I

Pst I digestion of the 16S rRNA PCR product of isolates 1120 & 5525. Isolate 1120 produced no digest products, 5525 produced 2 digest products (MW ~ 850 & 350bp), visualised here on a one percent agarose gel. Lane 1: Negative control (*Pst* I digest reaction with no DNA), Lane 2: Isolate 1120 (-ve control, no *Pst* I), Lane 3: Isolate 1120 after *Pst* I digestion, Lane 4: Isolate 5525 (-ve control, no *Pst* I), Lane 5: Isolate 5525 after *Pst* I digestion.

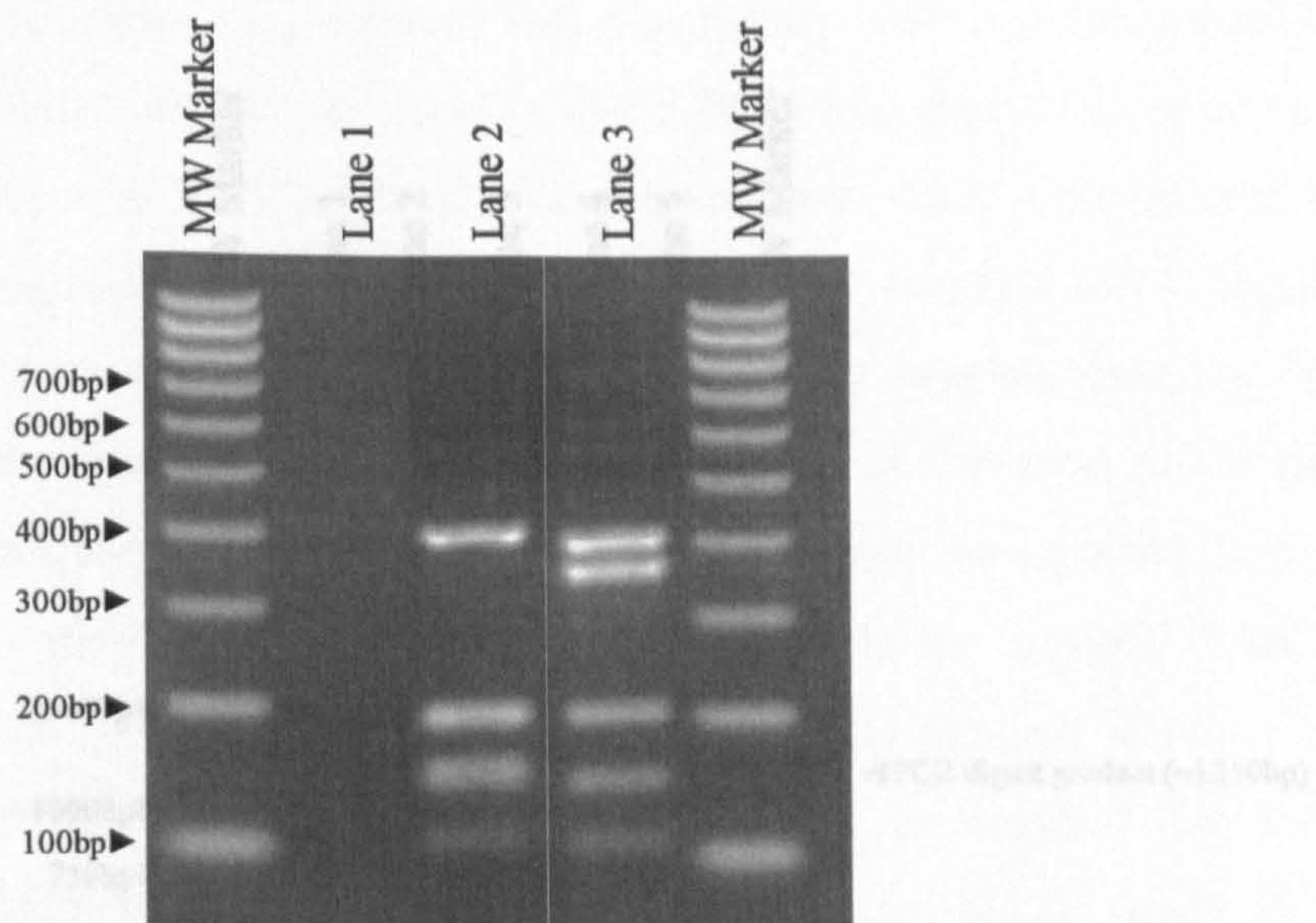


Figure 3.14: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolates 1120 & 5525 After Endonuclease Restriction With *Rsa* I

Rsa I digestion of the 16S rRNA PCR product of isolates 1120 & 5525. Isolate 1120 produced 7 digest products (MW ~ 110, 160, 170, 180, 205, 380 & 405), 5525 produced 5 digest products (MW ~ 110, 160, 205, 380 & 405bp), visualised here on a three percent agarose gel. Lane 1: Negative control (*Rsa* I digest reaction with no DNA), Lane 2: Isolate 1120 after *Rsa* I digestion, Lane 3: Isolate 5525 after *Rsa* I digestion.

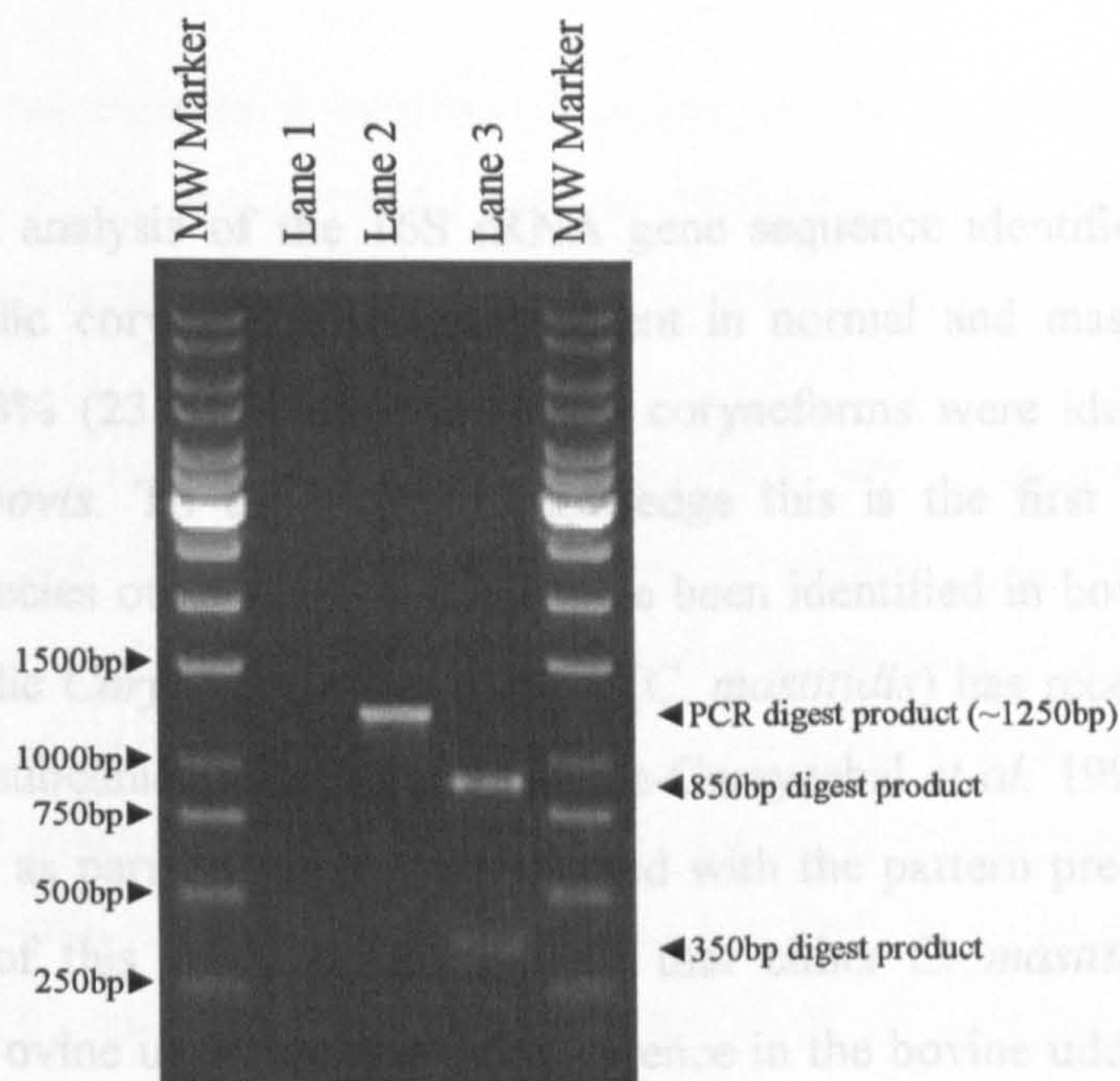


Figure 3.15: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolate 5724 After Endonuclease Restriction With *Pst* I

Pst I digestion of the 16S rRNA PCR product of isolate 5724 produced 2 digest products (MW ~ 850 & 350bp), visualised here on a one percent agarose gel. Lane 1: Negative control (*Pst* I digest reaction with no DNA), Lane 2: Isolate 5724 (-ve control, no *Pst* I), Lane 3: Isolate 5724 after *Pst* I digestion.

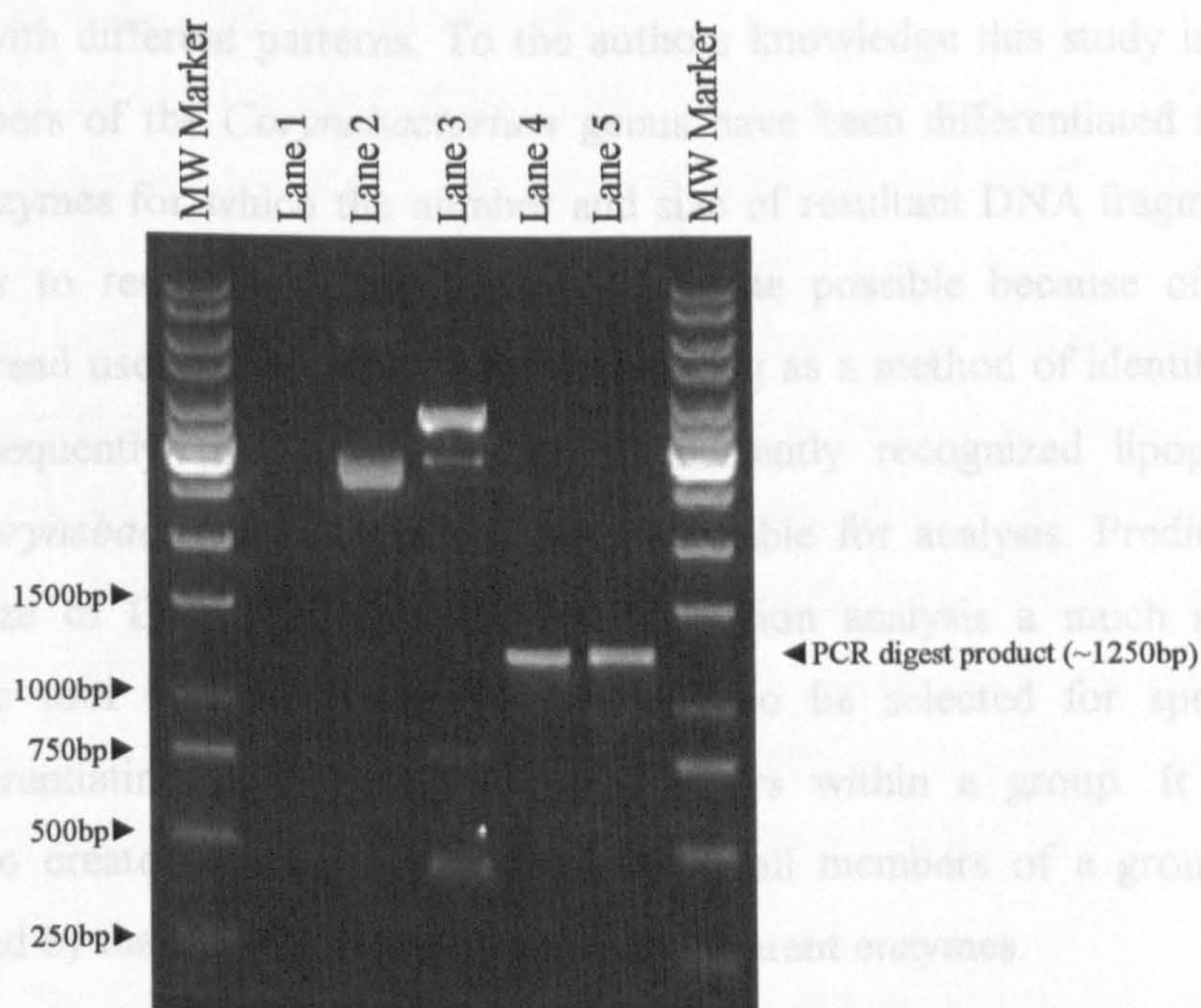


Figure 3.16: Agarose Gel Electrophoresis Image of the 16S rRNA PCR Product of Isolate 5724 After Endonuclease Restriction With *Pvu* II

Pvu II digestion of the 16S rRNA PCR produced no digest products, visualised here on a one percent agarose gel. Lane 1: Negative control (*Pvu* II digest reaction with no DNA), Lane 2: Positive control (pSport 1 (a plasmid), no *Pvu* II), Lane 3: Positive control (pSport I, after *Pvu* II digestion), Lane 4: Isolate 5724 (-ve control, no *Pvu* II), Lane 5: Isolate 5724 after *Pvu* II digestion.

3.4. DISCUSSION

Endonuclease restriction analysis of the 16S rRNA gene sequence identified 97.2% (781 of 804) of lipophilic coryneform isolates present in normal and mastitic milk samples as *C. bovis*. 2.8% (23 of 804) of lipophilic coryneforms were identified as species other than *C. bovis*. To the authors knowledge this is the first time that lipophilic coryneform species other than *C. bovis* have been identified in bovine milk, although a novel lipophilic *Corynebacterium* species (*C. mastitidis*) has recently been identified in sheep with subclinical mastitis (Fernandez-Garayzabal *et al.* 1997). None of the isolates examined as part of this study restricted with the pattern predicted for this organism. Results of this study would suggest that either *C. mastitidis* is an organism specific to the ovine udder or that its prevalence in the bovine udder is very low or restricted to certain geographical locations.

The enzymes *Alu* I, *Cfo* I, *Rsa* I (Vaneechoutte *et al.* 1995) *Hpa* I and *Pst* I (Wattiau *et al.* 2000) have been used to investigate the *Corynebacterium* genus by previous research workers. In both these cases the authors made no attempt to predict the outcome of restriction with the enzymes they used; they simply relied on different species restricting with different patterns. To the authors knowledge this study is the first in which members of the *Corynebacterium* genus have been differentiated from each other using enzymes for which the number and size of resultant DNA fragments was predicted prior to restriction. This has only become possible because of the increasingly widespread use of 16S rRNA gene sequencing as a method of identifying new species. Consequently the sequences for all currently recognized lipophilic members of the *Corynebacterium* genus are freely available for analysis. Predicting the number and size of DNA fragments makes restriction analysis a much more powerful diagnostic tool because it enables enzymes to be selected for specific purposes *e.g.* differentiating one species from all others within a group. It also becomes possible to create decision trees, which allow all members of a group or genus to be identified by the progressive application of different enzymes.

Twenty of the isolates identified as species other than *C. bovis* appeared to be very closely related, possibly representing a single species (similar colony morphology and colour and identical restriction pattern with four restriction enzymes), although the

possibility remains they represented a number of similar or closely related species. They could not be identified as any currently recognised lipophilic *Corynebacterium* species by endonuclease restriction analysis and will be considered in more detail in Chapter 4.

Three isolates restricted in patterns that suggested they represented three different species. One of these three (5724) demonstrated a restriction pattern identical to *"C. genitalium"* accession number U87821, a *Corynebacterium* species currently not validated. *"C. genitalium"* was first isolated in 1974 from a male and female patient with urethritis (Furness and Evangelista 1976). Initial characterisation of the new species was poor; only morphological and basic biochemical information was described. The 16S rRNA gene sequences for seven strains of *"C. genitalium"* have been published (www.ncbi.nlm.nih.gov). All of these strains produced different restriction patterns when examined within the database used for predicting the results of endonuclease restriction in this study (Table 3.1). Based on this analysis it would appear that *"C. genitalium"* currently contains more than one species or represents one species with a high degree of 16S rRNA gene sequences variability. Further work is needed in this area to characterize this apparently diverse "species" and confirm or refute its presence within the *Corynebacterium* genus.

The restriction patterns of the remaining two isolates (1120 & 5525) did not match that of any species present within the database, so could not be identified by this method.

Although the possibility remains that these three isolates represented infections of the quarters from which they were originally isolated, the more likely explanation for their presence, considering their prevalence, (one isolate in over 800 lipophilic coryneforms) is as sample contaminants from the environment. Based on the information gathered during this study it is impossible to assess their significance. Further work may be justified in the future if they are isolated from multiple quarters or from single quarters at multiple sampling time points. They will not be considered further in this thesis.

Alternatively the 23 lipophilic coryneforms that did not restrict in the manner predicted for *C. bovis* could be species members with atypical 16S rRNA gene sequences. A single base pair change to the sequence (within the area of recognition for any given enzyme) can either delete or add a restriction site. However as the number of enzymes employed increases the chances that random point changes have

occurred at every point of enzyme recognition decreases, in these cases it is much more likely that the sequence changes are real and the isolate represents another species with a different sequence. Based on the endonuclease restriction work presented here, it remains a possibility (though remote) that these atypical coryneforms are in fact *C. bovis.*

The endonuclease restriction analysis method outlined here is relatively simple to perform; however it does require equipment that not all bacteriology laboratories may possess (*e.g.* thermocycler, UV transilluminator). 16S rRNA gene sequencing is currently regarded as the "reference" method of identification (Watts *et al.* 2000), although it currently remains out with the capabilities of all but the very best equipped laboratories. At the time of writing, sequencing a 16S rRNA gene from PCR product costs approximately £180 per isolate (three pairs of primers on triplicate PCR product at £10 per run), making it too expensive for routine use or for processing large numbers of samples. It is likely to become much more widely used in the future as the cost falls. Until that time restriction analysis remains a viable alternative.

Endonuclease restriction analysis of the 16S rRNA gene sequence relies on being able to accurately predict the outcome of digesting the 16S rRNA sequence with restriction enzymes. The quality and quantity of available sequence data will influence the accuracy and validity of these predictions. The sensitivity and specificity of the technique will improve as the number of published 16S rRNA gene sequences from lipophilic *Corynebacterium* species increases. Forty nine *C. bovis* 16S rRNA gene sequences were suitable for inclusion in the database prepared for this study (Tables 3.1 and AIII.I). The endonuclease restriction patterns could be predicted with a high degree of certainty for this species and thus the sensitivity of the technique is likely to be high.

Sma I and *Hind* III were identified as the two enzymes that could be used to differentiate *C. bovis* from all other currently recognised lipophilic species. Of the 49 *C. bovis* 16S rRNA gene sequences suitable for analysis, 47 had identical predicted restriction pattern after digestion with these two enzymes, only two did not (Tables 3.1 and AIII.I). One sequence (AF311397, Table AIII.I) contained an extra *Sma* I restriction site and another (AF311419, Table AIII.I) contained an extra *Hind* III restriction site. No lipophilic coryneform isolate analysed during the course of this

study restricted in the pattern predicted for either of these sequences. If the extra restriction sites identified in these two sequences are real, it would appear that the prevalence of strains carrying these sequence variations are low, or they may represent strains more prevalent in other geographical locations (As far as it is possible to ascertain most *C. bovis* 16S rRNA sequences are from isolates originally identified in the USA). It remains a possibility however, that these extra restriction sites may in fact be due to PCR, sequencing or data handling errors during the sequencing process.

It is noteworthy that 45 of the 49 *C. bovis* sequences suitable for analysis were published after the work described here had been completed. The additional 45 sequences were incorporated into the database, actually improving its specificity.

The specificity of this technique depends upon its ability to differentiate all other lipophilic species from *C. bovis*. The database created contains all currently recognised lipophilic *Corynebacterium* species. The results of restriction with *Hind* III and *Sma* I will differentiate all these species from *C. bovis*. It is impossible to know whether all lipophilic species were represented in the database (currently unrecognised species may exist). The results of this study would suggest they were not (22 isolates, possibly representing three or more species could not be identified). The possibility remains that other species with identical *Hind* III and *Sma* I restriction patterns and similar cultural characteristics may exist which would not be differentiated from *C. bovis* by this method. In this case they would be incorrectly identified as *C. bovis*, which would reduce the specificity of the technique. This must be acknowledged as a flaw in the current methodology, but it is relatively unlikely and many other diagnostic methods that rely on the comparison of results to reference data (*e.g.* sugar fermentation profiles) suffer from the same problem.

Increasing the number of enzymes used could increase the specificity of the method employed. Endonuclease restriction enzymes bind to short and very specific sequences of bases within the gene sequence. Increasing the number of enzymes used decreases the possibility that two similar species will have identical restriction patterns because the likelihood of them containing all the appropriate enzyme sites decreases. It will however also increase the chances of members of the same species having different restriction patterns. 16S rRNA gene sequences are not identical even within the same species (Data available at www.ncbi.nlm.nih.gov). It is therefore possible for two members of the same species to restrict with different patterns if there

is only a single base alteration between the two. Some regions of the 16S sequence are very highly conserved both within and between species (Stackebrandt and Goebel 1994). Ideally species-specific restrictions sites should be situated in regions that are highly conserved between members of the same species. This will only be possible to assess if large numbers of the species in question have already been sequenced.

Results of restriction analysis of the 16S rRNA gene sequence suggest that it is not safe to assume that all lipophilic coryneforms isolated from milk or clinical mastitis samples are *C. bovis*. Lipophilic coryneforms other than *C. bovis* can infect the bovine mammary gland. Three percent (23 in 804) of isolates were identified as species other than *C. bovis* by this method. For routine clinical purposes this error is of little consequence and therefore a presumptive diagnosis of *C. bovis* based on phenotypic and growth characteristics (slow growing lipophilic coryneforms) is adequate. However for epidemiological studies, investigations of *C. bovis* "clinical mastitis" outbreaks and studies relating to the properties, characteristics and pathogenic interactions of *C. bovis*, it is not safe to assume that all lipophilic coryneforms of milk origin are *C. bovis*.

CHAPTER 4: IDENTIFICATION OF A NOVEL LIPOPHILIC *CORYNEBACTERIUM* SPECIES.

4.1. INTRODUCTION

The results of endonuclease restriction analysis of 804 lipophilic coryneform species isolated during the teat sealer study demonstrated that 23 did not have a restriction profile consistent with that predicted for *C. bovis*. Based on restriction patterns and colony morphology, 20 of these appeared to represent a single species. The restriction profile identified for these 20 isolates did not match predictions for any currently recognized lipophilic *Corynebacterium* species. These isolates therefore represented either a currently recognized species that restricted in an unusual manner, a new currently unrecognized species, or were lipophilic members of a coryneform genus other than *Corynebacterium*, which were not included in the endonuclease restriction database.

The *Corynebacterium* genus is expanding rapidly. A recent literature search revealed 60 recognised species (Table 4.3), compared to 28 that were documented in a paper published in 1997 (Funke *et al.* 1997). Six species were defined in 2001 alone, *C. freneyi* (Renaud *et al.* 2001), *C. mooreparkense*, *C. casei* (Brennan *et al.* 2001), *C. capitovis* (Collins *et al.* 2001), *C. testudinoris* and *C. felinum* (Collins *et al.* 2001). No new *Corynebacterium* species have been reported from cattle, although a new lipophilic species (*C. mastitidis*) has recently been isolated from sheep with subclinical mastitis (Fernandez-Garayzabal *et al.* 1997).

One of the principal reasons for this rapid expansion has been the improvement of diagnostic tools, particularly molecular methods that allows accurate differentiation and classification of closely related and previously unidentified isolates. One such tool, which has rapidly become the "reference" method for categorically identifying novel species (used in all of the peer reviewed papers above), is 16S rRNA gene sequencing. The 16S rRNA gene is responsible for the production of the small subunit of the prokaryotic ribosome. Large parts of the 16S rRNA gene sequence are highly conserved although small numbers of base variations do occur, primarily within hypervariable regions (Stackebrandt and Goebel 1994).

The development of rapid and relatively inexpensive methods of gene sequencing allows a comparison of the 16S rRNA sequences of closely related organisms to be made. The phylogenetic relationship between organisms can be elucidated using algorithms that predict the most likely relationships and the order in which organisms have diverged from common ancestors, based on the similarity and differences between gene sequences. The 16S rRNA gene sequence within the *Corynebacterium* genus is highly variable compared to genera such as *Staphylococcus* in which the sequence is largely conserved (Vaneechoutte *et al.* 1995). The gene sequence contains a number of hypervariable regions which differ markedly between closely related members of the genus (Pascual *et al.* 1995; Barrett *et al.* 2001); differences in these regions can be used to differentiate closely related species (Barrett *et al.* 2001).

4.2. MATERIALS AND METHODS

4.2.1. Analysis of Database

The identity of the 20 similar isolates which could not be identified by endonuclease restriction analysis were cross referenced back through the database produced during the teat sealer trial to identify the quarters, cows and farms from which they were originally identified.

4.2.2. Production of Sequencing Template by PCR

PCR products were produced in independent PCR reactions in triplicate, from extracted DNA template as described previously (Section 3.2.7.) using the universal forward and reverse primers pA and pH* (Figure 4.1) (Funke *et al.* 1997; Collins *et al.* 1999) which correspond to positions 8-28 and 1522-1542 of the 16S rRNA gene sequence (*E. coli* numbering) (Fernandez-Garayzabal *et al.* 1997).

4.2.3. DNA Quantification

The concentration of DNA in the PCR products was estimated by comparison to a known standard marker in a DNA molecular weight ladder (GeneRuler 1Kb DNA

Ladder, MBI Fermentas) after electrophoresis in a one percent agarose gel as previously described (Section 3.2.9.). Serial dilutions of DNA were performed to allow accurate estimation of concentration as necessary.

Figure 4.1: Oligonucleotide Primers, "pA" and "pH*"

Forward Primer

"pA" 5' – AGA GTT TGA TCC TGG CTC AG – 3'

Reverse Primer

"pH*" 5' – AAG GAG GTG ATC CAG CCG CA – 3'

4.2.4. Internal Primer Design

The original primers used during the *C. bovis* typing study (Coryneallfor and Coryneallrev, Figure 3.1) were selected as the first pair of internal primers for sequencing because they were already available.

Omiga (Version 2.0, Oxford Molecular Ltd) was used to identify all universal internal forward and reverse primers meeting specified criteria (20 – 24 bases long, GC% >50, melting temperature 55-65°C) within the *Corynebacterium* 16S gene sequence. Two pairs of primers were selected and synthesised based on their universality for all lipophilic species, relative positions within the 16S gene sequence and melting temperatures (Figure 4.2, Invitrogen custom primers, Invitrogen life technologies).

Figure 4.2: Oligonucleotide Primers, "midcorynefor", "Coryneendfor", "midcorynerev" and "Coryneendrev"

Forward Primers

"midcorynefor" 5' – CGC AGA TAT CAG GAG GAA CAC C – 3'

"Coryneendfor" 5' – CAT CAT GCC CCT TAT GTC CAG – 3'

Reverse Primers

"midcorynerev" 5' – GTT TAC GGC ATG GAC TAC CAG G – 3'

"Coryneendrev" 5' – CAT TAC CCC ACC AAC AAG CTG – 3'

4.2.5. Sequence Analysis

Primers were adjusted to 3.2 micromolar by dilution; DNA concentration was estimated to ensure the supply of at least 75ng of DNA (Minimum requirement 50ng per Kb to be sequenced, PCR product here is 1.5Kb in length) per sample submitted for sequencing. Triplicate PCR products were submitted for sequencing with all six primers (*i.e.* 18 sequencing runs in total) by the automated fluorescent method (The Sequencing Service, School of Life Science, University of Dundee).

Resultant sequences were imported into multiple sequence alignment software (Assembly LIGN 1.0.9c, Oxford Molecular). Sequences were trimmed at the 5' and 3' ends to exclude poor and inconclusive data generated at the start and end of the sequence run. Conflicting bases and point insertions were checked and corrected manually by visualisation of the chromatogram in Chromas 1.45 (Conor McCarthy, Queensland, Australia, freeware available at www.technelysium.com.au /chromas.html). A consensus sequence running between external primers pA and pH* was produced.

The resultant consensus sequence was exported to the "Blast" (Basic Local Alignment Search Tool) search engine in the NCBI database (Available at www.ncbi.nlm.nih.gov) and compared to all DNA sequence data held. Results of the search allowed the identification of the 16S rRNA gene sequences with the greatest similarity to the test sequence. Sequences with the greatest similarity were downloaded from the NCBI database and trimmed at either ends, approximately between bases 107 and 1438 (*E. coli* numbering), for uniformity of length and to reduce alignment uncertainties associated with hypervariable region V1 at the 5' end of the gene sequence (Pascual *et al.* 1995; Watts *et al.* 2001). Trimmed sequences (approximately 1325bp in length) together with the test sequence were aligned with OMIGA using the alignment parameters outlined in Figure 4.3 (Hall 2001).

Aligned sequences were imported into Clustal X Multiple Sequence Alignment Program (Version 1.8, freeware available at http://fttp-igbmc.u-strasbg.fr/pub/ClustalX). The alignment was corrected manually if obvious improvements could be made after visual inspection. Distance matrix data and a phylogenetic tree were generated by the Neighbor Joining distance method with boot strap analysis to assess reliability (500 replicants, (Collins *et al.* 2001)) in Phylip (Phyologeny Inference Package, version 3.5c, Joseph Felsenstein and the University of Washington, freeware available at http://evolution.genetics.washington.edu/phylip

.html) using SeqBoot, DNADist, Neighbor and Consense (Pascual *et al.* 1995; Fernandez-Garayzabal *et al.* 1998; Collins *et al.* 1999). The resultant tree was exported to and manipulated within Treeview (Version 1.6.6, Roderic DM Page, freeware available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and PowerPoint 2000 (Microsoft) for improved visual representation.

Figure 4.3: Alignment Parameters Specified for the Multiple Sequence Alignment

Pairwise Alignment:

Alignment Method – Slow

Open Gap Penalty – 10.00

Extended Gap Penalty – 0.10

Multiple Alignment:

Open Gap Penalty – 10.00

Extended Gap Penalty – 0.20

Delay Divergent Sequences – 30%

Transition – Weighted

The 20 unknown isolates were biochemically characterised using the API Coryne system (API bioMerieux, Marcy l'Etiole, France), a commercially available speciation system, which differentiates members of the coryneform group to genus or species level, based on 20 biochemical reactions. The API Coryne test strip was prepared according to the manufacturers recommendations; results were read from the strip after 24 hours incubation at 37°C.

4.3. RESULTS

4.3.1. Origin of 20 Similar but Unknown Coryneform Isolates

The 20 unknown coryneform isolates originated from the quarters of 14 cows on eight farms (Table 4.1). Three of the isolates originated from the same quarter of the same cow (169 B), one from each of the three sampling time points (drying off, calving and post calving). Five of the isolates originated from three quarters of the same cow (112 G) with at least one isolate coming from each of the three sampling time points.

Table 4.1: Origin (Farm, Cow, Quarter and Sampling Time Point) of 20 Similar but Unknown Coryneform Isolates

Sample reference	Farm reference letter	Cow identification	Quarter *	Sampling time point
4465†	B	169	RF	Drying Off
5613	B	169	RF	Calving
5669	B	169	RF	Post Calving
2358‡	G	112	RH	Drying Off
4375‡	G	112	LF	Calving
4376	G	112	LH	Calving
4519	G	112	LF	Post Calving
4520	G	112	LH	Post Calving
5789	G	A8	RF	Calving
5330b‡	H	53N	RH	Calving
4890b	H	65M	RH	Calving
5393b	H	57N	RF	Post Calving
5639	L	800	LF	Post Calving
4662	N	295	RH	Calving
4859b	N	353	LF	Calving
5109b	N	351	RF	Calving
M378	T	312	RF	Clinical Mastitis
M218‡	V	761	LF	Clinical Mastitis
563	Z	767	LF	Drying Off
4396	Z	407	LH	Drying Off

**R – Right, L – Left, F – Fore, H – Hind.*
†4465 was selected as the representative isolate for complete sequencing of the 16S rRNA gene.
‡Four further isolates were selected for partial sequencing of the 16S rRNA gene.

4.3.2. 16S rRNA Gene Sequence Analysis of Five Similar but Unknown Coryneform Isolates

Sample reference number 4465 was selected as being representative of the 20 isolates based on colony morphology and the quarter from which it was first isolated (Table 4.1). The almost complete 16S rRNA gene sequence between primers pA and pH*

was elucidated for this organism (Figure 4.4). The sequence was 1475 base pairs in length.

Figure 4.4: The Almost Complete 16S rRNA Gene Sequence for Isolate 4465

5' – GAT GAA CGC TGG CGG CGT GCT TAA CAC ATG CAA GTC GAA CGG AAA GGC CCC TGC TTG CAG GGG
TAC TCG AGT GGC GAA CGG GTG AGT AAC ACG TGG GTG ATC TGC CTT GTA CTT CGG GAT AAG CCT GGG
AAA CTG GGT CTA ATA CCG GAT AGG ACC AAT CTT TAG TGT GGT TGG TGG AAA GTT TTG TCG GTA CGA
GAT GAG CCC GCG GCC TAT CAG CTT GTT GGT GGG GTA ATG GCC TAC CAA GGC GAC GAC GGG TAG CCG
GCC TGA GAG GGT GTA CGG CCA CAT TGG GAC TGA GAC ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT
GGG GAA TAT TGC ACA ATG GGC GGA AGC CTG ATG CAG CGA CGC CGC GTG AGG GAT GAC GGC CTT CGG
GTT GTA AAC CTC TTT CGC TAG GGA AGA AGC CTT TTT GGG TGA CGG TAC CTG GAT AAG AAG CAC CGG
CTA ACT ACG TGC CAG CAG CCG CGG TAA TAC GTA GGG TGC GAG CGT TGT CCG GAA TTA CTG GGC GTA
AAG AGC TCG TAG GTG GTT TGT CGC GTC GTT AGT GAA AGC CCG GGG CTT AAC TCC GGG TCT GCT GGC
GAT ACG GGC ATA ACT TGA GTG CTG TAG GGG AGA CTG GAA TTC CTG GTG TAG CGG TGG AAT GCG CAG
ATA TCA GGA GGA ACA CCG ATG GCG AAG GCA GGT CTC TGG GCA GTA ACT GAC GCT GAG GAG CGA AAG
CAT GGG TAG CGA ACA GGA TTA GAT ACC CTG GTA GTC CAT GCC GTA AAC GGT GGG CGC TAG GTG TGG
GGG TTT TTC ACG ACT TCC GTG CCG TAG CTA ACG CAT TAA GCG CCC CGC CTG GGG AGT ACG GCC GCA
AGG CTA AAA CTC AAA GGA ATT GAC GGG GGC CCG CAC AAG CGG CGG AGC ATG TGG ATT AAT TCG ATG
CAA CGC GAA GAA CCT TAC CTG GGC TTG ACA TGT ACG GGA TCG GCG TAG AGA TAC GTT TTC CCT TGT
GGC TCG TAT ACA GGT GGT GCA TGG TTG TCG TCA GCT CGT GTC GTG AGA TGT TGG GTT AAG TCC CGC
AAC GAG CGC AAC CCT TGT CTT GTG TTG CCA GCA CGT GAT GGT GGG GAC TCG CGA GAG ACT GCC GGG
GTT AAC TCG GAG GAA GGT GGG GAT GAC GTC AAA TCA TCA TGC CCC TTA TGT CCA GGG CTT CAC ACA
TGC TAC AAT GGT CGG TAC AGT GGG TTG CGA TAC CGT GAG GTG GAG CTA ATC CCT TAA AGC CGG TCT
CAG TTC GGA TTG GAG TCT GCA ACT CGA CTC CAT GAA GTC GGA GTC GCT AGT AAT CGC AGA TCA GCA
ACG CTG CGG TGA ATA CGT TCC CGG GCC TTG TAC ACA CCG CCC GTC ACG TCA TGA AAG TTG GTA ACA
CCC GAA GCC AGT GGC CCA AAC TCG TTA GGG AGC TGT CGA AGG TGG GAT CGG CGA TTG GGA CGA AGT
CGT AAC AAG GTA GCC GTA CCG GAA GG – 3'

The partial 16S rRNA gene sequence (approximately 1150 bp) of four other isolates 2358, 4375, 5330b and M218 (see Table 4.1) were sequenced as previously described except only two sets of internal primers were used (Coryneallfor & Coryneallrev (Figure 3.1) and midcorynefor & midcorynerev (Figure 4.2)). Comparison of all four sequences to that of isolate 4465 demonstrated that M218 was identical, 4375 and 5330b had a single base alteration and 2358 had a single base insertion (Appendix IV, Figures AIV.I, AIV.II, AIV.III and AIV.IV, pages 210 and 211), indicating that they were all members of the same species.

4.3.3. Similarity of Isolate 4465 to Other *Corynebacterium* Species

A blast search of the NCBI database revealed that the known sequences with the greatest similarity to that of 4465 were all members of the *Corynebacterium* genus.

The sequence with the greatest similarity to that of 4465 belonged to *C. auriscanis*. The 20 closest related 16S rRNA sequences in descending order of similarity are outlined in Table 4.2.

Table 4.2: The 20 Species With the Greatest 16S rRNA Gene Sequence Similarity to Isolate 4465

Rank	Organism	Score (bits)*	E-value†	Rank	Organism	Score (bits)	E value
1	*C. auriscanis*	2672	0.0	11	*C. simulans*	2214	0.0
2	*C. falsenii*	2587	0.0	12	*C. tuberculostericum*	2198	0.0
3	*C. jeikeium*	2494	0.0	13	*C. striatum*	2185	0.0
4	*C. bovis*	2426	0.0	14	*C. felinum*	2173	0.0
5	*C. urealyticum*	2418	0.0	15	*C. ammoniagenes*	2163	0.0
6	*C. testudinoris*	2302	0.0	16	*C. vitarumen*	2161	0.0
7	*C. diphtheriae*	2300	0.0	17	*C. segmentosum*	2159	0.0
8	*C. ulcerans*	2250	0.0	18	*C. callunae*	2151	0.0
9	*C. pseudotuberculosis*	2230	0.0	19	*C. fastidiosum*	2147	0.0
10	*C. flavescens*	2228	0.0	20	*C. glutamicum*	2105	0.0

**Score (bits) – A system that assigns a score for base matches and no score or a penalty for mismatches or gaps, the higher the score the better the match between sequences.*
†E value – An estimation of the probability that two sequences are similar by chance, the lower the score the more likely it is that the similarity is "real", values of 0.0 indicate sequences are extremely unlikely to be similar by chance.

Because all the closest related sequences to 4465 were members of the *Corynebacterium* genus it was aligned with all currently recognised members of the genus to elucidate its evolutionary position.

Levels of sequence similarity between 4465 and all currently recognised *Corynebacterium* species were determined using DNAdist (PHYLIP 3.5c, Joseph Felsenstein & University of Washington) (Table 4.3). The closest related species was *C. auriscanis* with a similarity value of 99.1%. All *Corynebacterium* species had over 90% similarity; the member of the genus with the least similarity was *C. durum* with a similarity value of 91.3%.

4.3.4. Phylogenetic Analysis of Isolate 4465

A phylogram produced by the neighbor joining distance method, which demonstrates the relationship between isolate 4465 and all other currently recognised members of

the *Corynebacterium* genus is shown in Figure 4.5. Isolate 4465 lies well inside the robust grouping that contains all *Corynebacterium* species and was most closely related to *C. auriscanis.*

Table 4.3: Levels of Sequence Similarity Between Isolate 4465 and All Other Currently Recognised Members of the *Corynebacterium* Genus

Species	Accession Number	16S rRNA sequence similarity (%)	Species	Accession Number	16S rRNA sequence similarity (%)
C. auriscanis	AJ243820	99.1	*C. singulare*	Y10999	93.8
C. falsenii	Y13024	96.8	*C. glutamicum*	X84257	93.7
C. jeikeium	X84250	96.6	*C. fastidiosum*	X84245	93.7
C. urealyticum	X84439	96.4	*C. renale*	X84249	93.7
*C. bovis**	X84444	96.0	*C. macginley*	X80499	93.6
*C. bovis**	X82051	95.9	*C. camporealensis*	Y09569	93.6
C. diphtheriae	X84248	95.9	*C. segmentosum*	X84437	93.6
C. ulcerans	X84256	95.8	*C. mastitidis*	Y09806	93.6
C. pseudotuberculosis	X84255	95.6	*C. afermentans*	X81874	93.5
C. terpenotabidum	AB004730	95.4	*C. amycolatum*	X84244	93.5
C. variabile	X53185	95.3	*C. phocae*	Y10076	93.4
C. testudinoris	AF295841	95.1	*C. asperum*	X82050	93.2
C. felinum	AJ401282	95.0	*C. confusum*	Y15886	93.2
C. vitaeruminis	X84680	94.8	*C. ammoniagenes*	X84440	93.1
C. flavescens	X84441	94.5	*C. riegelii*	Y14651	93.1
C. freneyi	AJ292762	94.5	*C. mooreparkense*	AF267148	92.9
C. xerosis	X84446	94.4	*C. accolens*	X80500	92.8
C. kutscheri	X81871	94.2	*C. kroppenstedtii*	Y10077	92.8
C. mucifaciens	Y11200	94.2	*C. imitans*	Y09044	92.7
C. lipophiloflavum	Y09045	94.2	*C. sundsvallensis*	Y09044	92.6
C. tuberculostearicum	X84247	94.2	*C. thomssenii*	AF010474	92.6
C. argentoratense	X83955	94.1	*C. casei*	AF267152	92.4
C. striatum	X84442	94.1	*C. propinquum*	X84438	92.3
C. coyleae	X96497	94.1	*C. cystitidis*	X84252	92.3
C. auris	X81873	94.0	*C. pseudodiphtheriticum*	X84258	92.3
C. simulans	AJ012838	94.0	*C. glucuronolyticum*	X86688	92.2
C. acetoacidophilum	X84240	93.9	*C. pseudogenitalium*	X81872	92.2
C. minutissiumum	X82064	93.9	*C. capitovis*	AJ297402	92.1
C. pilosum	X84246	93.9	*C. matruchotii*	X84443	92.0
C. mycetoides	X84241	93.8	*C. durum*	Z97069	91.3
C. callunae	X84251	93.8			

**Two* C. bovis *isolates included because both isolates have been previously used in papers investigating the phylogenetic analysis of the* Corynebacterium *genus (*C. bovis *X84444 (Pascual et al. 1995) and X82051 (Ruimy et al. 1995)).*

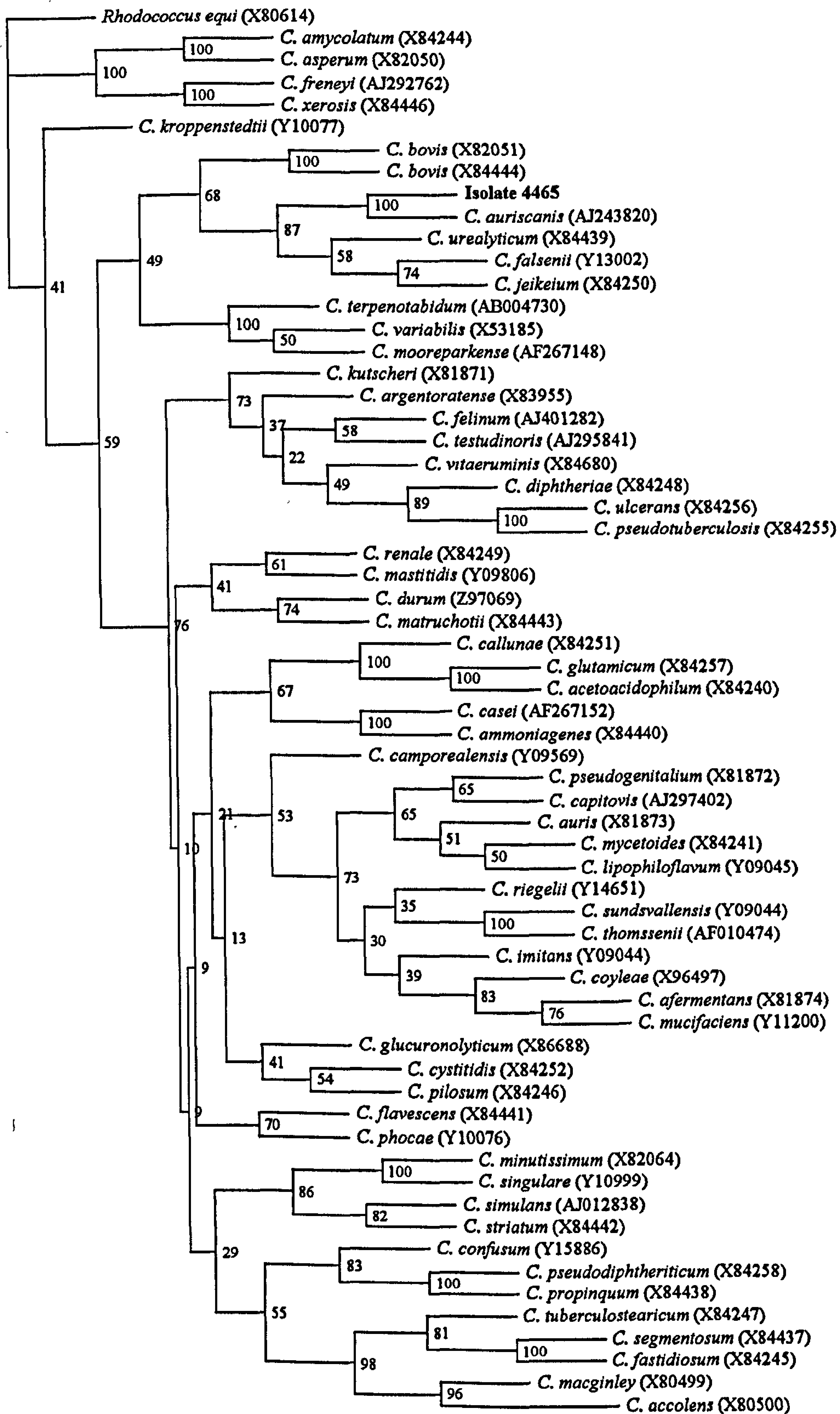


Figure 4.5: Phylogram Demonstrating the Relationship Between Sequence 4465 and Members of the Genus *Corynebacterium*

The tree was conducted using the neighbor joining distance method and rooted using *Rhodococcus equi* as the outgroup. Bootstrap values, as a percentage of 500 replicants are given at the branching points.

Biochemical Characteristics of 20 Unknown Coryneform Isolates

Results of biochemical characterisation of 20 strains of the unknown coryneform with the API Coryne system are outlined in Table 4.4. A consensus profile and the results from the type strain of *C. auriscanis*, are included for comparison. Acid production from glucose was variable, acid was not produced from ribose, xylose, mannitol, maltose, lactose, sucrose or glycogen. Eusculin and gelatine were not hydrolysed. Nitrate was not reduced. Alkaline phosphatase, urease and catalase activity were detected. Detection of Pyrrolidonyl Arylamidase was variable. Pyrazinamidase, beta-Glucuronidase, beta-Galactosidase, alpha-Glucosidase and N-Acetyl-beta-Glucosaminidase activity were not detected.

Table 4.4: Biochemical Properties of 20 Similar but Unknown Coryneform Isolates and the Type Strain of *C. auriscanis*

Isolate Reference Number	Nitrate reduction	Pyrazinamidase	Pyrrolidonyl Arylamidase	Alkaline Phosphatase	beta-Glucuronidase	beta-Galactosidase	alpha-Glucosidase	N-Acetyl-beta-Glucosaminidase	Esculin	Urease	Gelatine hydrolysis	O	Glucose	Ribose	Xylose	Mannitol	Maltose	Lactose	Sucrose	Glycogen	Catalase
4465	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
5613	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
5669	-	-	?	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
2358	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+
4375	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
4376	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
4519	-	-	-	+	-	-	-	-	-	+	-	-	?	-	-	-	-	-	-	-	+
4520	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
5789	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
5330b	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
4890	-	-	?	+	-	-	-	-	-	+	-	-	?	-	-	-	-	-	-	-	+
5393b	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
5639	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
4662	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+
4859b	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
5109b	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
M378	-	-	-	+	-	-	-	-	-	+	-	-	?	-	-	-	-	-	-	-	+
M218	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
563	-	-	?	+	-	-	-	-	-	+	-	-	?	-	-	-	-	-	-	-	+
4396	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+
Consensus	-	-	V	+	-	-	-	-	-	+	-	-	V	-	-	-	-	-	-	-	+
*C. auriscanis**	-	-	-	-	-	-	-	-	V	-	-		+	-	-	-	-	-	-	-	+

** Reactions of the type strain of C. auriscanis (CCUG 39938) described previously (Collins et al. 1999).*
? = Unknown, V = Variable, Consensus – A consensus biochemical profile for the 20 unknown isolates.

4.4. DISCUSSION

The 16S rRNA gene sequence of 4465 showed the greatest similarity with *C. auriscanis* (AJ243820) after both a "BLAST" search of the NCBI database (bit score 2672) and completion of a distance matrix (99.1%). *C. auriscanis* was first recognized and described as a new species by Collins *et al* (1999). Of six isolates first identified (all from canine samples), three were isolated from the ears of dogs with otitis. The type strain of *C. auriscanis* (CCUG 39938) is non-lipophilic (Collins *et al.* 1999), whereas the 20 isolates identified here were lipophilic (they have a definitive growth requirement which is met by the addition of one percent v/v Tween 80 to the basal growth media). Therefore the 20 isolates identified during the teat sealer study can be differentiated simply and conclusively from their closest known relative, *C. auriscanis*. The 20 isolates represent a currently unknown or previously uncharacterised species. "*Corynebacterium langfordii*" (From "Langford" the site of Bristol Veterinary Schools clinical site where this organism was first identified) is proposed as a temporary name for this organism whilst final typing is completed and to allow ease of identification during this study.

"*C. langfordii*" was identified from the same quarter of the same cow (right fore quarter of cow 169, farm B) at all three sampling time points (drying off, calving and post calving). There were a total of 70 days between first isolation at drying off and third isolation in the post calving sample. Five isolates of "*C. langfordii*" were identified from three quarters of the same cow (cow 112, farm G) with at least one sample isolated at all three sampling time points. There was a total of 76 days between first isolation at drying off and third isolation in the post calving sample. Both cows (169B and 112G) received the non-antibiotic teat sealer as DCT.

Isolation of the same organism from the same quarter at different sampling time points confirms that the isolates identified represented IMI and were not sample contaminants from the environment. "*C. langfordii*" was able to establish long term IMI capable of surviving the dry period and should be regarded as a pathogen of the bovine mammary gland. Unfortunately, it was not possible to follow infection further into the following lactation to assess IMI dynamics during lactation, because results of restriction analysis were not available until well over 12 months after isolates were identified in the calving and post calving samples. Interestingly, isolation from either side of the dry period may not have been possible if an antibiotic DCT had been used

on all animals. Further work is needed to definitively demonstrate that "*C. langfordii*" (isolated during this study) is capable of establishing infection in bacteriologically negative quarters after experimental challenge.

Percent similarity between the 16S rRNA sequences of different isolates describes the amount of common sequence that organisms share and is therefore a reflection of their evolutionary relationship (the greater the similarity the closer the relationship). All currently recognised members of the *Corynebacterium* genus shared similarity values of greater than 90% with "*C. langfordii*". It would appear that "*C. langfordii*" is closely related to other members of the genus.

16S rRNA similarity values of less than 97% have been described as necessary for the definition a new species (Stackebrandt and Goebel 1994). Based on this definition, *C. auriscanis* and "*C. langfordii*" should not be differentiated from each other. However, as previously mentioned, the two can be simply distinguished based on their differing growth requirements for free fatty acid. Other members of the *Corynebacterium* genus have been differentiated from each other based on biochemical properties and defined as separate species despite very high similarity values *e.g. C. propinquum, C. pseudodiptheriticum* (>99% (Pascual *et al.* 1995; Ruimy *et al.* 1995)), *C. acetoacidophilum, C. glutamicum* (>99% (Pascual *et al.* 1995)), *C. diptheriae, C. pseudotuberculosis, C. ulcerans* (>98% (Pascual *et al.* 1995; Ruimy *et al.* 1995)), *C. coyleae* and *C. afermentans* subsp. *lipophilum* (>98% (Funke *et al.* 1997)). Sjödén *et al* (1998) concluded that there is no single 16S rRNA sequence divergence value for differentiating and describing new species.

A phylogenetic tree produced by the neighbor joining distance method and tested for reliability by bootstrap analysis (500 replications) confirmed that sequence 4465 lay inside the grouping containing all members of the *Corynebacterium* genus and within a previously recognised distinct monophyletic group containing, *C. bovis, C. auriscanis, C. urealyticum, C. falsenii* and *C. jeikeium* (Collins *et al.* 1999; Renaud *et al.* 2001).

Based on their lipid growth requirements *C. auriscanis* (non-lipophilic) and "*C. langfordii*" (lipophilic) can be distinguished. A *Corynebacterium* species has previously been described with two subspecies, which are differentiated solely on their lipid growth requirements, *C. afermentans* subsp *afermentans* (non-lipophilic) and *C. afermentans* subsp. *lipophilum* (lipophilic) (Riegel *et al.* 1993). Like *C.*

auriscanis and "*C. langfordii*" the two sub-species of *C. afermentans* have a very high 16S rRNA similarity percentage (99.8% (Ruimy *et al.* 1995)); however, they were indistinguishable based on their biochemical properties (Riegel *et al.* 1993). Comparison between the biochemical profiles of *C. auriscanis* and the consensus profile of "*C. langfordii*" revealed that the two could be differentiated simply. "*C. langfordii*" possessed both alkaline phosphatase and urease activity, which *C. auriscanis* does not (Table 4.4). Thus despite very high 16S rRNA sequence similarity values, *C. langfordii* and *C. auriscanis* can be differentiated based on their lipid growth requirements and biochemical properties. It is therefore proposed that they should be considered separate species.

The pathogenicity of "*C. langfordii*" is difficult to assess based on the information available for the 20 isolates identified in this study. Eighteen of these isolates came from "screening" samples, apparently causing subclinical disease. Two originated from cases of clinical mastitis and in both cases "*C. langfordii*" was the only organism isolated. "*C. langfordii*" could have been responsible for the clinical disease seen, alternatively it could have been an incidental finding from a case of mastitis in which the actual causal pathogen was not identified.

C. bovis has rarely been identified as a cause of clinical mastitis in the past (Cobb and Walley 1962; Counter 1981; Hillerton *et al.* 1995; Morin and Constable 1998). In all of these cases the method of speciation is not clearly described or inadequate. Identification may have been based on the isolation of a lipophilic Gram-positive coryneform organism from milk samples. Thus the possibility remains that a coryneform organism other than *C. bovis* may have caused the clinical mastitis described in these cases. "*C. langfordii*" is also a lipophilic coryneform of milk origin. It or an as yet unidentified organism with similar characteristics could have been responsible for the clinical disease described in these papers. Further work is necessary to assess both the dynamics of "*C. langfordii*" IMI during lactation and its pathogenicity and clinical significance. The affect of "*C. langfordii*" IMI on quarter SCC will be considered further in Chapter 5.

"*C. langfordii*" probably remained unrecognised because of its close morphological similarity to *C. bovis* and its seemingly limited pathogenicity within the bovine mammary gland. *C. bovis* is considered a pathogen of limited significance in the

bovine mammary gland and so little work has been carried out on this organism since its detailed description in the 1960's (Cobb and Walley 1962; Cobb 1966; Harrigan 1966). The limited significance and high prevalence of *C. bovis* in clinical samples limits the diagnostic effort that most laboratories will undertake to definitively identify lipophilic coryneforms of milk origin. Most consider the identification of slow growing Gram-positive coryneforms that produce tiny colonies after 48-72 hours on blood agar adequate for the identification of *C. bovis* (Pers Obs). The possibility remains that *C. langfordii* is a new organism not present in bovine milk samples until recently, however it is perhaps more likely that its presence in bovine milk samples has simply been overlooked because of its close morphological similarity to *C. bovis*.

In 1995 Vaneechoutte *et al* predicted "For taxonomical purposes, endonuclease restriction analysis could be used for rapid screening of large collections of strains in order to select strains with different 16S rDNA restriction patterns for further studies, including 16S rDNA sequencing". This methodology has proved ideal for the screening and identification of *C. bovis* in large numbers of lipophilic coryneforms isolated from the bovine mammary gland. It also allowed the differentiation of 20 unusual isolates, which have been identified as a currently unrecognized species, by 16S rRNA gene sequencing. Further work is currently being undertaken on "*C. langfordii*" to broaden the definition of its biochemical characteristics and to confirm its status within the genus prior to its publication and description as a novel species.

CHAPTER 5: AN EVALUATION OF THE PREVALENCE AND SIGNIFICANCE OF *C. BOVIS* IMI IN AND AROUND THE DRY PERIOD.

5.1. INTRODUCTION

Data from the teat sealer study (Chapter 2) provided information on new IMI rates during the dry period in two groups of animals, who received either an internal teat sealer with no antibiotic properties or a long acting antibiotic DCT. Employing a method based on endonuclease restriction of the 16S rRNA gene sequence, *C. bovis* was speciated and differentiated from other coryneform organisms isolated during the study (Chapter 3). With this information it is possible to assess the impact of IMI with *C. bovis* on quarter SCC and the rate of IMI with other organisms before, during and after the dry period.

Many studies have previously investigated the impact of IMI with *C. bovis* on the subsequent rate of new IMI with other pathogens. Most have concentrated on evaluating the role of *C. bovis* on the subsequent rate of new IMI with other pathogens during lactation, although two previous studies have investigated the role of *C. bovis* during the dry period. A small trial conducted as part of a larger study by Pankey *et al* (1985) and more recently a four farm study by Berry and Hillerton (2002) have demonstrated no difference in the new dry period IMI rate between quarters infected with *C. bovis* at drying off compared with those that were not.

In light of the information provided by speciation of *C. bovis* by 16S rRNA endonuclease restriction analysis, the database generated during the teat sealer study was re-visited to assess the impact of *C. bovis* on quarter SCC, dry period IMI rate with other pathogens and clinical mastitis during the first 100 days of the next lactation.

5.2. METHODOLOGY

5.2.1. Data Collation and Statistical Analysis

The database produced from the results of the teat sealer study was re-evaluated in light of the results of the speciation of coryneform isolates described in Chapter 4. Data were collated and analysed using Excel and Access 2000 (Microsoft Corporation), Stata version 6 (Stata Corporation, Texas), Epi-Info version 6.04b (CDC, Atlanta) and Egret version 2.0.3 (Cytel Software Corporation, Cambridge, MA). The χ^2 test was used to compare proportions; the unpaired T test was used to compare SCC after log transformation to normalise the data. The significance probability was set at $P \leq 0.05$ for a two -tailed test. All farms were used in all analyses.

All available dry period data (drying off, calving and post calving samples) were used for all analyses.

In collaboration with Martin Green (University of Warwick), unconditional logistic regression was used to model the interactions between *C. bovis* and other pathogen IMI, employing a method similar to that previously described (Section 2.2.18.). The effects of quarters retaining a *C. bovis* IMI during the dry period (culture positive at drying off and calving) compared to those that were not infected at either sampling time point on the number of new dry period IMI caused by a major pathogen was investigated. Farms B, F, G, J, K & L dropped out from the analysis because no cows on these farms retained a *C. bovis* IMI during the dry period.

5.2.2. Definition of Terms Used for Analysis

Definitions of terms used for analysis were identical to those laid out in previously (Section 2.2.19.). The following additional terms were specified and utilized.

5.2.2.1. Corynebacterium bovis

A lipophilic coryneform isolate identified as *C. bovis* by endonuclease restriction analysis of the 16S rRNA gene sequence

5.2.2.2. *"Corynebacterium langfordii"*

A lipophilic coryneform isolate identified as "*C. langfordii*" by endonuclease restriction analysis and sequencing of the 16S rRNA gene sequence.

5.2.2.3. *Non-Lipophilic Coryneform*

A coryneform isolate identified as non-lipophilic based on growth characteristics on media with and without one percent v/v Tween 80.

5.2.2.4. *Other Coryneform*

All coryneform isolates other than *C. bovis*, *i.e.* other lipophilic coryneforms and non-lipophilic coryneforms together.

5.3. RESULTS

5.3.1. Prevalence of *C. bovis* at Drying Off

The overall prevalence of *C. bovis* in the 16 herds at drying off was 21.2% (429 of 2020), 38.0% (192 of 505) and 81.3% (13 of 16) at the quarter, cow and farm levels respectively. On individual farms the prevalence ranged from 0% (Farms B, F & K) to 74% (Farm S) at the quarter level (Figure 5.1) and 0% (Farms B, F & K) to 100% (Farm S) at the cow level (Figure 5.2).

Figure 5.1: Proportion of Quarters Infected with *C. bovis* at Drying Off on Each Farm

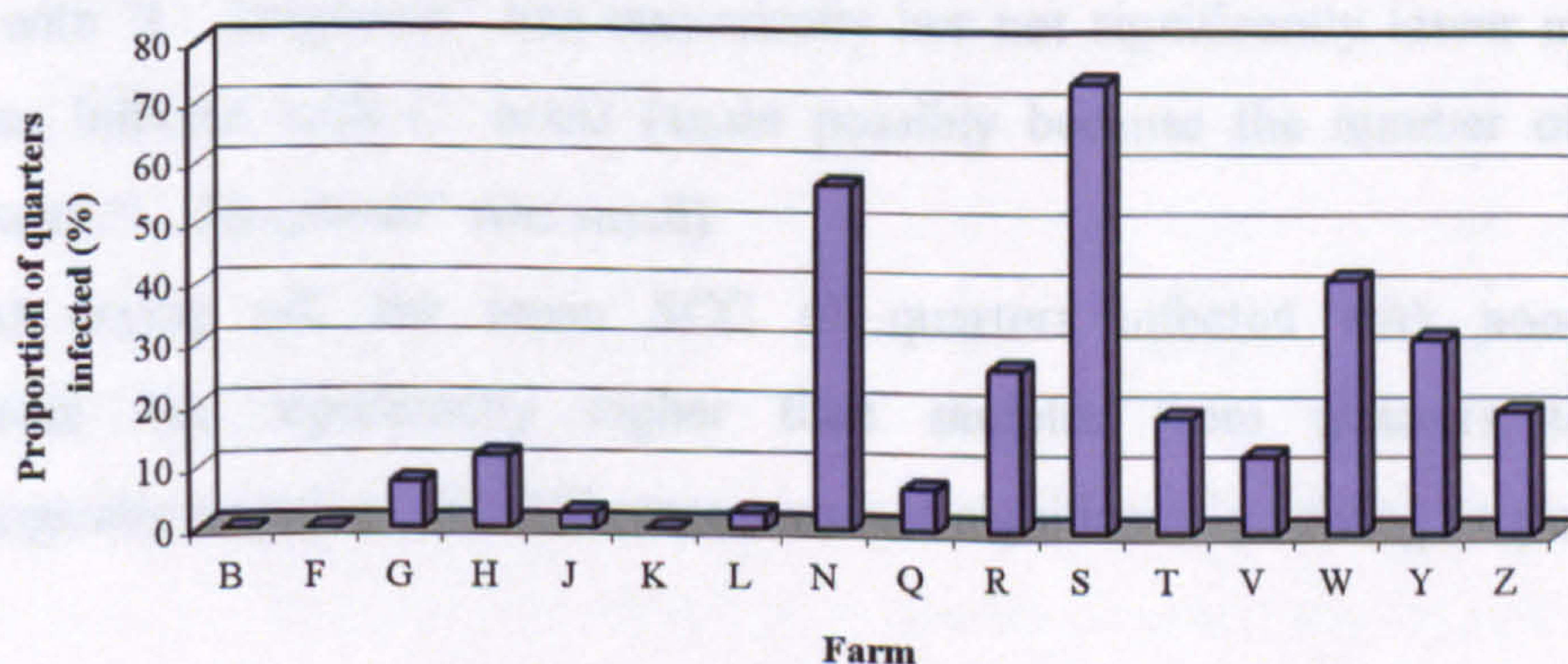
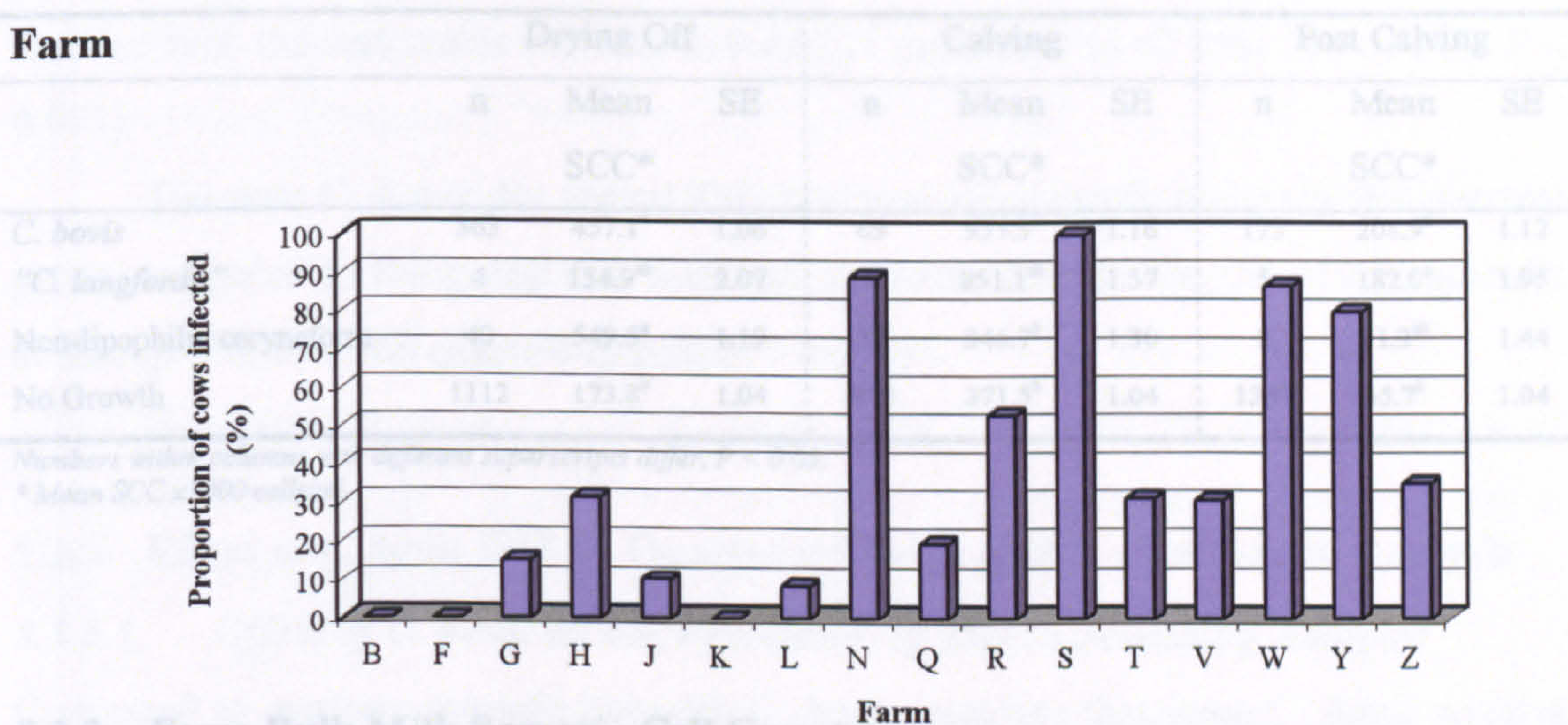


Figure 5.2: Proportion of Cows Infected with *C. bovis* at Drying Off on Each Farm



5.3.2. Affect of Coryneform IMI on Quarter Somatic Cell Count

Compared to quarters that yielded no growth after bacterial culture, quarters infected with *C. bovis* had significantly higher mean SCC at all three sampling time points (Table 5.1).

Seven to 14 days after calving, quarters infected with "*C. langfordii*" had significantly higher mean SCC than quarters that were bacteriologically negative. Despite large numerical differences at calving the difference was not significant at drying off or calving (propably because the number of quarters infected was small). The mean SCC of quarters infected with "*C. langfordii*" were similar to those infected with *C. bovis* in calving and post calving screening samples. At drying off quarters infected with "*C. langfordii*" had numerically but not significantly lower mean SCC than those infected with *C. bovis* (again possibly because the number of quarters infected with "*C. langfordii*" was small).

At drying off, the mean SCC of quarters infected with non-lipophilic coryneforms was significantly higher than samples from quarters that were bacteriologically negative; the difference was not significant in calving or post calving samples.

Table 5.1: Somatic Cell Count of Quarters Infected With *C. bovis*, "*C. langfordii*", Non-Lipophilic Coryneforms and Those Yielding No Growth at Three Sampling Time Points

	Drying Off			Calving			Post Calving		
	n	Mean SCC*	SE	n	Mean SCC*	SE	n	Mean SCC*	SE
C. bovis	363	457.1^{a}	1.06	69	933.3^{a}	1.16	173	208.9^{a}	1.12
"*C. langfordii*"	4	154.9^{ab}	2.07	9	851.1^{ab}	1.57	5	182.0^{a}	1.95
Non-lipophilic coryneform	40	549.5^{a}	1.19	38	346.7^{b}	1.30	8	91.2^{ab}	1.44
No Growth	1112	173.8^{b}	1.04	917	371.5^{b}	1.04	1337	45.7^{b}	1.04

Numbers within columns with different superscripts differ, $P < 0.05$.
** Mean SCC x1000 cells/mL*

5.3.3. Farm Bulk Milk Somatic Cell Counts

The bulk milk somatic cell counts of the 16 farms at the start of the teat sealer study are shown in Table 5.2. The four farms with the lowest prevalence of *C. bovis* at the quarter level (B, F, J & K) had the 1st, 10th, 14th and 15th highest BMSCC; the four farms with highest prevalence (N, S, W & Y) had the 2nd, 8th, 11th and 16th highest BMSCC.

Table 5.2: BMSCC, BMSCC Rank and Percent of Quarters Infected With *C. bovis* at Drying Off on the 16 Study Farms

Farm	BMSCC (cells/ml)*	BMSCC Rank (Low-High)	*C. bovis* positive quarters (%)	Farm	BMSCC (cells/ml)	BMSCC Rank (Low-High)	*C. bovis* positive quarters (%)
B	51,000	1st	0	Z	111,000	9th	19.9
Y	63,000	2nd	32.0	J	115,000	10th	2.5
G	87,000	3rd	7.5	S	117,000	11th	74.0
V	91,000	4th	12.5	Q	120,000	12th	7.0
L	94,000	5th	2.6	T	131,000	13th	18.8
H	101,000	6th	11.7	K	143,000	14th	0
R	102,000	7th	26.5	F	148,000	15th	0
N	119,000	8th	56.9	W	163,000	16th	41.7

**BMSCC at the start of the study*

5.3.4. Dry Period Cure and New IMI Rates During the Dry Period

The overall dry period cure rate of *C. bovis* IMI was 83.0%. It was however significantly higher in the group which received antibiotic DCT compared to those that received the teat sealer (99.5%, 210 of 211 compared to 67.0%, 146 of 218, $P < 0.001$).

The new *C. bovis* dry period IMI rate was significantly higher in the teat seal group compared to the group that received antibiotic DCT (4.0%, 30 of 752 quarters at risk *cf.* 0%, 0 in 765 quarters at risk, $P < 0.001$)

5.3.5. Effect of *C. bovis* IMI on Quarter Infection Status - Univariate Analysis

5.3.5.1. Effect of C. bovis on the Prevalence of IMI in Screening Samples

Compared to quarters not infected with *C. bovis*, quarters that were *C. bovis* positive at drying off were significantly less likely to be concurrently infected with *Micrococcus* spp. (21 in 1591 *cf.* 0 in 429, $P = 0.01$), Coagulase negative Staphylococci (185 in 1591 *cf.* 24 in 429, $P < 0.001$), other coryneforms (59 in 1591 *cf.* 0 in 429, $P < 0.001$), a minor pathogen (262 in 1591 *cf.* 24 in 429, $P < 0.001$), coagulase positive Staphylococci (21 in 1591 *cf.* 0 in 429, $P = 0.01$), *Enterococcus* spp. (17 in 1591 *cf.* 0 in 429, $P = 0.03$) and a major pathogen (61 in 1591 *cf.* 6 in 429, $P = 0.02$), Table 5.3.

Compared to quarters not infected with *C. bovis*, quarters that were *C. bovis* positive at calving were significantly less likely to be concurrently infected with coagulase negative Staphylococci (419 in 1824 *cf.* 0 in 103, $P = 0.005$), other coryneforms (122 in 1824 *cf.* 0 in 103, $P = 0.01$), a minor pathogen (535 in 1824 *cf.* 12 in 103, $P < 0.001$), all *Enterobacteriaceae* species (71 in 1824 *cf.* 0 in 103, $P = 0.03$) and a major pathogen (273 in 1824 *cf.* 5 in 103, $P = 0.005$), Table 5.4.

Compared to quarters not infected with *C. bovis*, quarters which were *C. bovis* positive in post calving samples were significantly less likely to be concurrently infected with coagulase positive Staphylococci (37 in 1722 *cf.* 0 in 209, $P = 0.03$) and a major pathogen (96 in 1722 *cf.* 2 in 209, $P = 0.007$), Table 5.5.

Coagulase positive Staphylococci were isolated in 21 drying off samples, 33 calving samples and 37 post calving samples. No single coagulase positive Staphylococcal isolate was ever found in a quarter concurrently infected with *C. bovis*.

5.3.5.2. Effect of C. bovis on New IMI During the Dry Period

Quarters that retained a *C. bovis* IMI during the dry period (culture positive at drying off and calving) were significantly less likely to acquire a new dry period IMI caused by all major pathogens ($P = 0.04$), compared to quarters that were not infected at either sampling time point (Table 5.6, 3 / 73 *cf.* 174 / 1362).

Quarters that were *C. bovis* positive at calving were significantly less likely to have acquired a new dry period IMI caused by coagulase negative Staphylococci (10 in 103 *cf.* 336 in 1824, $P = 0.04$), other coryneforms (0 in 103 *cf.* 104 in 1824, $P = 0.02$), a minor pathogen (11 in 103 *cf.* 443 in 1824, $P = 0.002$) all *Enterobacteriaceae* species (0 / 103 *cf.* 63 / 1824, $P = 0.05$) and a major pathogen (5 in 103 *cf.* 243 in 1824, $P = 0.02$), compared with those that were not, Table 5.7.

5.3.5.3. Effect of Other Coryneforms on New IMI During the Dry Period

Quarters which acquired a new coryneform IMI other than *C. bovis* during the dry period were significantly more likely to acquire a new dry period IMI caused by *Acinetobacter* spp. (5 in 104 *cf.* 23 in 1823, $P = 0.015$) and Non fermenters (4 in 104 *cf.* 18 in 1823, $P = 0.03$), compared to quarters that did not. Results against all major pathogens combined demonstrated a trend toward significance (20 in 104 *cf.* 228 in 1823, $P = 0.07$), Table 5.8.

5.3.5.4. Effects of C. bovis on Clinical Mastitis in the First 100 Days of Lactation

There was no difference in the number of quarters which suffered clinical mastitis during the first 100 days of lactation in quarters that were *C. bovis* positive or negative in drying off (10 in 429 *cf.* 44 in 1591), calving (5 in 103 *cf.* 49 in 1824) or post calving (10 in 429 *cf.* 44 in 1591) samples.

Quarters that retained a dry period *C. bovis* IMI were no more or less likely to suffer clinical mastitis during the first 100 days of lactation compared to those that did not (2 in 70 *cf.* 55 in 1850).

Quarters that acquired a new dry period *C. bovis* IMI were no more or less likely to suffer clinical mastitis during the first 100 days of lactation compared to those that did not (3 in 29 *cf.* 53 in 1866).

Table 5.3: Number of Quarter IMI Present at Drying Off, in Quarters Positive or Negative for *C. bovis* at Drying Off, by Pathogen

Diagnosis	*C. bovis* culture positive quarters at drying off (n = 429)	*C. bovis* culture negative quarters at drying off (n = 1591)
Coagulase +ve Staphylococci	0[c]	21[d]
S. dysgalactiae	0	0
S. uberis	0	2
Streptococcus spp. (Other)	2	5
Enterococcus spp.	0[a]	17[b]
E. coli	1	3
Other *Enterobacteriaceae* spp.	0	2
All *Enterobacteriaceae*	1	5
Pseudomonas spp.	1	0
Acinetobacter spp.	0	3
Non fermenters	1	1
Bacillus spp.	1	8
Aspergillus spp.	0	2
Yeast spp.	0	1
All Major Pathogens	6[a]	61[b]
Micrococcus spp.	0[c]	21[d]
Coag –ve Staphylococci	24[e]	185[f]
Other Coryneforms	0[e]	59[f]
All Minor Pathogens	24[e]	262[f]

[ab] Numbers within rows with different superscript differ $P \leq 0.05$; [cd] Numbers within rows with different superscript differ $P \leq 0.01$; [ef] Numbers within rows with different superscript differ $P \leq 0.001$.

Table 5.4: Number of Quarter IMI Present at Calving, in Quarters Positive or Negative for *C. bovis* at Calving, by Pathogen

Diagnosis	*C. bovis* culture positive quarters at calving (n = 103)	*C. bovis* culture negative quarters at calving (n = 1824)
Coagulase +ve Staphylococci	0	33
S. dysgalactiae	0	2
S. uberis	0	23
Streptococcus spp. (Other)	0	28
Enterococcus spp.	2	55
E. coli	0	55
Other *Enterobacteriaceae* spp.	0	17
All *Enterobacteriaceae*	0^{a}	71^{b}
Acinetobacter spp.	3	25
Chryseomonas spp.	0	1
Aerococcus spp.	0	2
Ochrobacter spp.	0	3
Non fermenters	0	23
Bacillus spp.	0	21
Other non speciated organisms	0	1
Aspergillus spp.	0	3
Yeast spp.	0	1
Mucur spp.	0	4
All Major Pathogens	5^{c}	273^{d}
Micrococcus spp.	1	32
Coag -ve Staphylococci	11^{c}	419^{d}
Other Coryneforms	0^{c}	122^{d}
All Minor Pathogens	12^{e}	535^{f}

[ab] Numbers within rows with different superscript differ $P \leq 0.05$; [cd] Numbers within rows with different superscript differ $P \leq 0.01$; [ef] Numbers within rows with different superscript differ $P \leq 0.001$.

Table 5.5: Number of Quarter IMI Present Seven to Fourteen Days After Calving, in Quarters Positive or Negative for *C. bovis* at that Time, by Pathogen

Diagnosis	*C. bovis* culture positive quarters post calving (n = 209)	*C. bovis* culture negative quarters post calving (n = 1722)
Coagulase +ve Staphylococci	0^a	37^b
S. dysgalactiae	0	7
S. uberis	1	14
Streptococcus spp. (Other)	0	6
Enterococcus spp.	0	13
E. coli	1	11
Other *Enterobacteriaceae* spp.	0	2
All *Enterobacteriaceae*	1	13
Acinetobacter spp.	0	3
Non fermenters	0	7
Bacillus spp.	0	3
Yeast spp.	0	2
Mucur spp.	0	2
All Major Pathogens	2^c	96^d
Micrococcus spp.	0	11
Coag -ve Staphylococci	17	133
Other Coryneforms	0	17
All Minor Pathogens	17	157

[ab] Numbers within rows with different superscript differ $P \leq 0.05$; [cd] Numbers within rows with different superscript differ $P \leq 0.01$.

Table 5.6: Number of New Dry Period IMI Acquired in Quarters Retaining a *C. bovis* IMI During the Dry Period, Compared to those that were not Infected, by Pathogen

Diagnosis	Quarters retaining a *C. bovis* IMI during the dry period (n = 73)	Quarters *C. bovis* culture negative at drying off and calving (n = 1362)
Coagulase +ve Staphylococci	0	14
S. dysgalactiae	0	2
S. uberis	0	16
Streptococcus spp. (Other)	0	16
Enterococcus spp.	1	41
E. coli	0	41
Other *Enterobacteriaceae* spp.	0	10
All *Enterobacteriaceae*	0	51
Acinetobacter spp.	2	12
Chryseomonas spp.	0	1
Aerococcus spp.	0	1
Ochrobacter spp.	0	2
Non fermenters	0	15
Bacillus spp.	0	11
Other non speciated organisms	0	1
Aspergillus spp.	0	2
Yeast spp.	0	1
Mucur spp.	0	4
All Major Pathogens	3[a]	174[b]
Micrococcus spp.	1	23
Coag –ve Staphylococci	7	241
All Minor Pathogens	8	259

[ab] *Numbers within rows with different superscript differ P ≤ 0.05.*

Table 5.7: Number of New Dry Period IMI Acquired in Quarters that were Culture Positive or Negative for *C. bovis* at Calving, by Pathogen

Diagnosis	*C. bovis* culture positive quarters at calving (n = 103)	*C. bovis* culture negative quarters at calving (n = 1824)
Coagulase +ve Staphylococci	0	17
S. dysgalactiae	0	2
S. uberis	0	23
Streptococcus spp. (Other)	0	25
Enterococcus spp.	2	53
E. coli	0	55
Other *Enterobacteriaceae* spp.	0	17
All *Enterobacteriaceae*	0^{a}	63^{b}
Acinetobacter spp.	3	25
Chryseomonas spp.	0	1
Aerococcus spp.	0	2
Ochrobacter spp.	0	2
Non fermenters	0	22
Bacillus spp.	0	13
Other non speciated organisms	0	1
Aspergillus spp.	0	3
Yeast spp.	0	1
Mucur spp.	0	4
All Major Pathogens	5^{a}	243^{b}
Micrococcus spp.	1	31
Coag -ve Staphylococci	10^{a}	336^{b}
Other Coryneforms	0^{a}	104^{b}
All Minor Pathogens	11^{c}	443^{d}

[ab] Numbers within rows with different superscript differ $P \leq 0.05$; [cd] Numbers within rows with different superscript differ $P \leq 0.01$.

Table 5.8: Number of New Dry Period IMI, in Quarters Acquiring an IMI Caused by *Corynebacterium* Species Other than *C. bovis* During the Dry Period, by Pathogen

Diagnosis	Quarters acquiring an IMI caused by *Corynebacterium* species other than *C. bovis* during the dry period (n = 104)	Quarters not acquiring an IMI caused by *Corynebacterium* species other than *C. bovis* during the dry period (n = 1823)
Coagulase +ve Staphylococci	0	17
S. dysgalactiae	0	2
S. uberis	2	21
Streptococcus spp. (Other)	2	23
Enterococcus spp.	2	53
E. coli	5	50
Other *Enterobacteriaceae* spp.	1	16
All *Enterobacteriaceae*	5	58
Acinetobacter spp.	5^{a}	23^{b}
Chryseomonas spp.	0	1
Aerococcus spp.	0	2
Ochrobacter spp.	0	2
Non fermenters	4^{a}	18^{b}
Bacillus spp.	0	13
Other non speciated organisms	0	1
Aspergillus spp.	1	2
Yeast spp.	0	1
Mucur spp.	0	4
All Major Pathogens	20*	228*
Micrococcus spp.	2	30
Coag –ve Staphylococci	21	322
Other Coryneforms	0	30
All Minor Pathogens	22	374

*[ab] Numbers within rows with different superscript differ $P \leq 0.05$; [cd] Numbers within rows with different superscript differ $P \leq 0.01$. * $P = 0.07$.*

5.3.6. Effect of *C. bovis* IMI on Quarter Infection Status - Multivariate Analysis

5.3.6.1. Effect of C. bovis on New IMI During the Dry Period

Compared to quarters that were *C. bovis* negative at drying off and calving, quarters that retained a dry period *C. bovis* IMI (culture positive at drying off and calving) were significantly less likely to acquire a new dry period IMI caused by a major pathogen (Table 5.9, Odds ratio = 0.28, 95% confidence interval 0.07 – 1.10, P = 0.04).

Table 5.9: Logistic Regression Model to Assess the Effect of Quarters that Retained a Dry Period *C. bovis* IMI (Compared to those that were not Infected) on the Number of New Major Pathogen Dry Period IMI

Variable	Coefficient	SE	Odds Ratio	95% CI	*P* value
Outcome variable – New dry period IMI caused by a major pathogen					
CONSTANT	-4.17	1.14	0.02	0.00 - 0.15	< 0.001
CONFOUNDERS					
FARM					
G	0.00	1.00	-	-	
H	0.91	0.64	2.48	0.70 - 8.74	0.157
N	-0.13	0.64	0.88	0.25 - 3.07	0.839
Q	0.25	0.58	1.28	0.41 - 4.01	0.672
R	-0.17	0.89	0.85	0.15 - 4.81	0.851
S	-1.14	1.28	0.32	0.03 - 3.90	0.372
T	-1.64	1.23	0.19	0.02 - 2.17	0.183
V	-0.63	0.76	0.53	0.12 - 2.34	0.402
W	0.16	0.93	1.17	0.19 - 7.23	0.862
Y	0.34	0.72	1.40	0.34 - 5.77	0.642
Z	0.27	0.67	1.32	0.36 - 4.88	0.681
DRYING OFF SCC (LOG)					
Every increase in 1	0.69	0.30	2.00	1.12 - 3.57	0.019
YIELD					
Every increase in 1 litre	0.01	0.04	1.01	0.94 - 1.08	0.839
LACTATION NUMBER					
Lactation three or greater	0.00	1.00	-	-	
Lactation two	-0.20	0.32	0.82	0.44 - 1.55	0.543
EXPOSURE VARIABLE					
No *C. bovis* IMI at drying off or calving (Ref)	-	-	-	-	-
Retained *C. bovis* IMI during dry period	-1.26	0.69	0.28	0.07 - 1.10	0.044

** % SCL is a scalar term for the random effect of cow (Egret Manual Version 2.0.3, Cytel Statistical Software Corp, USA)*

5.4. DISCUSSION

Quarters infected with *C. bovis* had a significantly higher SCC than quarters that were bacteriologically negative. This was true at all three sampling time points and is in agreement with other authors (Honkanen-Buzalski *et al.* 1984; LeVan *et al.* 1985; Erskine *et al.* 1987; Lam *et al.* 1997). The reported average increase in SCC in *C. bovis* positive quarters compared to those yielding no growth, is approximately 50,000 cells/ml (section 1.3.5.1.). Increases were much higher in this study probably because SCC tends to be higher at drying off and around calving (Barkema *et al.* 1999). Samples collected at this time may not be representative of the lactation as a whole and perhaps the most appropriate samples to compare in this study are those collected seven to 14 days after calving. In these samples, quarters infected with *C. bovis* had a cell count 163,000 cells/ml higher than culture negative quarters. This is still above the average level detailed in the literature but very similar to that reported by some authors (Erskine *et al.* 1987). SCC may have been elevated in these samples because many of them were collected in the morning shortly after milking; residual strippings from quarters have higher SCC (Holdaway *et al.* 1996).

The prevalence of *C. bovis* between farms showed huge variation at both the quarter (0-74%) and cow levels (0-100%). At the quarter level, the four farms with the lowest prevalence (B, F, J & K) had the 1st, 10th, 14th and 15th highest BMSCC and the four farms with highest prevalence (N, S, W & Y) had the 2nd, 8th, 11th and 16th highest BMSCC. There seems no association between BMSCC and prevalence of *C. bovis*. This is in agreement with data presented by Hillerton *et al* (1995) that demonstrated on one farm where the prevalence of *C. bovis* increased from less than 30% to greater than 70% over a five year period there was no increase in the BMSCC.

Farm S had a quarter and cow level prevalence of 74 and 100%, yet their BMSCC was well below the national average (117,000 cells/ml). It would therefore appear possible to have a very high prevalence of *C. bovis* yet still maintain BMSCC within acceptable limits.

The link between the prevalence of *C. bovis* and BMSCC appears complex. Quarters infected with *C. bovis* have higher SCC and it is therefore logical to assume that an increase in the prevalence of *C. bovis* would lead to an increase in BMSCC but this does not always appear to be the case. Quarter SCC is raised substantially more by subclinical IMI with major pathogens (Lam *et al.* 1997). It can be postulated if *C.*

bovis protects quarters from infection with other pathogens, that herds with a high prevalence of *C. bovis* may have a lower prevalence of subclinical major pathogen IMI. Consequently BMSCC will be mainly raised by the effect of *C. bovis* (high quarter prevalence but small increase in SCC). The converse may also be true, that herds with a low prevalence of *C. bovis* may have a higher prevalence of subclinical major pathogen IMI (no "protective" effect present). In this case the BMSCC will be mainly elevated by the effect of major pathogens (low prevalence but large increase in SCC).

Based on the data collected during this study it is difficult to draw any firm conclusions because of the subset of cows investigated (low SCC) and the limited number of samples collected. Further research is required to investigate this potentially very interesting and clinically important relationship. An "ideal" balance point may exist, at which the prevalence of *C. bovis* limits the number of quarters sub-clinically infected with major pathogens yet the effect of *C. bovis* on BMSCC is limited. This ideal balance point may be the point at which the majority of quarters most susceptible to infection have acquired a *C. bovis* IMI that protects them from subsequent supra-infection with other more pathogenic organisms. The number of at risk quarters will probably vary from farm to farm depending on factors such as milk yield, teat end condition, genetic make up of the herd, level of challenge (*i.e.* prevalence of subclinical IMI) and environmental conditions.

In calving and post calving screening samples the mean SCC of quarters infected with "*C. langfordii*" was higher than bacteriologically negative quarters although the difference was only significant in the post calving samples. An elevated SCC implies that quarters infected with "*C. langfordii*" are mounting an immune response, implying that "*C. langfordii*" IMI are not sample contaminants from the environment. In calving and post calving screening samples the SCC elevation was similar to that in quarters infected with *C. bovis*. This initial work based on small numbers of infected quarters implies that the degree of quarter inflammation caused by "*C. langfordii*" is similar to that caused by *C. bovis*.

At drying off quarters infected with "*C. langfordii*" had significantly lower SCC than quarters infected with *C. bovis*, in fact quarters infected with "*C. langfordii*" had numerically lower SCC than those quarters which were bacteriologically negative. The number of quarters infected with "*C. langfordii*" at drying off was small (four);

the conclusions that can be drawn from such a small number of infections are limited. The possibility remains that these infections were sample contaminants from the environment. However, as previously discussed (Section 4.4), this would seem unlikely because at least two of these "*C. langfordii*" IMI established prolonged infections that were still present after the dry period. Further work is needed to assess the prevalence and significance of "*C. langfordii*" IMI, including an assessment of the impact of infection on SCC.

The potential protective effect afforded to a quarter by infection with *C. bovis* can be investigated in two ways, either *in vivo* or *in vitro*. *In vitro* methods will be discussed further in Chapters 6 and 7. Reports of *in vivo* work from the literature can be broadly divided into two types; those employing artificial infection techniques (of *C. bovis*, mastitis pathogen or both) and those employing either cross sectional or longitudinal study designs to assess natural IMI dynamics in the field. Both have flaws, which must be borne in mind whilst designing studies and interpreting the results that they produce.

Artificial infection studies rely on the selection of a strain or strains from either personal (Brooks and Barnum 1984; Pankey *et al.* 1985) or type culture collections. In one recent study, Watts *et al* (2000) demonstrated that 29 of 212 (13.7%) isolates held in lab collections and one isolate held in type culture collections were in fact not *C. bovis*. Great care must therefore be taken to ensure the validity of *C. bovis* strains selected for artificial infection studies. Selection of single strains also assumes that the characteristics of that strain are representative of the species as a whole. Large differences in pathogenicity between strains of *C. bovis* have been demonstrated in the mouse mammary gland (Anderson *et al.* 1985). If differences in pathogenicity occur, it is possible that strain differences in protective effect may also occur, especially if some or all of the mode of protection is via initiation of an intra-mammary inflammatory response. In this case selecting single strains with which to work may bias results produced if a markedly pathogenic/non-pathogenic or protective/non-protective strain is selected.

The second problem with artificial infection studies is the way in which artificial infections are established. The principal site of *C. bovis* IMI is the teat canal. Previous studies have induced artificial infection by dipping the quarter in *C. bovis* cultures (Brooks and Barnum 1984; Pankey *et al.* 1985) or inoculating them into the

teat canal (Brooks and Barnum 1984) or teat cistern (Brooks and Barnum 1984). With any of these methods it is impossible to know whether the IMI dynamics and thus potential protective effects afforded are similar to those produced by natural infections, especially in the case of inoculation through the teat canal.

Similarly, artificial infection studies also involve inducing major pathogen IMI in quarters either artificially (Brooks and Barnum 1984; Pankey *et al.* 1985) or naturally infected with *C. bovis* (Doane *et al.* 1987; Schukken *et al.* 1999). Inducing artificial infections in quarters naturally infected with *C. bovis* avoids the problems outlined above, however it does not avoid the similar problems associated with inducing major pathogen IMI. As with the selection of single strains of *C. bovis*, selection of single pathogen strains relies on accurate identification and assumes that the pathogenicity of the test organism is representative of the species as a whole. Some authors have tried to minimise this affect by using established infection models which employ strains of known pathogenicity *e.g. S. aureus* strain Newbould 305 (Brooks and Barnum 1984; Pankey *et al.* 1985; Schukken *et al.* 1999). However all artificial infection studies must employ a method of infection that may not replicate the situation in the field; either challenge dipping quarters in solutions containing many millions of organisms (Brooks and Barnum 1984; Pankey *et al.* 1985) or inoculation into the teat cistern (Brooks and Barnum 1984; Doane *et al.* 1987; Schukken *et al.* 1999) (which immediately circumvents the principal site of *C. bovis* colonisation). It is difficult to know how accurately results gained from artificial infection / challenge studies reflect the situation following natural infection in the field.

Studies based on naturally acquired field IMI avoid the problems of artificial infection work already discussed. There are however a number of problems also inherent with this methodology. *C. bovis* is an extremely infectious organism (Pankey *et al.* 1985), which spreads contagiously from cow to cow, especially during the milking process. However, even in herds where the prevalence is high, not all quarters become infected (Farm S, cow prevalence 100%, quarter prevalence 74%), which suggests that some quarters are naturally more prone to infection than others; this has been demonstrated in the field (Zadoks *et al.* 2001). If this is the case quarters that are more or less resistant to infection with *C. bovis* may also be more or less resistant to infection with major pathogens, a conclusion reached by Lam *et al* (1997). Thus studies that investigate quarters naturally infected with *C. bovis* in the field may in

fact be selecting a subpopulation of quarters naturally more prone to infection with other pathogens. Even if IMI with *C. bovis* does confer some degree of protection to these quarters, it may not be enough to overcome the inherent susceptibility of the quarter to acquire infection with other organisms. If this is the case it may explain some of the conflicting results present in the literature.

Field studies are of two principal types; cross sectional and longitudinal. Cross-sectional studies examine the protective effects of *C. bovis* by comparing the number of quarters infected with other pathogens in *C. bovis* positive and negative quarters at the time of sampling. Whilst providing good supporting evidence for an effect, finding fewer major pathogen IMI in *C. bovis* positive quarters is not a causal relationship as the order in which IMI occurred is impossible to elucidate. Longitudinal studies on the other hand are a much more powerful study design. They allow quarter infection status and hence disease incidence to be studied over time. This allows risk/protective factors to be studied in the field. Compared to cross sectional studies, longitudinal studies tend to be more expensive and time consuming to perform.

The complexity of natural IMI dynamics is also a problem in large field studies. Study results can be confounded by many factors such as parity, stage of lactation, yield, genetics, co-existing disease and nutritional status. The reliability of results depends on careful study design (which control for the effects of confounding factors), large studies and if possible the use of multivariate statistical techniques that can take into account the influence of confounding variables. All of these precautions were taken during the study described here.

Results of both cross sectional and longitudinal analysis were available for analysis during this study. Examination of the results of cross sectional analysis indicate that quarters infected with *C. bovis* were significantly less likely to be concurrently infected with other pathogens at all three sampling time points. Results were significant for coagulase positive Staphylococci, *Enterococcus* spp., all major pathogens, *Micrococcus* spp., Coagulase negative Staphylococci, other coryneforms and all minor pathogens at drying off; all *Enterobacteriaceae* species, all major pathogens, coagulase negative Staphylococci, other coryneforms and all minor pathogens at calving; coagulase positive Staphylococci and all major pathogens in post calving samples. Quarters that were *C. bovis* positive at calving were

significantly less likely to have acquired a new dry period IMI caused by all *Enterobacteriaceae* species, all major pathogens, coagulase negative Staphylococci, other coryneforms and all minor pathogens.

Results of longitudinal analysis indicate that quarters that retained a *C. bovis* IMI during the dry period were significantly less likely to acquire a new dry period IMI caused by a major pathogen compared to quarters that were not infected with *C. bovis* at drying off or calving. The difference was significant after both univariate and multivariate analysis. The logistic regression model was improved if the confounding effects of farm, drying off SCC, last recorded yield before drying off and lactation number were included in the model. The model was not improved by the addition of DCT *i.e.* the results were not confounded by this variable. Considering that the *C. bovis* new IMI rate during the dry period and the dry period cure rate were significantly different between the two treatment groups this finding was surprising. The likely explanation is that only one quarter in the antibiotic group retained a *C. bovis* IMI during the dry period compared to 72 in the teat sealer group. Because of this disparity all the protective effect of quarters retaining a *C. bovis* IMI during the dry period occurred in the teat sealer group limiting the confounding influence of the antibiotic DCT on the effect.

The author accepts that based on available data it is impossible to be sure that these quarters actually represent retained IMI and are not in fact quarters that were cured and then became re-infected with *C. bovis* at a later date. However, the new dry period IMI rate with *C. bovis* was low (30 of 1517 of quarters, 2%). Unless quarters that have previously been infected with *C. bovis* are significantly more prone to re-infection at a later date, it is logical to assume that quarters that cured and then became re-infected only represent two percent of all the quarters that retained a dry period *C. bovis* infection. This number of quarters is unlikely to substantially influence the results or subsequent analysis.

Taken as a whole these results would suggest that in this study, quarters infected with *C. bovis* were "protected" against infection with a wide range of other major and minor pathogens. This study, therefore, agrees with the results of other natural infection studies (Black *et al.* 1972; Rainard and Poutrel 1988; Pociecha 1989; Lam *et al.* 1997; Zadoks *et al.* 2001). It is also the first to demonstrate that quarters infected with *C. bovis* during the dry period are significantly less likely to become infected with other pathogens. The only other studies that investigated the affect of *C.*

bovis at this time did not show any statistically significant difference in the new dry period IMI rate (Pankey *et al.* 1985; Berry and Hillerton 2002) and a third only demonstrated protection against clinical mastitis in the following lactation (Green *et al.* 2002).

A number of potential issues arise from the animals selected for inclusion in this study, which need to be addressed. Firstly, the subset of cows selected were all low SCC animals largely uninfected at drying off which may not be representative of the population as a whole. These animals may be inherently less prone to acquiring IMI so although the results reported here are pertinent to these animals it may not be safe to assume that the results can be extrapolated to the population as a whole. Similar work is needed on higher SCC animals to confirm or refute the findings of this study before it can be concluded categorically that quarters infected with *C. bovis* during and around the dry period are less prone to infection with other organisms.

Secondly, five farms (B, F, J, K & L) dropped out of the logistic regression model used to investigate the effect of quarters retaining a *C. bovis* IMI. No cows on these farms retained a *C. bovis* infection during the dry period. To calculate an odds ratio it is necessary to compare two events (Kleinbaum 1994) *i.e.* quarters that retained a *C. bovis* IMI and quarters that were not infected with *C. bovis* at drying off or calving. Farms on which one or other events did not occur were therefore excluded. An alternative approach would have been to include farm as a random effect in the model. However, because the five farms on which no quarters retained a *C. bovis* infection added no information to the model the outcome would have been the same. The smaller number of farms used for this analysis must be noted, but is of little consequence because the number involved is still considerable and the model demonstrated a significant protect effect against major pathogens ($P = 0.04$).

Quarters infected with *C. bovis* in the drying off, calving or post calving samples were no more or less likely to suffer a case of clinical mastitis during the first 100 days of lactation. These results differ from those of other authors that demonstrated quarters infected with *Corynebacterium* species at drying off were significantly more likely and those infected at and around calving significantly less likely to suffer clinical mastitis during the next lactation (Green *et al.* 2002).

Quarters that acquired a *C. bovis* infection during the dry period were no more or less likely to suffer a case of clinical mastitis during the next lactation, however the result approached significance ($P = 0.052$). Although not significant there was a very strong trend towards quarters that acquired a *C. bovis* IMI during the dry period suffering more clinical mastitis. This result is surprising in light of other findings, which suggest that a quarter infected with *C. bovis* is "protected" against supra-infection with other organisms. Only 30 of 1517 quarters (2.0%) became infected with *C. bovis* during the dry period and all of them were in the teat sealer group. The rate of new IMI was thus low, even in quarters that did not receive antibiotic treatment. The principal site of *C. bovis* IMI is the teat canal; the teat sealer used in this study will therefore not prevent infection with *C. bovis* as it sits inside the teat and has no antibacterial properties. Assuming exposure is equal it can be postulated that if IMI rate with *C. bovis* is low during the dry period, most quarters must have an inherent ability to resist infection during this time or exposure to the pathogen is low during this period. It can be postulated that those quarters that do become infected may have an inherent quarter or cow level susceptibility to IMI. If this is the case inherently susceptible quarters may also be more likely to acquire mastitis causing pathogens leading to clinical cases during lactation. A similar explanation has been proposed by other authors to explain the finding that quarters infected with *C. bovis* at drying off were significantly more likely to suffer clinical mastitis during the next lactation (Green *et al.* 2002). It does however remain a possibility that because the significance of this finding only approaches significance and it is in conflict with all other results, it may in fact represent a chance event. If the group sizes were larger this finding may disappear.

In this study *C. bovis* was identified based on colony morphology, Gram-stain and morphology, catalase test, demonstration of a lipid growth requirement and endonuclease restriction of the 16S rRNA gene sequence. Many reports in the literature that attribute either an increase or a decrease in the risk of subsequent supra-infection with other pathogens do not specify clearly how *C. bovis* was speciated or differentiated from other coryneforms isolated during the studies involved. Some give no details at all of microbiological technique in the methodology (Linde *et al.* 1980; Rainard and Poutrel 1988; Viseslava and Vera 1989) where as others quote that bacteriological technique was according to certain defined standards (Zadoks *et al.*

2001). In papers that do define technique some have based a diagnosis on colony morphology on blood agar alone (Black *et al.* 1972), or in combination with Gram-stain (and morphology) and/or limited biochemical characterization (Honkanen-Buzalski *et al.* 1984; Pociecha 1989). Only two papers have defined the lipid requirement of isolates (and therefore only diagnosed lipophilic isolates as *C. bovis*) based on stimulation of growth with Tween 80 (Doane *et al.* 1987; Berry and Hillerton 2002).

If studies do not specify the methods they have used to speciate *C. bovis*, it can be postulated that many have assumed that any lipophilic coryneform isolated in milk is *C. bovis*. In this case, because of the low prevalence of "*C. langfordii*" identified on 16 farms in SW England (2.9%) in this thesis, it is likely that the rate of misclassification will be low. The potential effects on results of misclassifying "*C. langfordii*" as *C. bovis* will be minimal, unless the prevalence of "*C langfordii*" shows large national, regional or farm-to-farm variations. This problem could confound results substantially in small (one or two farm) studies if the prevalence of "*C. langfordii*" is high on these farms and if it behaves differently to *C. bovis*.

Worse than classifying all lipophilic isolates as *C. bovis*, some previous studies may have assumed that any Gram-positive coryneform isolated from milk was *C. bovis* (*i.e.* including non-lipophilic species). In this case, the rate of misclassification will be much higher. More importantly, univariate analysis of results from this study have demonstrated that quarters, which acquired a coryneform IMI other than *C. bovis* during the dry period, were at significantly greater risk of acquiring a new dry period IMI caused by *Acinetobacter* spp. and non-fermenters. Results for all major pathogens together approached significance ($P = 0.07$). It was not possible to analyze the results using multivariate techniques, because the number of quarters involved was small. It therefore remains a possibility that these results are confounded by factors not taken into account by univariate statistical analysis. This remains an area in need of further research because it may be that in some circumstances coryneforms other than *C. bovis* actually predispose a quarter to supra-infection with another organism. In this case if the prevalence of non-lipophilic coryneforms is high on some farms, it could explain the results of studies which have not demonstrated a protective effect or those which showed that *C. bovis* IMI predisposed to disease. At the very least this will act as a serious confounding factor in studies that do not differentiate lipophilic from non-lipophilic species.

It is vital in any study involving *C. bovis* that the method of speciation and differentiation from other lipophilic and non-lipophilic coryneforms is clearly described. For artificial infection studies involving single strains of *C. bovis*, sequencing of the 16S rRNA gene sequence (the gold standard technique), or use of a reference strain for which the 16S rRNA gene sequence has been previously elucidated, should be considered the minimum standard required. For natural infections studies involving many hundreds of isolates, differentiation based on the catalase test, Gram stain and morphology and differentiation of lipophilic from non-lipophilic strains should by considered an absolute minimum (in this case the misclassification of other lipophilic isolates as *C. bovis* should be acknowledged). Preferably a diagnosis of "*C. bovis*" should be supported by other more specific tests such as sugar fermentation profiles or restriction analysis of the 16S rRNA gene sequence. If precautions such as these are taken the rate of misclassification will be minimised and any increase or decrease in the risk of subsequent supra-infections correctly attributed to *C. bovis*. To avoid confusion, in cases where only limited speciation has been carried out, authors should refer to isolates as Coryneform, *Corynebacterium* species or lipophilic *Corynebacterium* species depending on the diagnostic tests performed.

Elucidating any potentially protective effect that IMI with *C. bovis* may offer is undoubtedly a very complex process because of the many interactions that take place in any complex biological system such as this. Correctly identifying isolates as *C. bovis* and adequately controlling for the effects of confounding factors remains difficult and has almost certainly played a role in explaining some of the apparently conflicting results that the literature describes. This is the first study to report that quarters infected with *C. bovis* during the dry period are less prone to subsequent IMI with other pathogens at that time. The identity of *C. bovis* was confirmed by analysis of the 16S rRNA gene sequence and the results were analysed using a logistic regression model.

CHAPTER 6: AN ASSESSMENT OF THE INHIBITORY EFFECT OF *C. BOVIS* ON MASTITIS PATHOGENS ON SOLID MEDIA.

6.1. INTRODUCTION

Reanalysis of the database produced during the teat sealer study demonstrated that quarters infected with *C. bovis* for the duration of the dry period were less likely to acquire a major pathogen IMI compared to quarters uninfected with *C. bovis*. This concurs with the work of previous authors, who demonstrated that quarters infected with *C. bovis* were protected from IMI with other mastitis pathogens (Black *et al.* 1972; Brooks and Barnum 1984; Rainard and Poutrel 1988; Lam *et al.* 1997).

Many authors have considered that the most likely explanation for this effect is that the leucocytosis induced by a *C. bovis* IMI protects the quarter from subsequent infection with other mastitis pathogens (Forbes 1970; Black *et al.* 1972; Brooks and Barnum 1984; Rainard and Poutrel 1988). However, others have concluded that the protective effect appears to be partially independent of the elevation in SCC (Lam *et al.* 1997; Schukken *et al.* 1999).

Bacteria can produce a wide range of molecules and substances that can inhibit the growth of and in some circumstances kill other bacteria. These include: toxins; bacteriolytic enzymes *e.g.* lysostaphin; bacteriophages and defective bacteriophages; metabolic by products *e.g.* organic acids and hydrogen peroxide; antibiotic like substances *e.g.* bacitracin; bacteriocins (Jack *et al.* 1995).

Woodward *et al* (1987) investigated the effects of normal bacterial flora from the teat skin of cattle on the growth of mastitis pathogens (*E. coli*, *Klebsiella* sp., *A. pyogenes*, *S. aureus*, *S. epidermidis*, *S. bovis*, *S. dysgalactiae*, *S. agalactiae* and *S. uberis*) on solid media. Some isolates, including members of the *Corynebacterium* genus, produced factors that inhibited the growth of both Gram positive and Gram negative mastitis pathogens. The effects were most marked against *S. aureus* and *Streptococcus* species.

The most widely studied inhibitory factors are bacteriocins, small bactericidal proteins produced by some organisms. A diverse range of *Corynebacterium* species

have previously been shown to produce bacteriocins (Gross and Vidaver 1978; Karabekov *et al.* 1984; Kato *et al.* 1984; Carnio *et al.* 1999).

During the remaining two chapters of this thesis the hypothesis that *C. bovis* produces a factor or product, which is inhibitory to mastitis pathogens, will be explored using *in vitro* techniques on solid and in liquid media.

6.2. MATERIALS & METHODS

6.2.1. Bacterial Isolates

All bacteria used were isolated from screening milk samples (drying off, calving or post calving) collected during the teat sealer study and stored at –80°C. *C. bovis, S. aureus* and *E. coli* isolates were selected using randomised tables from all those collected. *S. aureus* was selected as a representative of Gram-positive organisms because it was never cultured from a quarter infected with *C. bovis* (Chapter 5). *E. coli* was selected because it was the most prevalent Gram-negative organism cultured and demonstrated the largest reduction in numbers of new dry period IMI during the teat sealer study.

C. bovis isolates used had reference numbers 147, 486, 727, 1513, 1537, 1539, 1721, 2093, 2129, 2294, 2775, 2811, 2879, 3009, 3290, 4373, 4622, 4786, 4851B, 4855, 5147, 5186, 5334, 5577, 5654, 5791.

S. aureus isolates used had reference numbers 1947, 2063, 3094, 3439, 4166, 4494, 5527, 5575.

E. coli isolates used had reference numbers 1081, 1126, 2491, 3247, 4259, 4362.

6.2.2. Bacterial Interaction on Solid Media

The affect of *C. bovis* on pathogen growth on solid media was studied employing a technique previously described (Abrehem and Zamiri 1983; Corbeil *et al.* 1985; Woodward *et al.* 1987). Ten centimetre square sterile petri dishes were prepared with 50mls brain heart agar containing one percent v/v Tween 80. A two centimetre broad band was marked down the centre of the plate on its base. *C. bovis* colonies from a

purity plate were inoculated into the band using a sterile cotton swab. Plates were incubated aerobically at 37°C for 24 hours. After 24 hours all visible growth was removed from the marked band using sterile cotton swabs. The surface of the agar was sterilised with chloroform vapour by inverting the plate over liquid chloroform in a sealed glass jar for one hour. Plates were aerated without lids for one hour, close to a Bunsen flame, to allow any remaining chloroform vapour to evaporate.

Bacterial growth (*S. aureus* / *E. coli*) was harvested from a purity plate and emulsified in one millilitre of PBS (Phosphate buffer saline) by vortexing. The optical density of the test emulsification was diluted in PBS to a transmission of 75% of that of pure PBS at 610nm (Woodward *et al.* 1987). *S. aureus* and *E. coli* test solutions were then diluted 1:2000 and 1:6000 respectively with PBS. Ten streaks per plate of diluted inoculum were applied at right angles to the initial *C. bovis* growth across its width with a sterile cotton swab. Plates were incubated aerobically at 37°C and read after 12 and 24 hours. A wide range of different effects were noted and are described graphically in Figure 6.1.

Figure 6.1: Graphical Representation of the Patterns of *S. aureus* and *E. coli* Growth Seen on Solid Media

C. bovis
streak

1
2
3
4
5
6

1 – No inhibition; 2 – Slight stimulation; 3 – Stimulation / Inhibition; 4 – Slight inhibition (S. aureus); 5 – Slight inhibition (E. coli); 6 – Marked inhibition.

6.3. RESULTS

6.3.1. Affect of *C. bovis* on the growth rate of *S. aureus* on solid media

The central streak on which *C. bovis* had previously been cultured had a wide range of effects on the growth response of the eight *S. aureus* isolates tested (Tables 6.1 and 6.2). The growth of three of the isolates was stimulated; this was manifest as an increase in the size of the *S. aureus* colonies within or at the edge of the *C. bovis* streak. The growth of five of the isolates was inhibited. Some isolates were inhibited within the *C. bovis* streak but stimulated at its borders. The degree of inhibition varied. Isolates 3094 and 5527 were markedly inhibited by a large number of the *C. bovis* isolates tested. Marked inhibition included complete absence of or very scant growth within the band on which *C. bovis* had previously been cultured. No single *C. bovis* isolate inhibited all the *S. aureus* isolates tested (Table 6.2). Photographic examples of inhibition and stimulation of *S. aureus* by *C. bovis* on solid media are demonstrated in Figure 6.2.

Table 6.1: Number of *C. bovis* Isolates Causing Specified Alterations in the Growth Pattern of Eight *S. aureus* Isolates

S. aureus isolate	Slight Stimulation	Stimulation / Inhibition	No Change	Slight Inhibition	Marked Inhibition	No Data
1947	14	2	5	2	1	2
2063				16	3	7
3094				14	11	1
3439	12	2	4	7		1
4166				16	3	7
4494				23	1	2
5527				2	17	7
5575	16	2	1			7

6.3.2. Affect of *C. bovis* on the growth rate of *E. coli* on solid media

The effects of the central streak on which *C. bovis* had previously been cultured had a much less profound effect on the growth of the six *E. coli* isolates tested (Tables 6.2 and 6.3). In general most of the *C. bovis* isolates demonstrated no effect although all *E. coli* isolates were slightly inhibited by at least one *C. bovis* isolate. This was manifest as a decrease in the number (but not the size) of *E. coli* colonies within the *C.*

bovis streak. No single *C. bovis* isolate inhibited all the *E. coli* isolates (Table 6.2), although *C. bovis* isolate 4622 inhibited four of five and isolate 5654 inhibited four of six of those tested.

Table 6.2: Effects of 26 *C. bovis* Isolates on the Growth Pattern of Eight *S. aureus* and Six *E. coli* Isolates on Solid Media

	S. aureus isolate								*E. coli* isolate					
C. bovis	1947	2063	3094	3439	4166	4494	5527	5575	1081	1126	2491	3247	4259	4362
147	nc		-	nc		-			nc	nc	nc	nc	nc	nc
486	nc	-	--	-	-	-	--	+	nc	nc	nc	nc	nc	nc
727	+/-		--	-		-			nc	nc	nc	nc	nc	nc
1513	nc		-	nc		-			nc	nc	nc	nc	nc	nc
1537	--		--	-		-			nc	nc	nc	-	nc	nc
1539	+	-	-	+	-	-	--	+	nc	nc	nc	nc	nc	nc
1721	+	-	-	+	-	-	--	+	nc	nc	nc	nc	nc	nc
2093	-	-	-	-	-	-	--	+	nc	nc	nc	-	nc	nc
2129			-	-		-			nc	nc	nc	nc	nc	nc
2294	+		-	+/-		-			nc	nc	nc	nc	-	nc
2775	nc	-	--	nc	-	-	--	+	nc	nc	nc	nc	nc	nc
2811	nc		-	nc		-			nc	nc	nc	nc	nc	nc
2879		-			-		--	nc	nc	nc	nc	nc	nc	nc
3009	+/-	-	-	+	-	-	-	+	nc	nc	nc	nc	nc	nc
3290	+	-	-	+	-	-	--	+	nc	nc	nc	nc	nc	nc
4373	-	--	--	-	--		--	+/-	nc	nc	nc	-	nc	nc
4622	+	-	--	+/-	-	--	--	+	-	nc	-	-	-	
4786	+	-	--	+	-	-	--	+	nc	nc	nc	nc	nc	nc
4851B	+	-	-	+	-	-	--	+	nc	nc	nc	nc	nc	-
4855	+	-	--	+	-	-	--	+	nc	-	nc	nc	nc	nc
5147	+	-	-	+	-	-	--	+	nc	nc	nc	-	nc	nc
5186	+	--	--	-	--	-	--	+/-	nc	nc	nc	nc	nc	nc
5334	+	-	-	+	-	-	-	+	nc	nc	nc	nc	nc	-
5577	+	-	-	+	-	-	--	+	nc	-	nc	-	nc	nc
5654	+	-	--	+	-	-	--	+	nc	-	-	-	nc	-
5791	+	--	--	+	--	-	--	+	nc	nc	nc	nc	nc	nc

+ Slight stimulation; +/- Stimulation / Inhibition; nc No change; - Slight inhibition; --Marked inhibition; Blank cell: No data.

Table 6.3: Number of *C. bovis* Isolates Causing Specified Alterations in the Growth Pattern of Six *E. coli* Isolates

E. coli isolate	No Change	Slight Inhibition	No Data
1081	25	1	
1126	23	3	
2491	24	2	
3247	19	7	
4259	24	2	
4362	22	3	1

Figure 6.2: Examples of Inhibition and Stimulation of *S. aureus* by *C. bovis* on Solid Media

Central 2cm streak – C. bovis 5791. Lane 1 – Slight stimulation, Lane 2 – Slight stimulation, Lane 3 – Slight inhibition (decreased colony numbers), Lane 4 – Slight inhibition, Lane 5 – Slight stimulation, Lane 6 – Marked inhibition, Lane 7 – Marked inhibition, Lane 8 – Slight inhibition.

Central 2cm streak – C. bovis 4373. Lanes 1 to 4 – Marked inhibition, Lane 5 – Marked inhibition, Lane 6 – slight inhibition, Lane 7 – Marked inhibition, Lane 8 – Slight inhibition within C. bovis streak and slight stimulation around boarders of C. bovis streak.

6.4. DISCUSSION

The agar band in which *C. bovis* had previously been cultured produced a broad range of effects on the growth rate of *S. aureus* and to a lesser extent of *E. coli*. *S. aureus* isolates tended to fall into one of two categories, either predominantly stimulated or predominantly inhibited by the range of *C. bovis* isolates assessed.

Stimulation of growth occurred predominantly within the two centimetre broad band in which *C. bovis* had previously been cultured, although in some instances the growth stimulation appeared to extend beyond its borders (but never greater than one centimetre from it). Growth stimulation beyond the borders of the band implies that a partially diffusible stimulatory factor was being produced. Stimulation of the growth of *Haemophillus somnus* and *Pasteurella* species by isolates from the normal flora of the bovine respiratory and reproductive tracts (including *Corynebacterium* species, but not *C. bovis*) has previously been demonstrated using a technique identical to this one (Corbeil *et al.* 1985). The authors made no attempt to postulate what the stimulatory factor may be; it could be a growth limiting metabolite *e.g.* an amino acid or a specific factor which acts to stimulate growth or the rate of bacterial multiplication.

Inhibition of growth occurred exclusively within the band in which *C. bovis* had been cultured in this series of experiments. The inhibitory factor was not able to diffuse beyond the band suggesting it was unable to move because of its physical properties or because it was strongly bound within the region in which it was produced. Inhibitory effects of *Corynebacterium* species isolated from the mammary integument and bovine respiratory and reproductive tracts on mastitis pathogens and *H. somnus* and *Pasteurella* species respectively has previously been demonstrated using a technique identical to the one employed here (Corbeil *et al.* 1985; Woodward *et al.* 1987). Woodward *et al* (1987) demonstrated that *Corynebacterium* species (no *C. bovis* isolates were tested) could inhibit the growth of *S. aureus* isolates cultured from clinical mastitis cases. The author's results concur with and extend the findings of these papers by demonstrating that *C. bovis* isolated from the bovine mammary gland can also inhibit the growth of *S. aureus* on solid media.

There were a small number of cases in which the growth of *S. aureus* was inhibited within the *C. bovis* band but stimulated at and beyond its periphery. It can be postulated that in this instance both the inhibitory and stimulatory factors were being

produced, but only the stimulatory factor was diffusible. Where both occurred within the *C. bovis* band the inhibitory factor dominated and caused growth inhibition; however, at the borders the stimulatory factor diffused beyond the inhibitory factor and stimulated growth at these points.

The inhibition of *S. aureus* (and to a lesser extent *E. coli*) seen may be caused by a product of *C. bovis* growth *e.g.* toxins; bacteriolytic enzymes *e.g.* lysostaphin; bacteriophages and defective bacteriophages; metabolic byproducts *e.g.* organic acids and hydrogen peroxide; antibiotic like substances *e.g.* bacitracin; bacteriocins (Jack *et al.* 1995). Alternatively, it may be caused by depletion of a non-diffusible limiting nutrient from the medium within the band in which *C. bovis* was originally cultured. Methods designed to exclude the potential confounding effects of nutrient depletion from the media have been developed (Tagg *et al.* 1976), although most rely on the inhibitory factor being freely diffusible.

The nature of both the inhibitory and stimulatory factors was impossible to elucidate based on the data available from this study. Further work was needed to characterise and isolate the factors identified. The potential modalities of the inhibitory effect were investigated further and will be discussed in Chapter 7.

CHAPTER 7: AN ASSESSMENT OF THE INHIBITORY EFFECT OF *C. BOVIS* ON MASTITIS PATHOGENS IN LIQUID MEDIA.

7.1. INTRODUCTION

An investigation into the effects of products of *C. bovis* metabolism on the growth rate of two mastitis pathogens on solid media indicated that *S. aureus* was both inhibited and stimulated and *E. coli* could be inhibited, but to a much lesser extent. A problem with the method employed in Chapter 6 was the inability to control for the effects of nutrient depletion from the band in which *C. bovis* had previously been cultured.

The effects of *C. bovis* metabolites on the growth rate of *S. aureus* and *S. agalactiae* in liquid media have been reported by other authors (Hogan *et al.* 1987). In this study *C. bovis* metabolites had no affect on the growth rate of the two pathogens studied, although the concentration of filter sterilized broth (in which *C. bovis* had previously been cultured) used during this investigation was low. During the final chapter of this thesis the affects of *C. bovis* metabolites (over a wide range of concentrations) on the growth of five mastitis pathogens is described.

7.2. MATERIALS & METHODS

7.2.1. Microbiology Workstation Bioscreen C

The growth rate of mastitis pathogens in filter sterilized media in which *C. bovis* had previously been cultured was investigated using a Microbiology Workstation Bioscreen C (Thermo Labsystems). The Bioscreen C automatically measures and records the optical density of cultures in two 100 well plates (*i.e.* 200 cultures simultaneously) over a given time frame (*e.g.* 24 – 72 hours) and at a given time interval (*e.g.* every ten minutes). It is therefore possible to assess the rate of bacterial growth (indirectly via an increase in optical density) under many different culture conditions or perform multiple repeats of a smaller number of culture conditions over

an extended time period. Data is recorded on a computer and can be directly exported to Excel (Microsoft) for analysis.

7.2.2. Preparation of "Used" *C. bovis* Broth

One *C. bovis* colony from a purity plate incubated at 37°C for 48 hours was inoculated into 150ml brain heart broth containing one percent Tween 80 v/v in a two litre conical flask and incubated at 37°C for 48 hours in an orbital shaker (200 rpm). Purity of the culture was checked twice at 24 and 48 hours by plating. After 48 hours the broth was cooled to 6°C and bacteria and cellular debris were removed from suspension by centrifugation. Supernatant broth was sterilised by passage through a 0.22μm sterile syringe driven filter (Millex-GP Sterilizing Filter Unit). Filter sterilized "used" broth was collected and refrigerated (for a maximum of ten days) at 4°C until required.

7.2.3. Preparation of Test and Control Culture Media

Test cultures with a final volume of 250μl were prepared to contain 90, 80, 70, 60, 50, 40, 30, 20, and 10 percent filter sterilized "used" *C. bovis* broth diluted with fresh brain heart broth containing one percent v/v Tween 80. Similarly, equivalent control broths were prepared substituting "used" *C. bovis* broth with sterile PBS as a negative control *e.g.* Table 7.1. Test and control cultures therefore contained equal quantities of fresh brain heart broth, to control for the nutrient affect. Cultures containing 100% (250μl) fresh brain heart broth, PBS and filter sterilized "used" *C. bovis* broth were included. At least triplicate cultures were prepared.

Table 7.1: Preparation of Test and Control Culture Media

	Test culture	*Control culture*
225μl (90%) "fresh" brain heart broth, plus...	25μl (10%) "used" *C. bovis* broth	25μl (10%) PBS
200μl (80%) "fresh" brain heart broth, plus...	50μl (20%) "used" *C. bovis* broth	50μl (20%) PBS
175μl (70%) "fresh" brain heart broth, plus...	75μl (30%) "used" *C. bovis* broth	75μl (30%) PBS
150μl (60%) "fresh" brain heart broth, plus...	100μl (40%) "used" *C. bovis* broth	100μl (40%) PBS
125μl (50%) "fresh" brain heart broth, plus...	125μl (50%) "used" *C. bovis* broth	125μl (50%) PBS
100μl (40%) "fresh" brain heart broth, plus...	150μl (60%) "used" *C. bovis* broth	150μl (60%) PBS
75μl (30%) "fresh" brain heart broth, plus...	175μl (70%) "used" *C. bovis* broth	175μl (70%) PBS
50μl (20%) "fresh" brain heart broth, plus...	200μl (80%) "used" *C. bovis* broth	200μl (80%) PBS
25μl (10%) "fresh" brain heart broth, plus...	225μl (90%) "used" *C. bovis* broth	225μl (90%) PBS

7.2.4. Preparation of Test Inoculum

Growth of test mastitis pathogen from a 24 hour purity plate was emulsified in 1.5ml of PBS by vortexing and adjusted to an optical density of 1.0 at 600nm by dilution with PBS. Standardized suspensions were then diluted 1:1,000 (*S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis*) or 1:10,000 (*E. coli*) with PBS and mixed by vortexing to provide approximately equal numbers of colony forming units per microlitre for all pathogens. Ten microlitres of diluted test bacterial suspension was inoculated into each well of test and control cultures.

The number of colony forming units in test inoculums was quantified by plating onto solid media. Ten microlitres of test inoculum contained approximately 1800 (*S. aureus*), 1300 (*E. coli*), 1700 (*S. agalactiae*), 900 (*S. dysgalactiae*) and 1400 (*S. uberis*) colony forming units.

Control cultures containing 100% fresh brain heart broth, 100% filter sterilized used *C. bovis* broth and 50% PBS + 50% fresh brain heart broth (100% PBS will not support bacterial growth) were left un-inoculated to assess the sterility of all culture components.

7.2.5. Bioscreen C Culture Conditions

All cultures containing *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* were incubated at 30°C for 48 hours unless otherwise stated; cultures containing *E. coli* were incubated at 30°C for 24 hours. The optical density of each well was measured at 600nm every ten minutes; the plates were automatically shaken for 30 seconds prior to optical density reading.

7.2.6. Data Processing

Data were exported to Excel 2000 (Microsoft) for manipulation and analysis. Where three (or more) wells contained identical cultures the mean OD at all measurement time points was calculated and used to produce the growth curve.

7.2.7. Selection of *C. bovis* and *S. aureus* Isolates for Experimental Optimisation

The effects of seven *C. bovis* isolates (three that had previously demonstrated inhibitory effects during the studies on solid media (486, 727 & 2879) and four

randomly selected isolates (122, 147, 1276 & 1513)) on the growth of two test pathogens in broth culture was assessed. The two *S. aureus* isolates (3094 & 5527) that had demonstrated the highest degree of inhibition on solid media (Chapter 6) were selected as test pathogens.

7.2.8. Affect of Time of *C. bovis* Incubation on *S. aureus* Inhibition

The affect of the length of initial *C. bovis* incubation at 37°C in an orbital shaker on the inhibitory effect on test pathogen was investigated. *C. bovis* isolate 122 and *S. aureus* isolate 3094 were used as the inhibitor and test pathogen strains respectively. 30mls of *C. bovis* broth was sterilely removed from a single culture flask after 24, 48, 72 and 96 hours. In a second experiment, 20mls of broth was removed after 12, 24, 36, 48, 60 and 72 hours of incubation. The purity of the flask culture was tested daily by plating onto solid media.

7.2.9. Affect of Heat Treating Filter Sterilized *C. bovis* Broth on *S. aureus* Inhibition

C. bovis isolate 122 was cultured in a conical flask in an orbital shaker at 37°C for 48 hours, as previously described. After filter sterilization, broth was heated to 60, 80 and 100°C for 30 minutes in a water bath. The degree of inhibition of the broth after each heat treatment was tested against *S. aureus* isolate 3094.

7.2.10. Affect of Treating Filter Sterilized *C. bovis* Broth with Proteinase K on *S. aureus* Inhibition

C. bovis isolate 122 was cultured in a conical flask in an orbital shaker at 37°C for 48 hours, as previously described. Filter sterilized broth was treated with ten percent v/v Proteinase K (20mg/ml (~600mAU/ml), Qiagen) and incubated at 37°C for 30 minutes. Proteinase K was then inactivated by heat treating to either 80 or 100°C for 30 minutes in a water bath. Control broths (no treatment negative control, heat treatment to 80°C only and heat treatment to 100°C only) were diluted ten percent v/v with PBS. The degree of inhibition of each broth was tested against *S. aureus* 3094.

7.2.11. Affect of Filter Sterilized *C. bovis* Broth (Isolate 122) on the Growth Rate of Six Isolates of Five Mastitis Pathogens

The inhibitory effect of filter sterilized *C. bovis* broth from isolate 122 (48 hours incubation at 37°C in an orbital shaker) on six isolates of five mastitis pathogens (*S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *E. coli*) was investigated. For *S. aureus*, *S. dysgalactiae*, *S. uberis* and *E. coli*, three isolates from screening samples (drying off, calving or post calving) and three isolated from clinical mastitis samples were randomly selected from those collected during the teat sealer study. No *S. agalactiae* isolates were identified during the study. Six isolates (all originally from the bovine mammary gland), were obtained from the culture collection held by the Veterinary Laboratories Agency, England.

In previous experiments, the most marked inhibition of test pathogens occurred at the highest concentrations of filter sterilized *C. bovis* broth. During this series of experiments only the growth of test pathogens in 225μl (90%) "used" *C. bovis* broth plus 25μl (10%) "fresh" brain heart broth compared to 225μl (90%) PBS plus 25μl (10%) "fresh" brain heart broth was investigated.

7.2.12. Affect of Six Filter Sterilized *C. bovis* Broths on the Growth Rate of One Isolate of Five Mastitis Pathogens

The affects of filter sterilized broth from six *C. bovis* isolates on the growth rate of one selected isolate of five mastitis pathogens was investigated. In addition to *C. bovis* isolate 122 (previously used as the "reference isolate"), five isolates were selected at random from those collected during the teat sealer study (345, 3045, 4143, 4361, 4861). One isolate from the five mastitis pathogens previously investigated (*S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *E. coli*) was selected if the broth of *C. bovis* 122 had inhibited them during previous experiments (Section 7.2.11.). Selected isolates were: *S. aureus* 453M, *E. coli* 1599, *S. agalactiae* 185, *S. dysgalactiae* 3616 and *S. uberis* 4192.

In a similar fashion to previously (Section 7.2.11), during this series of experiments only the growth of test pathogens in 225μl (90%) "used" *C. bovis* broth plus 25μl (10%) "fresh" brain heart broth compared to 225μl (90%) PBS plus 25μl (10%) "fresh" brain heart broth was investigated.

7.3. RESULTS

7.3.1. Selection of *C. bovis* and *S. aureus* Isolates for Experimental Optimisation

C. bovis isolate 122 demonstrated the most marked inhibitory effects on the growth of the two *S. aureus* test pathogens selected, this was particularly true for *S. aureus* isolate 3094. Typical results gained from repeated tests are demonstrated in Figure 7.1. The growth curves of *S. aureus* 3094 were almost identical at low concentration of filter sterilized *C. bovis* broth/PBS. However as the concentration of PBS/filter sterilized *C. bovis* broth increases a lag between the two growth curves becomes increasingly apparent. At the highest concentration of PBS/filter sterilized *C. bovis* broth (90%) there was a lag of approximately 20 hours between the growth curves. In comparison the inhibitory affects of *C. bovis* isolate 122 on *S. aureus* isolate 5527 were not so marked (Figure 7.2)

The inhibitory effect of *C. bovis* isolate 122 on *S. aureus* isolate 3094 was adopted as the "reference" effect and employed during further optimization of the experimental design.

7.3.2. Affect of Time of *C. bovis* Incubation on *S. aureus* Inhibition

Growth curves for *S. aureus* in cultures containing 40, 60, 80 and 100% filter sterilized *C. bovis* broth, when broth was removed from *C. bovis* culture every 24 hours, are demonstrated in Figure 7.3. Filter sterilized broth collected after 48 hours demonstrated the highest degree of inhibitory effect. Cultures incubated for more than 48 hours demonstrated progressively less inhibition against *S. aureus* isolate 3094.

Growth curves for *S. aureus* in cultures containing 40, 60, 80 and 100% filter sterilized *C. bovis* broth, when broth was removed from *C. bovis* culture every 12 hours, are demonstrated in Figure 7.4. The filter sterilized broths with the most inhibitory effects were those removed from culture after 36 and 48 hours. The inhibitory effects of broth removed after 60 and 72 hours were less than that removed after 36 and 48 hours. Filter sterilized broth removed after 12 and 24 hours had the least inhibitory effects.

Further work incubating *C. bovis* cultures for 36 and 48 hours indicated that compared to 36 hour cultures, those incubated for 48 hours demonstrated a more consistent degree of inhibition against *S. aureus* 3094 (data not shown). Incubation for

48 hours at 37°C on an orbital shaker (200rpm) was adopted as the "standard" culture conditions and employed in all further experimental work.

7.3.3. Affect of Heat Treating Filter Sterilized *C. bovis* Broth on *S. aureus* Inhibition

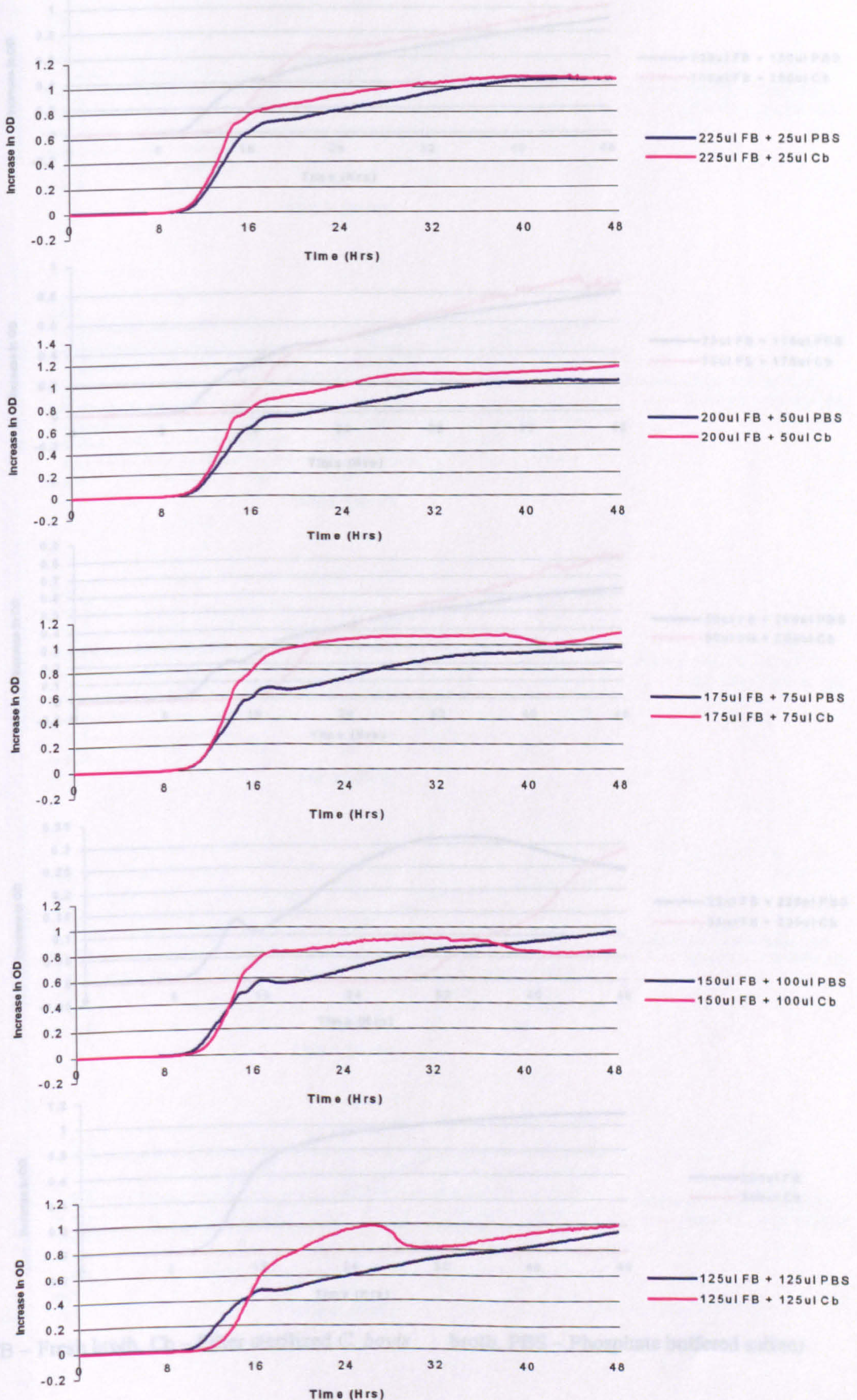
Growth curves for *S. aureus* in cultures containing 60, 70, 80, 90 and 100% filter sterilized *C. bovis* broth following no treatment or heat treatment to 60°C, 80°C and 100°C are demonstrated in Figure 7.5. Heat-treating filter sterilized *C. bovis* broth to 60 and 80°C had no affect on its inhibitory effects against *S. aureus* (isolate 3094). Although slightly variable (especially at high concentration of filter sterilized *C. bovis* broth) the growth curves produced in broth heated to these temperatures were very similar to that produced in no heat treatment control broth cultures. However, filter sterilized broth heated to 100°C for 30 minutes was markedly less inhibitory compared to the no treatment control broth. In the control broth no growth was seen in the culture containing 100% filter sterilized *C. bovis* broth after 48 hours. In the broth that had been heat treated to 100°C, growth in the 100% filter sterilized *C. bovis* broth began after approximately 24 hours.

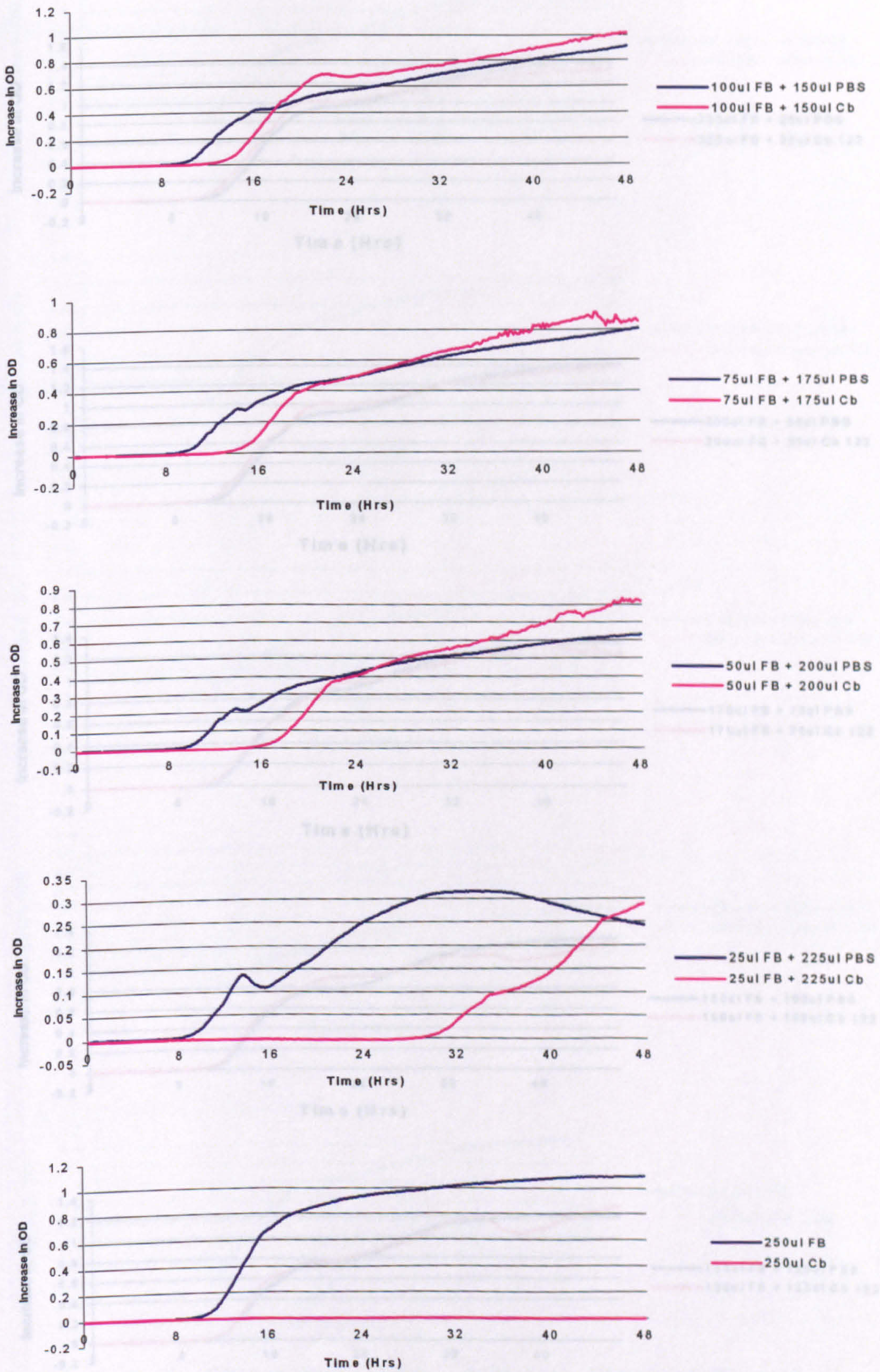
7.3.4. Affect of Treating Filter Sterilized *C. bovis* Broth with Proteinase K on *S. aureus* Inhibition

Growth curves for *S. aureus* in cultures containing 60, 70, 80, 90 and 100% filter sterilized *C. bovis* broth following no treatment or heat treatment to 60°C or 80°C with or without treatment with Proteinase K are demonstrated in Figure 7.6. Heat treatment to 80°C only had no affect on the degree of inhibition of *S. aureus* (3094) compared to a no treatment control. Heat treatment to 100°C partially reduced the inhibitory effects of *C. bovis* (122) broth.

Treatment of filter sterilized *C. bovis* broth (isolate 122) with ten percent v/v Proteinase K markedly, but not entirely, eliminated the inhibitory effects of *C. bovis* broth on *S. aureus* (3094). No growth was observed in 100% filter sterilized *C. bovis* broth after 48 hours in the no treatment control broth, where as after Proteinase K treatment growth began after approximately 14 hours. Similarly, in the culture containing 90% filter sterilized *C. bovis* broth, growth began after approximately 20 hours in the control broth and 12 hours in the Proteinase K treated broth.

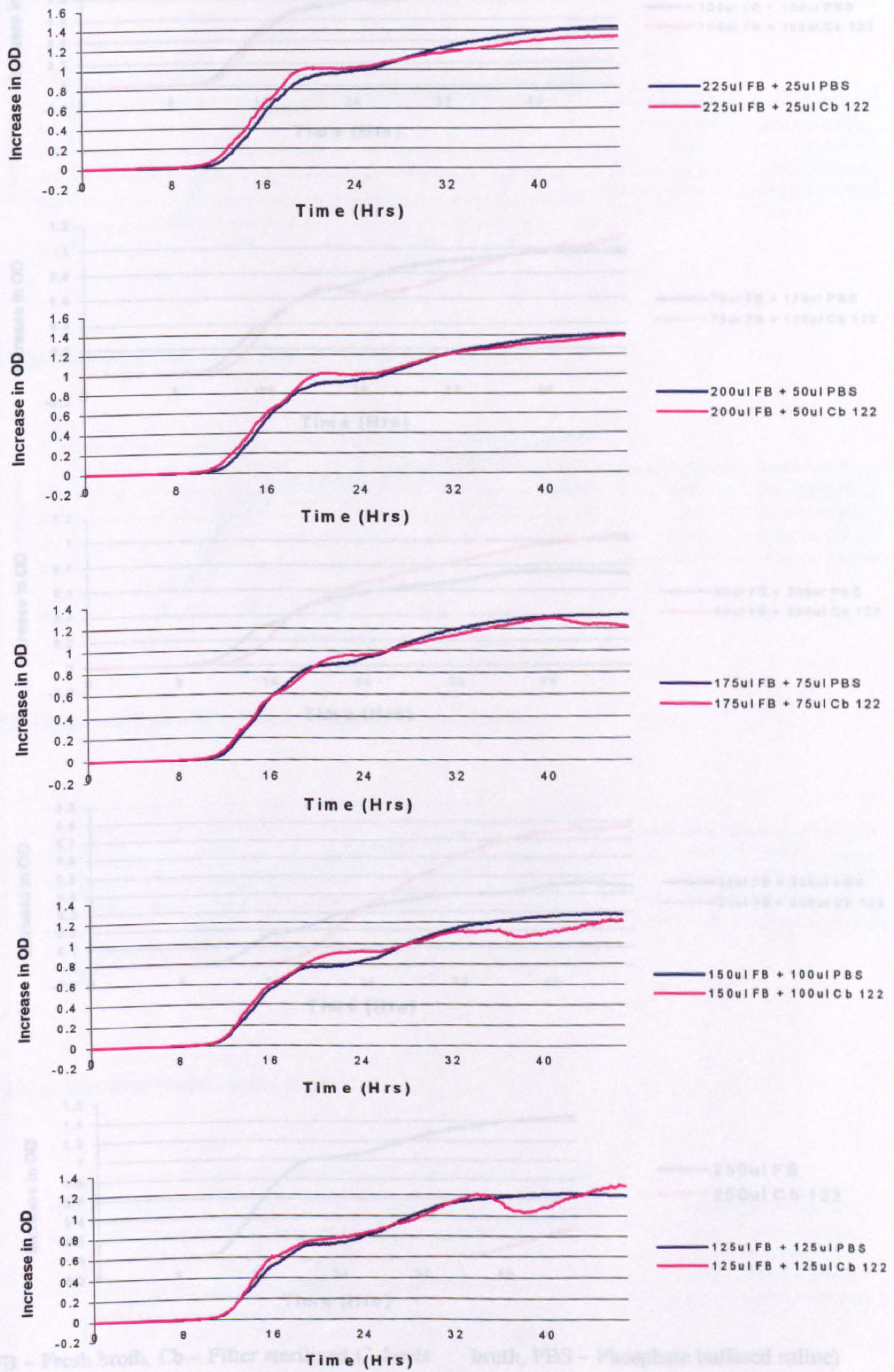
Figure 7.1: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of a *S. aureus* (3094) Isolate

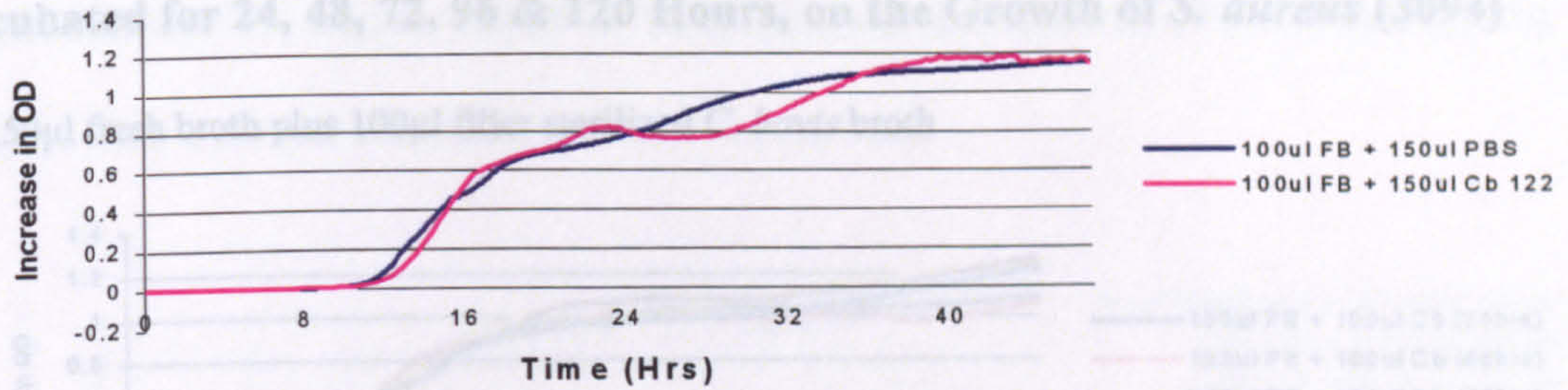


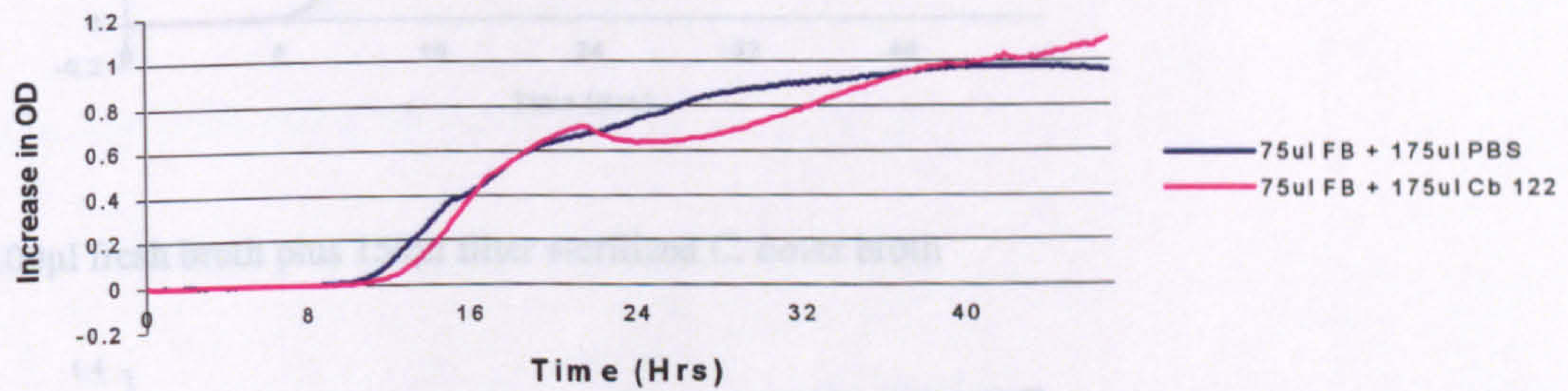


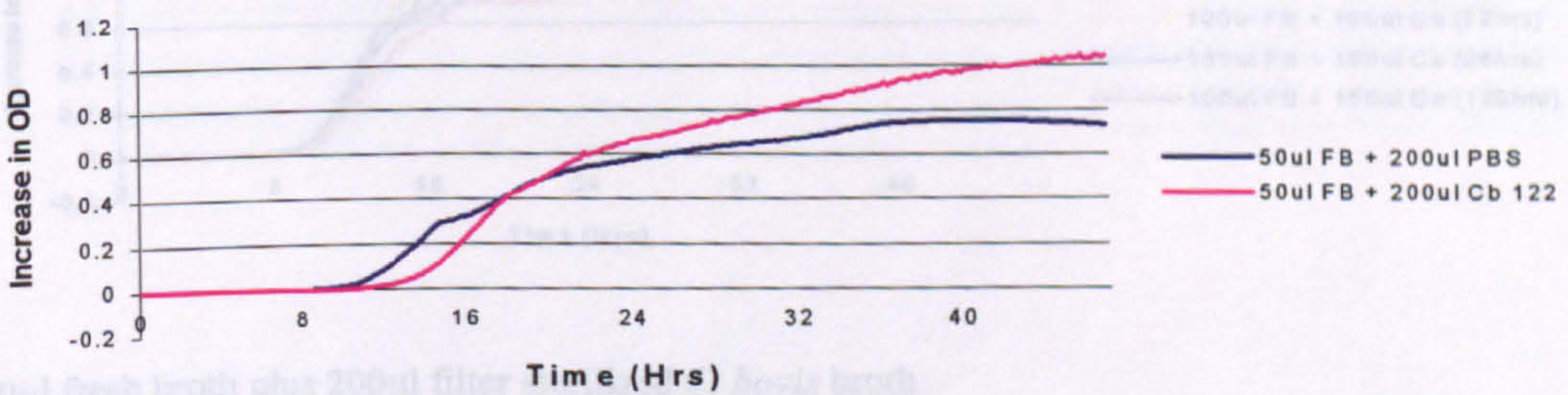
(FB – Fresh broth, Cb – Filter sterilized *C. bovis* broth, PBS – Phosphate buffered saline)

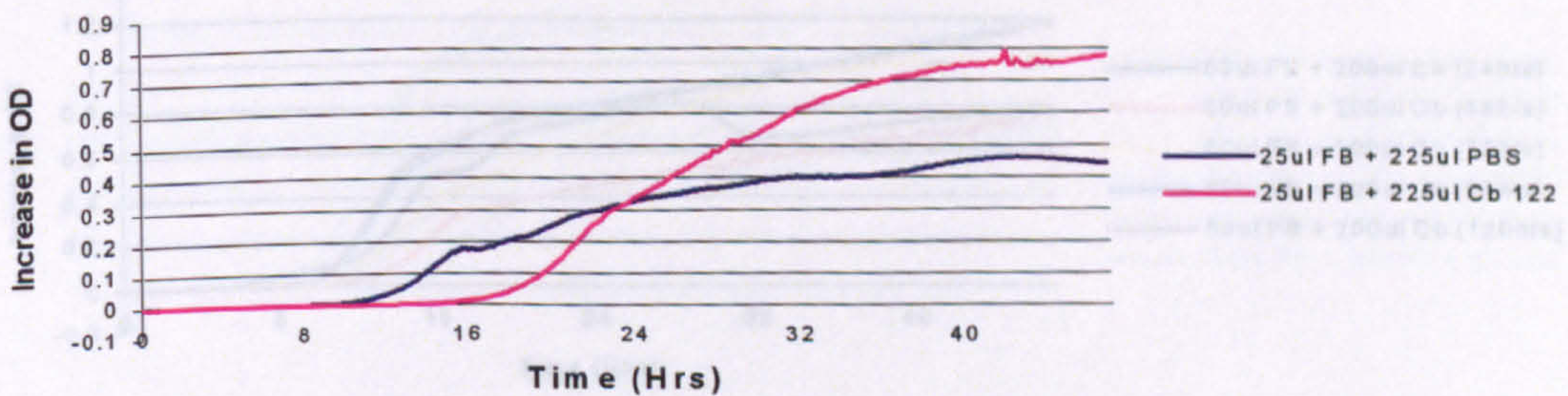
Figure 7.2: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of a *S. aureus* (5527) Isolate

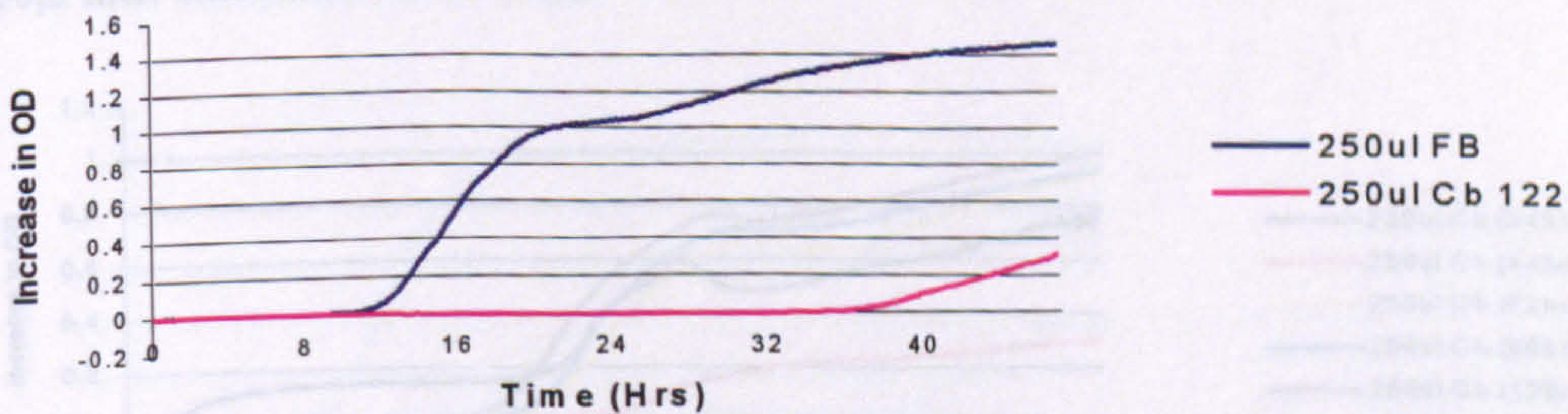








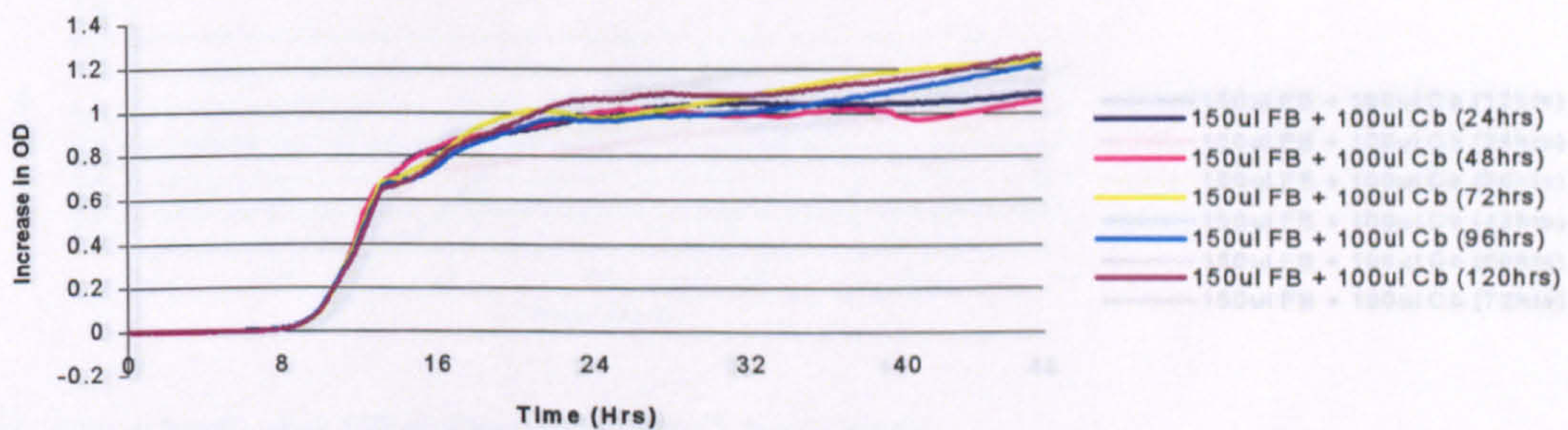




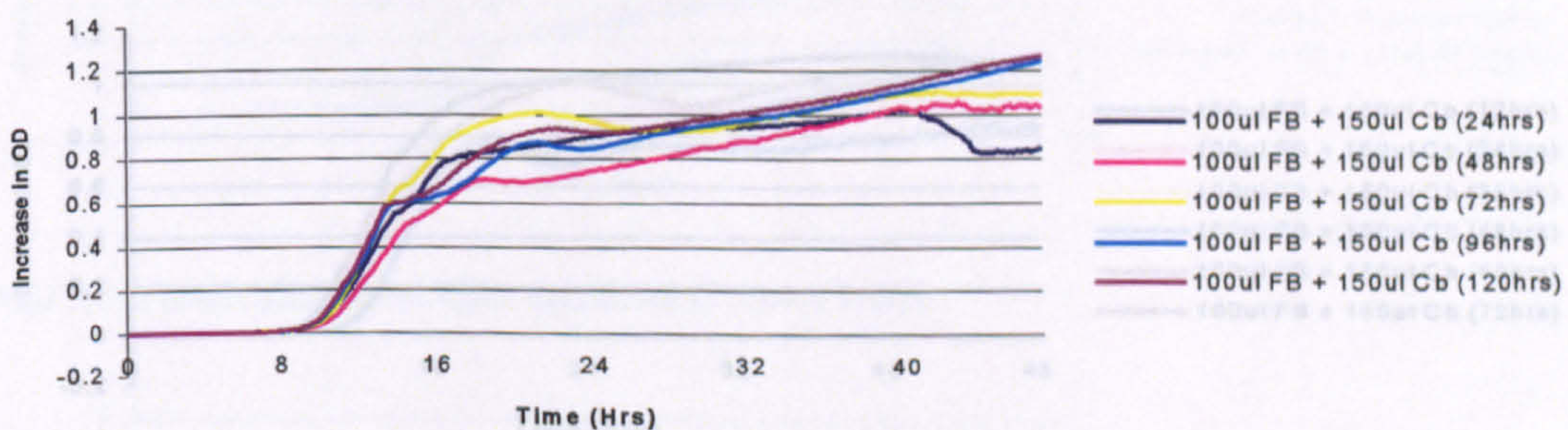
(FB – Fresh broth, Cb – Filter sterilized *C. bovis* broth, PBS – Phosphate buffered saline)

Figure 7.3: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth Incubated for 24, 48, 72, 96 & 120 Hours, on the Growth of *S. aureus* (3094)

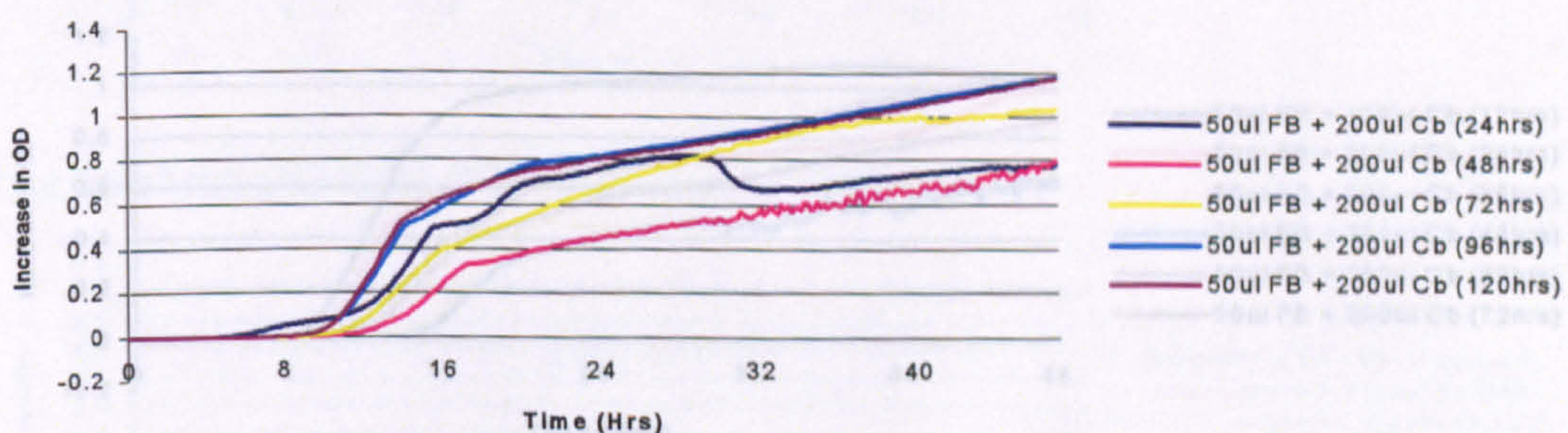
a. 150µl fresh broth plus 100µl filter sterilized *C. bovis* broth



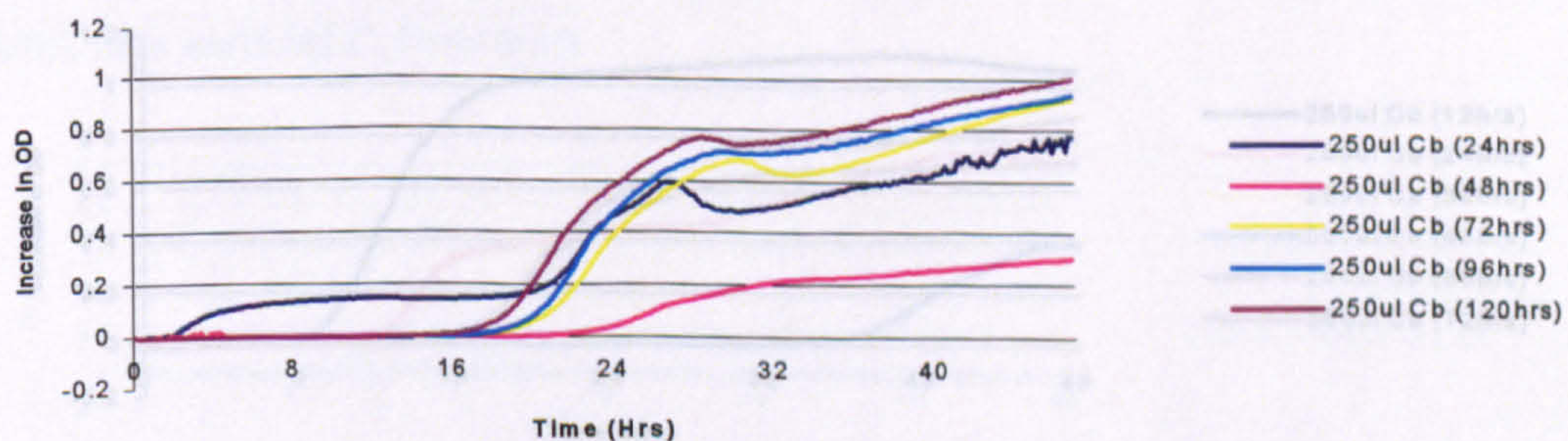
b. 100µl fresh broth plus 150µl filter sterilized *C. bovis* broth



c. 50µl fresh broth plus 200µl filter sterilized *C. bovis* broth



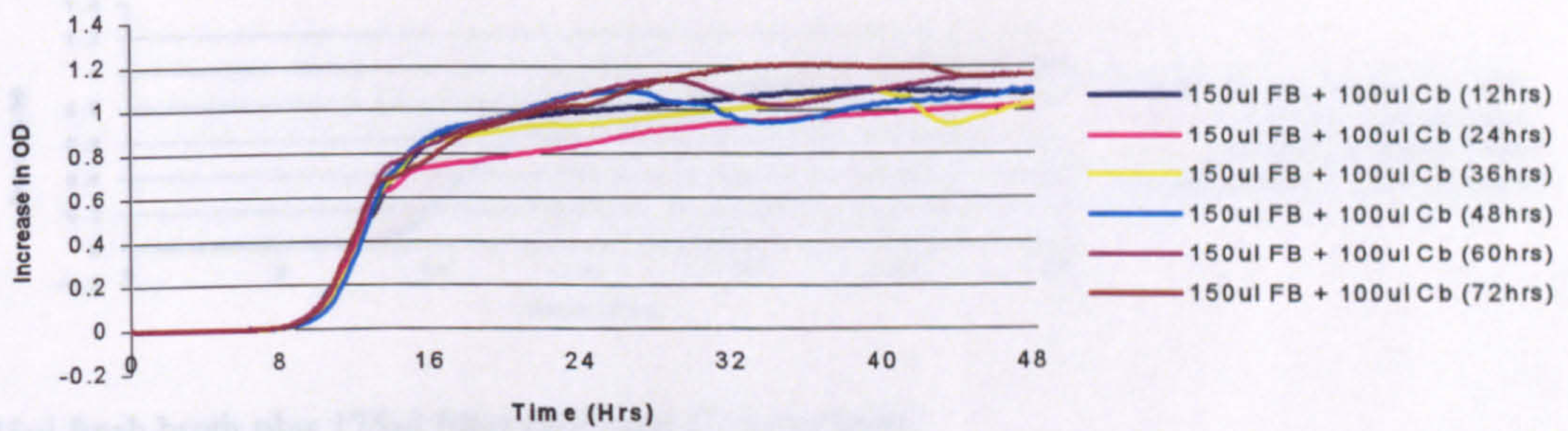
d. 250µl filter sterilized *C. bovis* broth



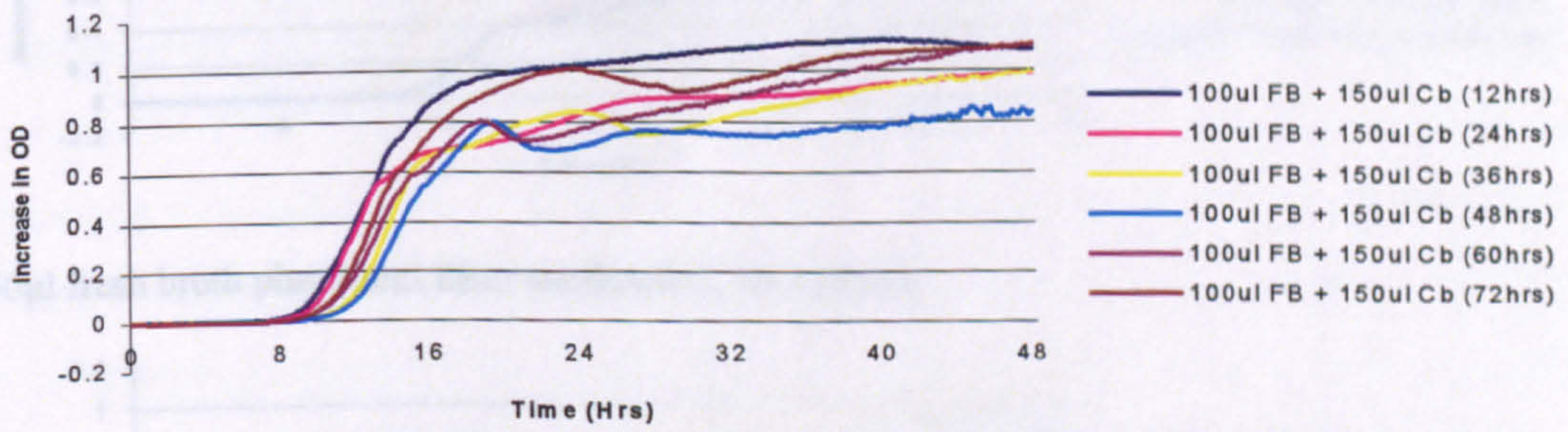
(Fb – Fresh broth, Cb – Filter sterilized *C. bovis* broth)

Figure 7.4: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth Incubated for 12, 24, 36, 48, 60 & 72 Hours, on the Growth of *S. aureus* (3094)

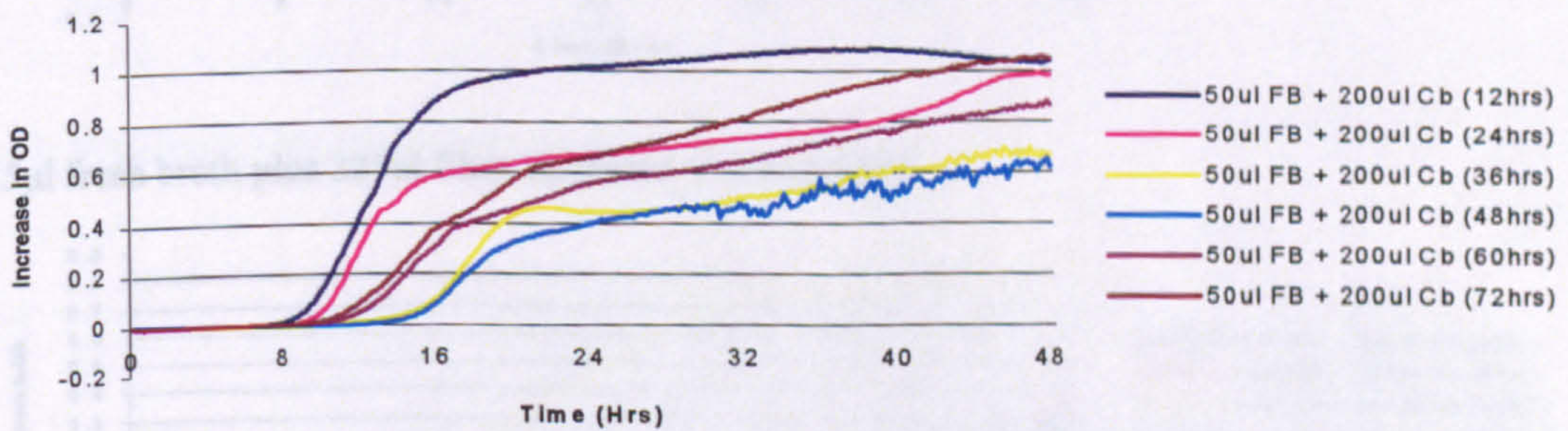
a. 150µl fresh broth plus 100µl filter sterilized *C. bovis* broth



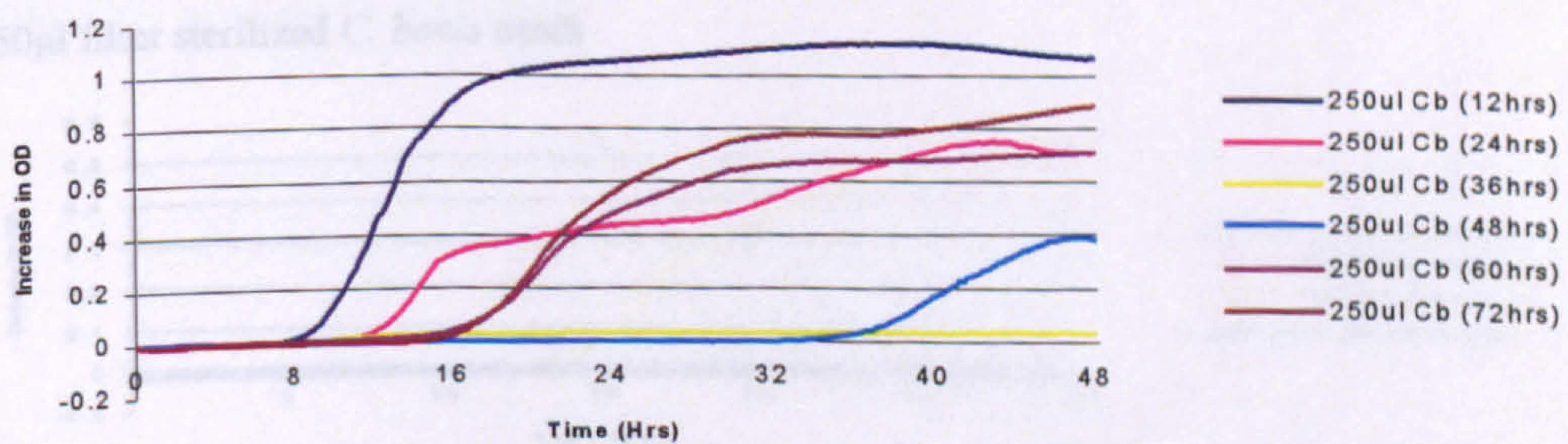
b. 100µl fresh broth plus 150µl filter sterilized *C. bovis* broth



c. 50µl fresh broth plus 200µl filter sterilized *C. bovis* broth



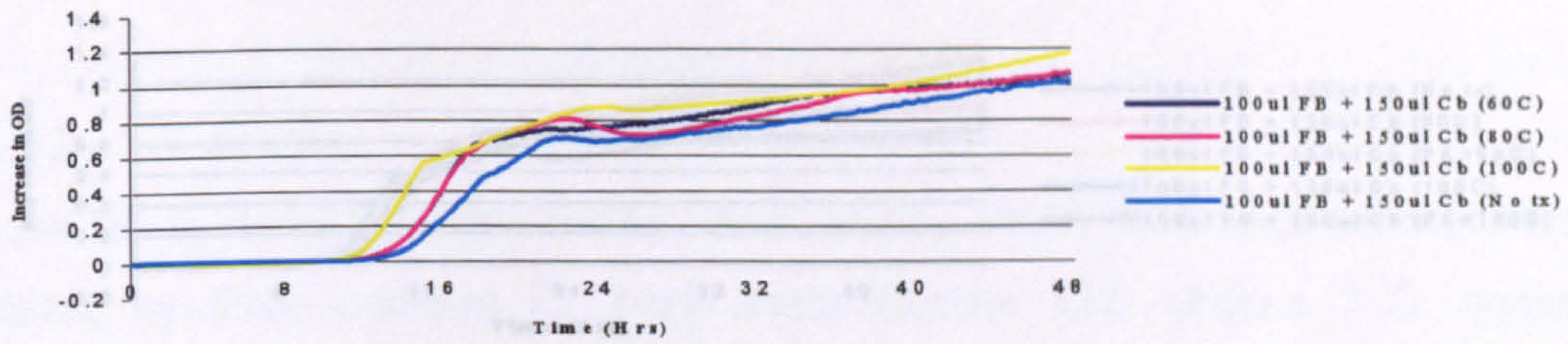
d. 250µl filter sterilized *C. bovis* broth



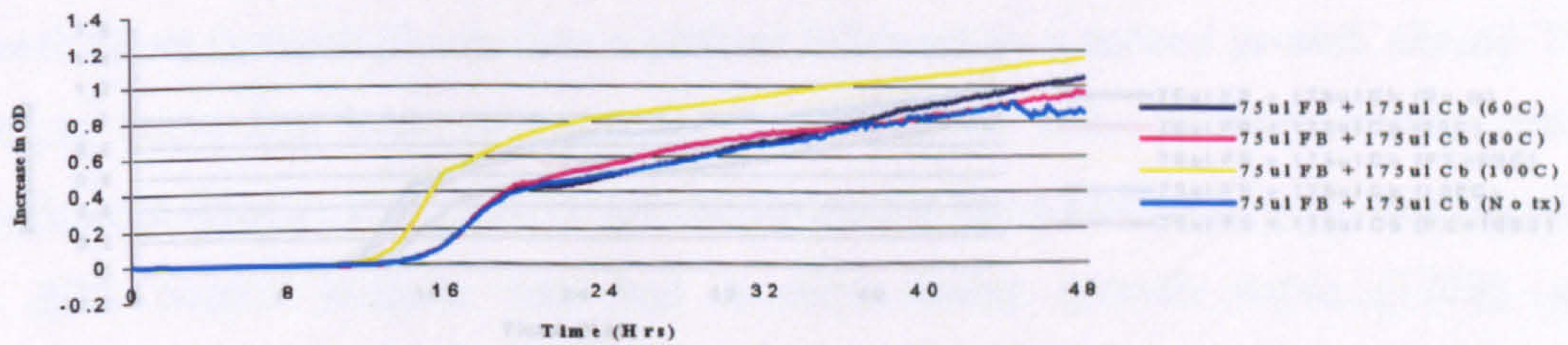
(Fb – Fresh broth, Cb – Filter sterilized *C. bovis* broth)

Figure 7.5: Inhibitory Effect of Filter Sterilized *C. bovis* (122) Broth After No Treatment or Heating to 60, 80 or 100°C on the Growth of *S. aureus* (3094)

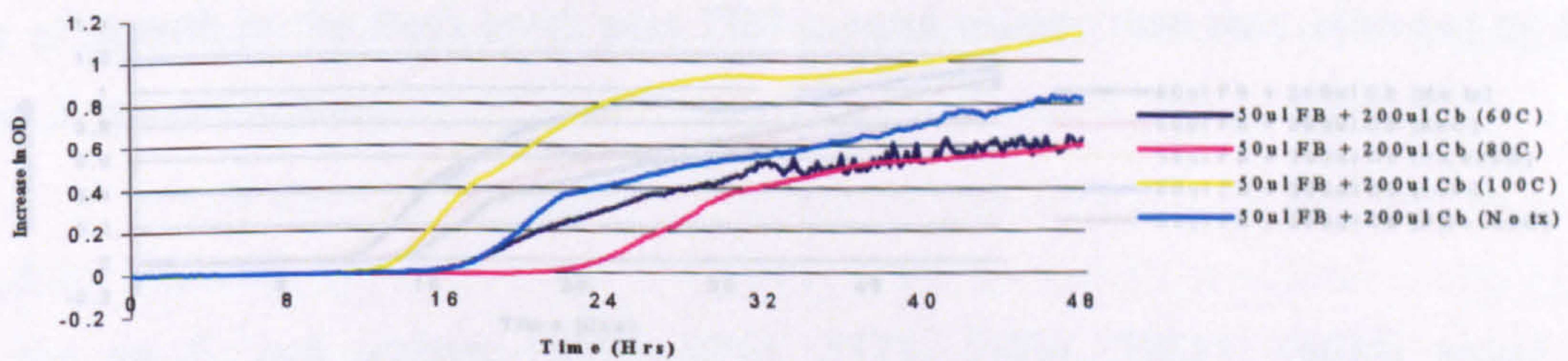
a. 100µl fresh broth plus 150µl filter sterilized *C. bovis* broth



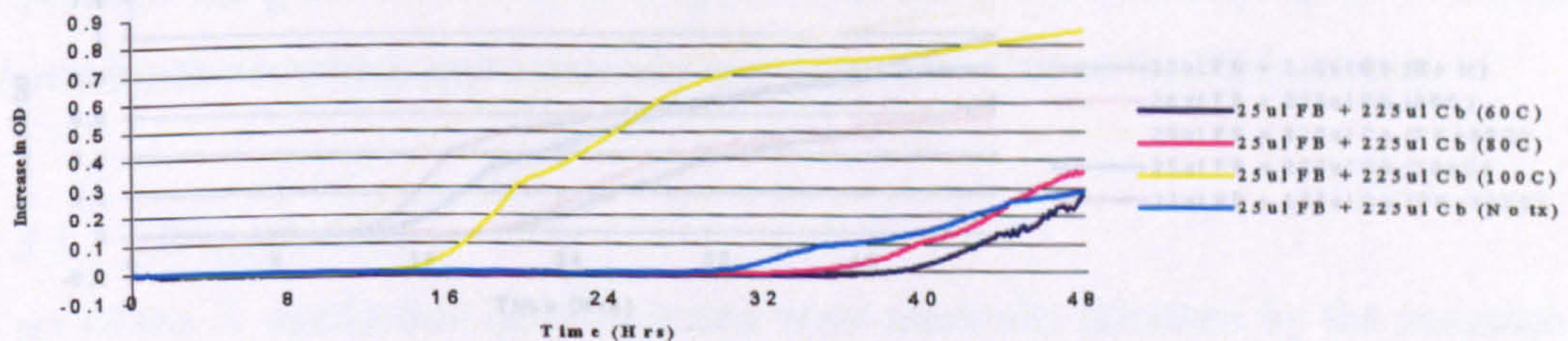
b. 75µl fresh broth plus 175µl filter sterilized *C. bovis* broth



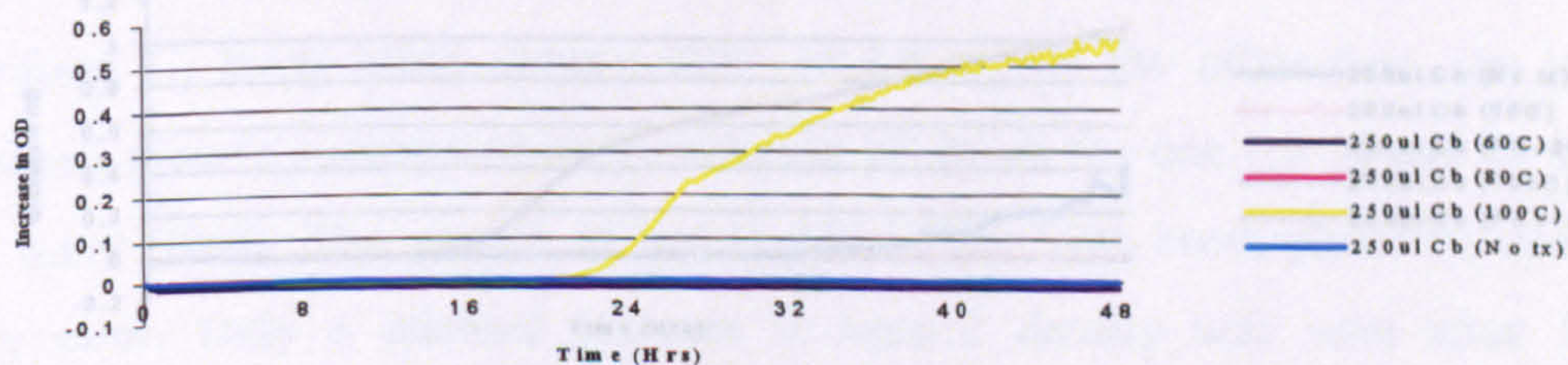
c. 50µl fresh broth plus 200µl filter sterilized *C. bovis* broth



d. 25µl fresh broth plus 225µl filter sterilized *C. bovis* broth



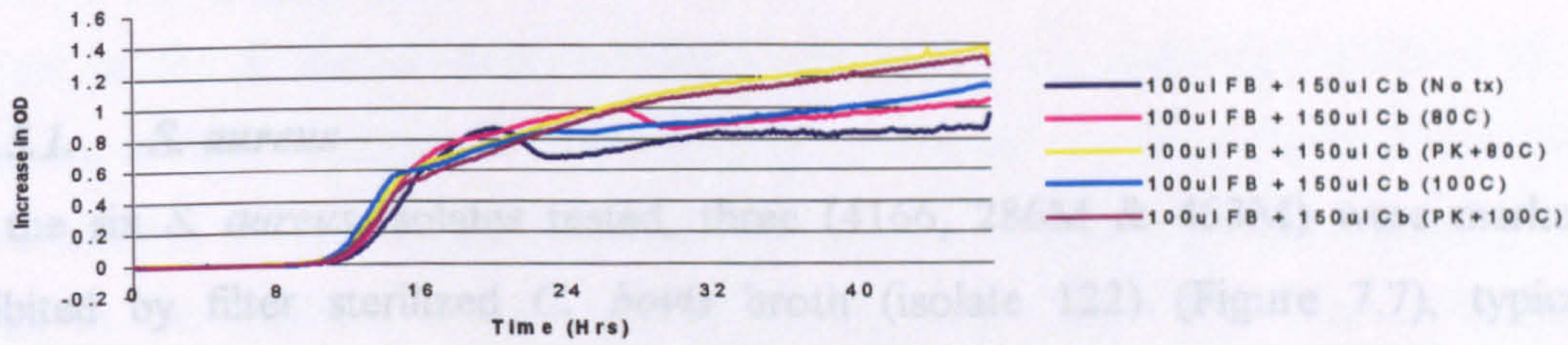
e. 250µl filter sterilized *C. bovis* broth



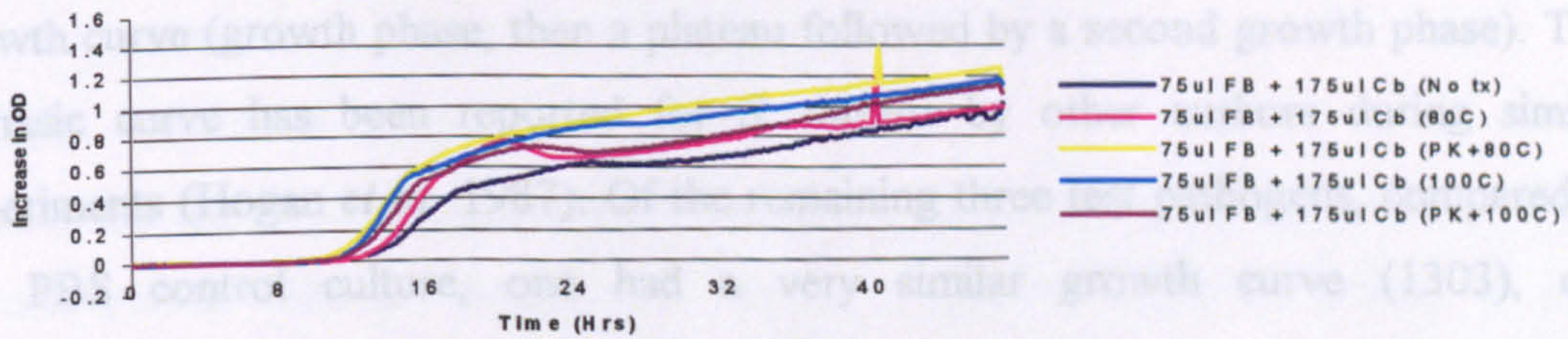
(FB – Fresh broth, Cb – Filter sterilized *C. bovis* broth, No tx – No treatment Control

Figure 7.6: Inhibitory Effect of Filter Sterilized *C. bovis* (122) Broth Heated to 80 or 100°C and Treated with Proteinase K, on the Growth of *S. aureus* (3094)

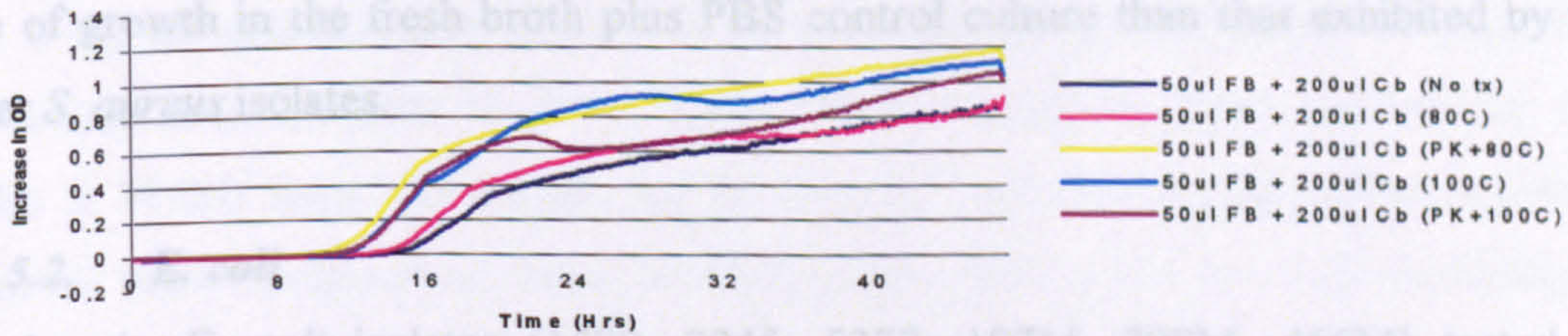
a. 100µl fresh broth plus 150µl filter sterilized *C. bovis* broth



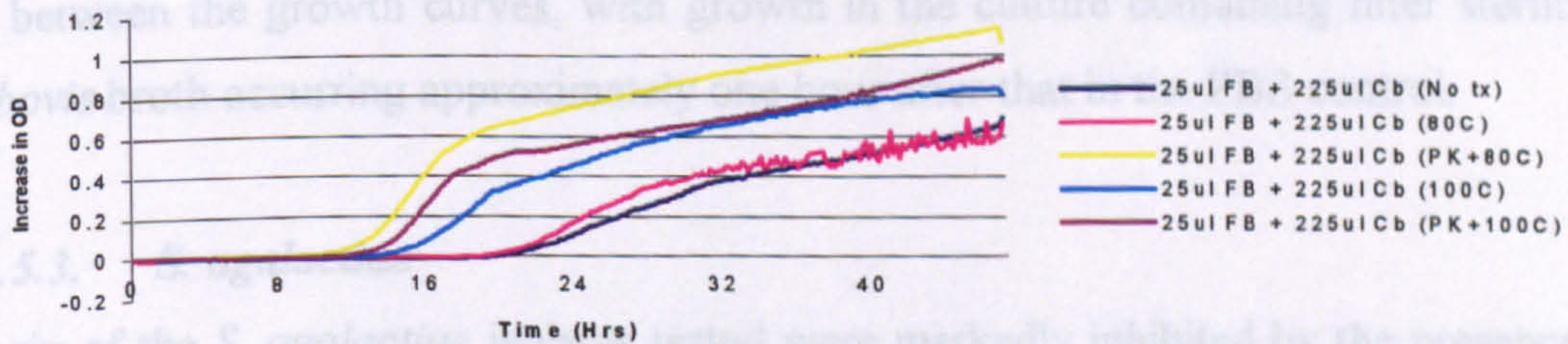
b. 75µl fresh broth plus 175µl filter sterilized *C. bovis* broth



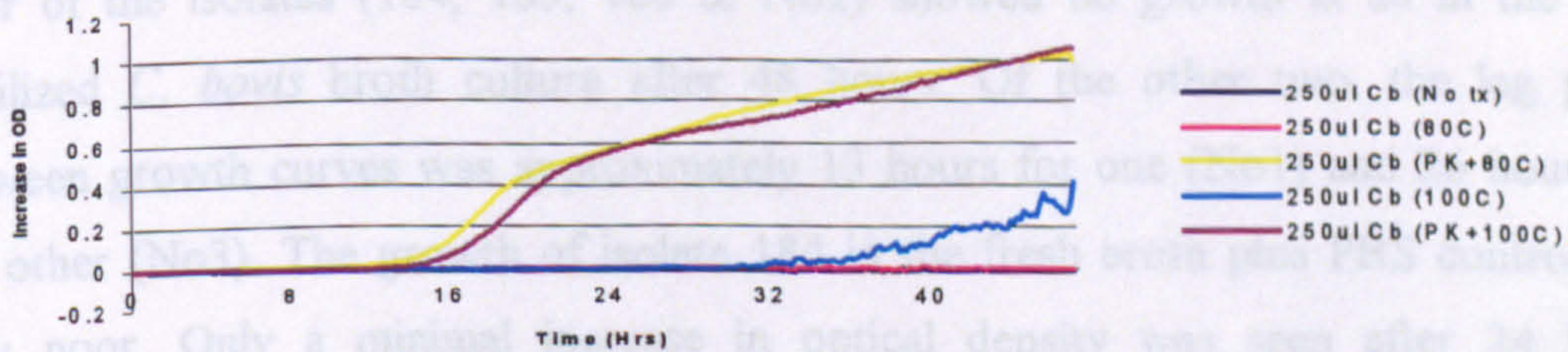
c. 50µl fresh broth plus 200µl filter sterilized *C. bovis* broth



d. 25µl fresh broth plus 225µl filter sterilized *C. bovis* broth



e. 250µl filter sterilized *C. bovis* broth



(FB – Fresh broth, Cb– Filter sterilized *C. bovis* broth, No tx– No treatment control, PK – Proteinase K)

7.3.5. Affect of Filter Sterilized *C. bovis* Broth (Isolate 122) on the Growth Rate of Six Isolates of Five Mastitis Pathogens

7.3.5.1. S. aureus

Of the six *S. aureus* isolates tested, three (4166, 286M & 453M) were markedly inhibited by filter sterilized *C. bovis* broth (isolate 122) (Figure 7.7), typically demonstrating a lag of approximately six hours. In all three cases the growth of the *S. aureus* isolates in the fresh broth plus PBS control culture demonstrated a biphasic growth curve (growth phase, then a plateau followed by a second growth phase). This biphasic curve has been reported for *S. aureus* by other authors during similar experiments (Hogan *et al.* 1987). Of the remaining three test pathogens, compared to the PBS control culture, one had a very similar growth curve (1303), one demonstrated a slight lag (81M) and one (4249) appeared to grow marginally faster in the filter sterilized *C. bovis* broth culture. This *S. aureus* strain demonstrated a slower rate of growth in the fresh broth plus PBS control culture than that exhibited by the other *S. aureus* isolates.

7.3.5.2. E. coli

Of the six *E. coli* isolates (1599, 2945, 5377, 197M, 298M, 466M) tested all demonstrated a very similar response (Figure 7.8). There was a small but consistent lag between the growth curves, with growth in the culture containing filter sterilized *C. bovis* broth occurring approximately one hour after that in the PBS control.

7.3.5.3. S. agalactiae

All six of the *S. agalactiae* isolates tested were markedly inhibited by the presence of filter sterilized *C. bovis* broth in the culture, compared to the PBS control (Figure 7.9). Four of the isolates (184, 185, 186 & No2) showed no growth at all in the filter sterilized *C. bovis* broth culture after 48 hours. Of the other two, the lag phase between growth curves was approximately 13 hours for one (No1) and 26 hours for the other (No3). The growth of isolate 184 in the fresh broth plus PBS control was very poor. Only a minimal increase in optical density was seen after 24 hours

incubation. Similar results were obtained in a previous experiment with this isolate (data not shown).

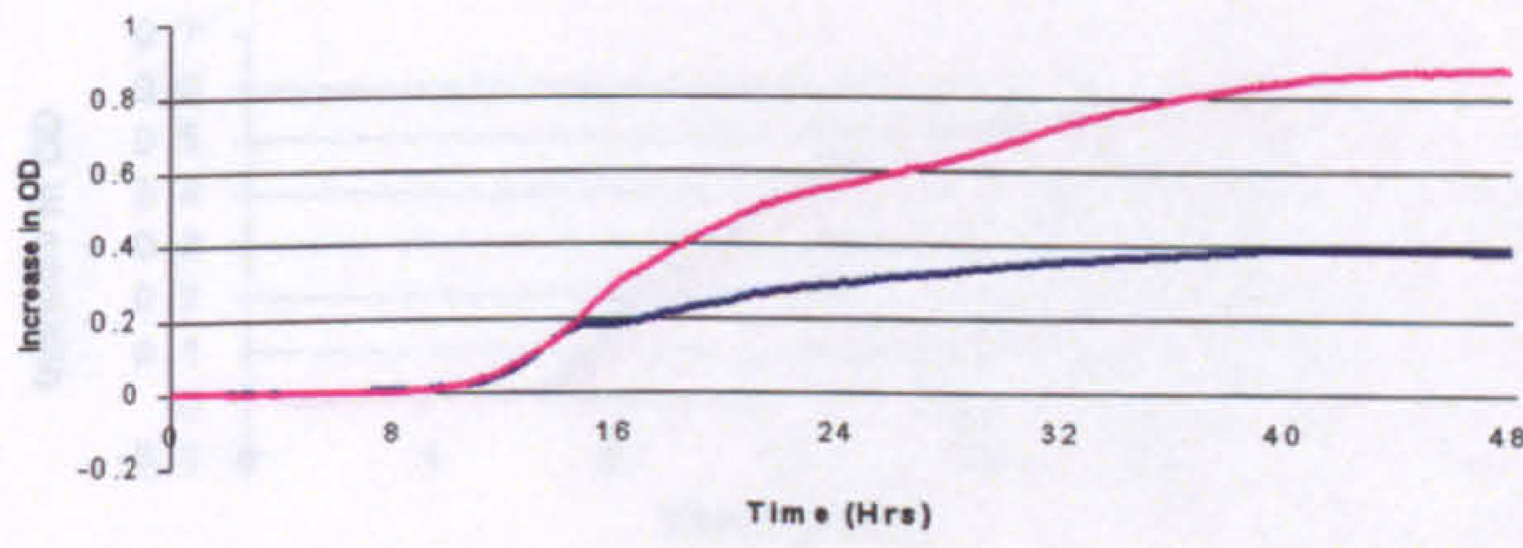
7.3.5.4. S. dysgalactiae

All six of the *S. dysgalactiae* isolates tested were very markedly inhibited by the presence of filter sterilized *C. bovis* broth in the culture medium (Figure 7.10). None of the isolates (3616, 4092D, 5861B, 55M, 108M & 421M) demonstrated any growth at all in the medium containing filter sterilized *C. bovis* broth after 48 hours incubation at 37°C. Growth in the control media containing PBS caused an increase in optical density after 12 to 16 hours.

7.3.5.5. S. uberis

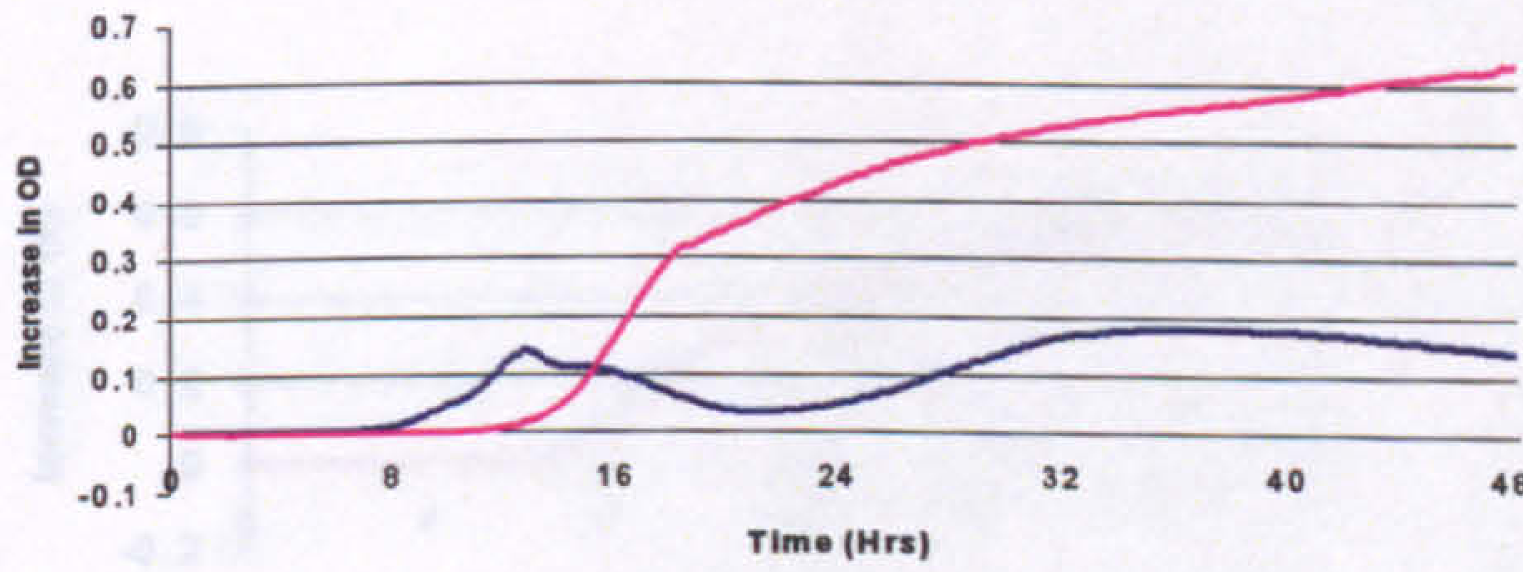
Results for the six *S. uberis* isolates tested are demonstrated in Figure 7.11. Three isolates (4190, 71M & 300M) were markedly inhibited by the presence of filter sterilized *C. bovis* broth in the culture media. Of these three, two (4190 & 71M) demonstrated no growth at all after 48 hours incubation and for the other (300M) there was a lag of approximately ten hours between the media containing PBS and that containing filter sterilized *C. bovis* broth. Of the remaining three isolates, for two (1086 & 399M) there was a slight lag between the cultures containing filter sterilized *C. bovis* broth compared to those containing PBS and for the last (2201), timing of initial growth was very similar in the test and control cultures.

Figure 7.7: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of Six *S. aureus* Isolates



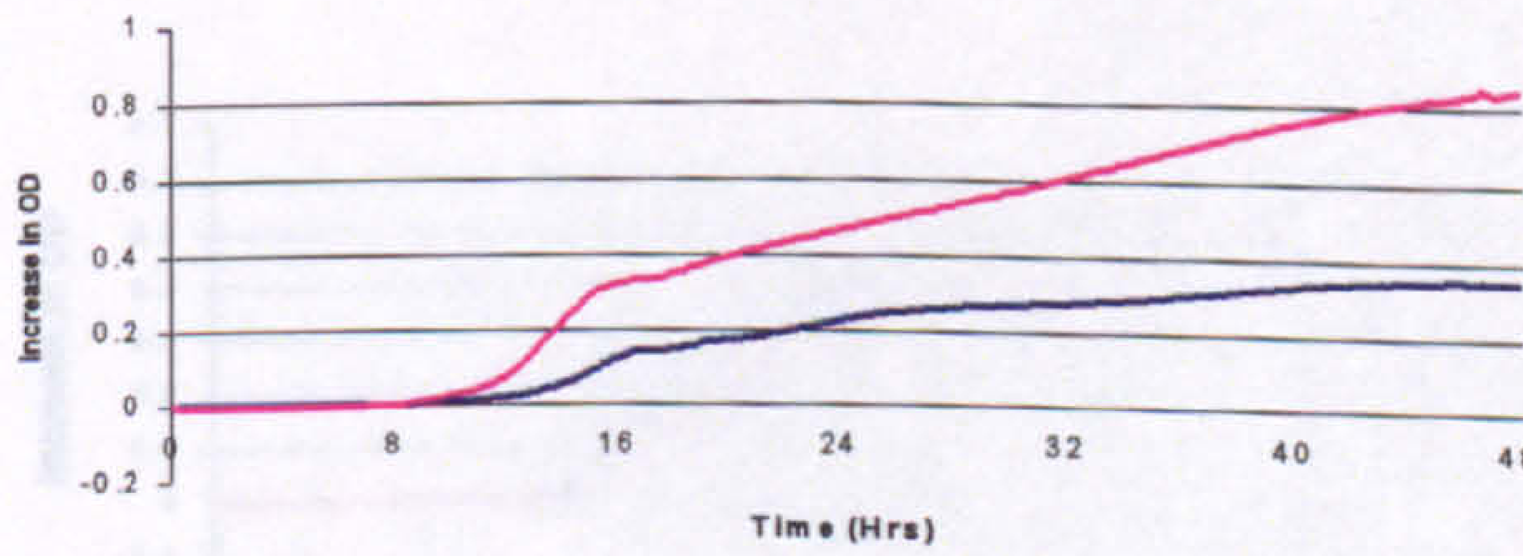
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. aureus 1303



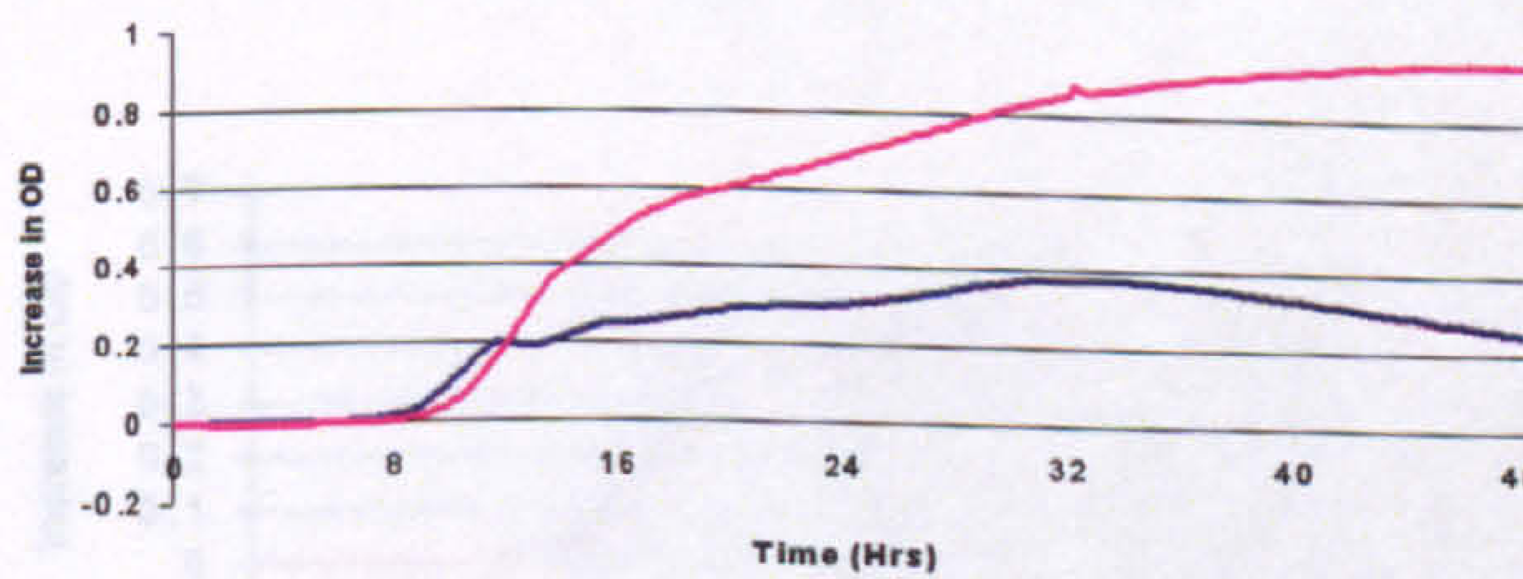
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. aureus 4166



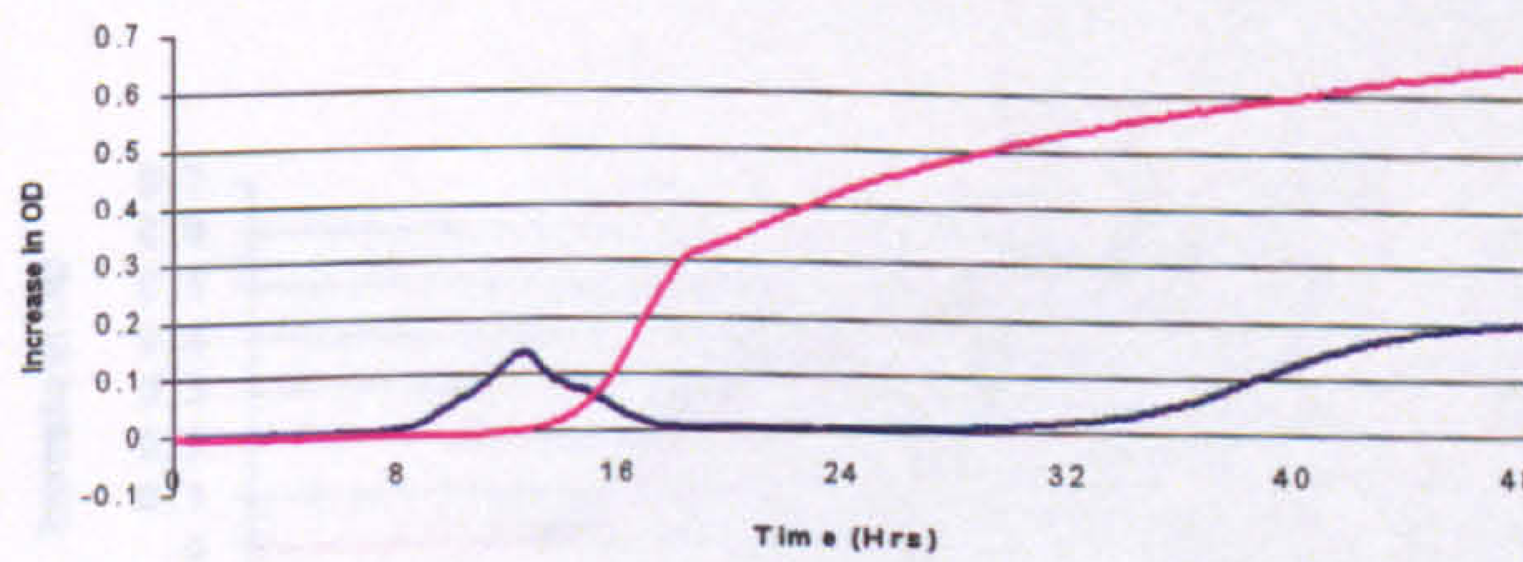
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. aureus 4249



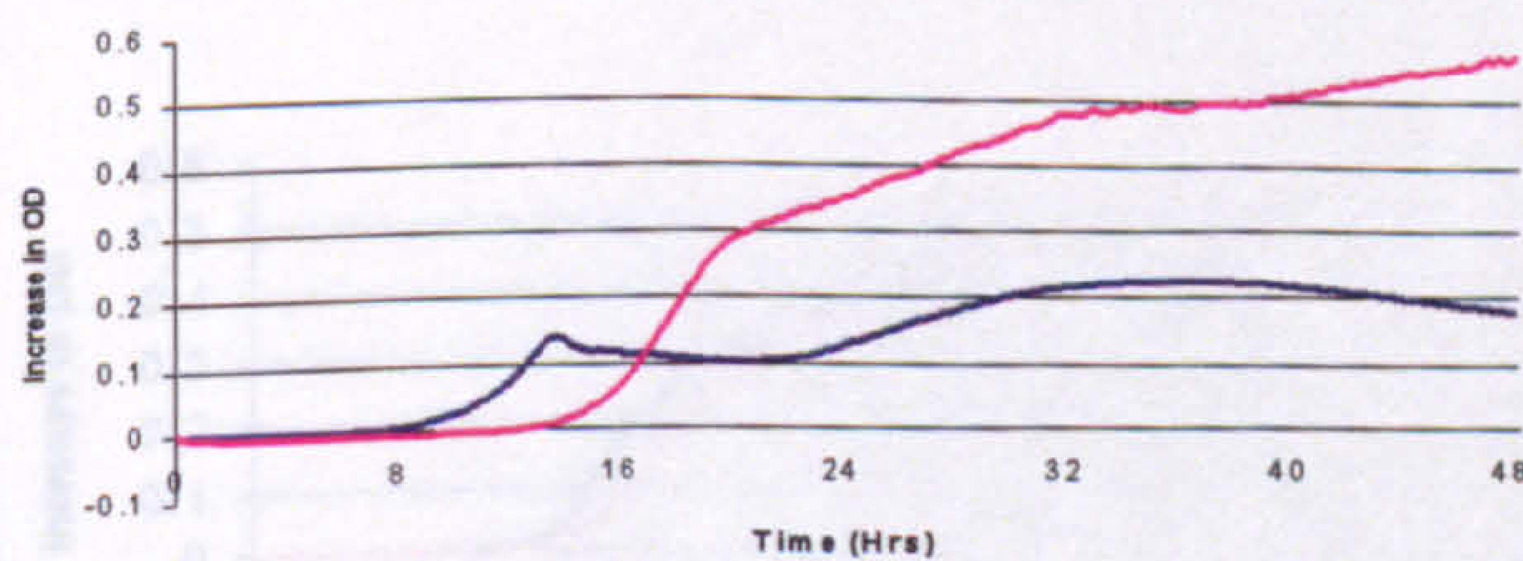
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. aureus 81M



25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. aureus 286M

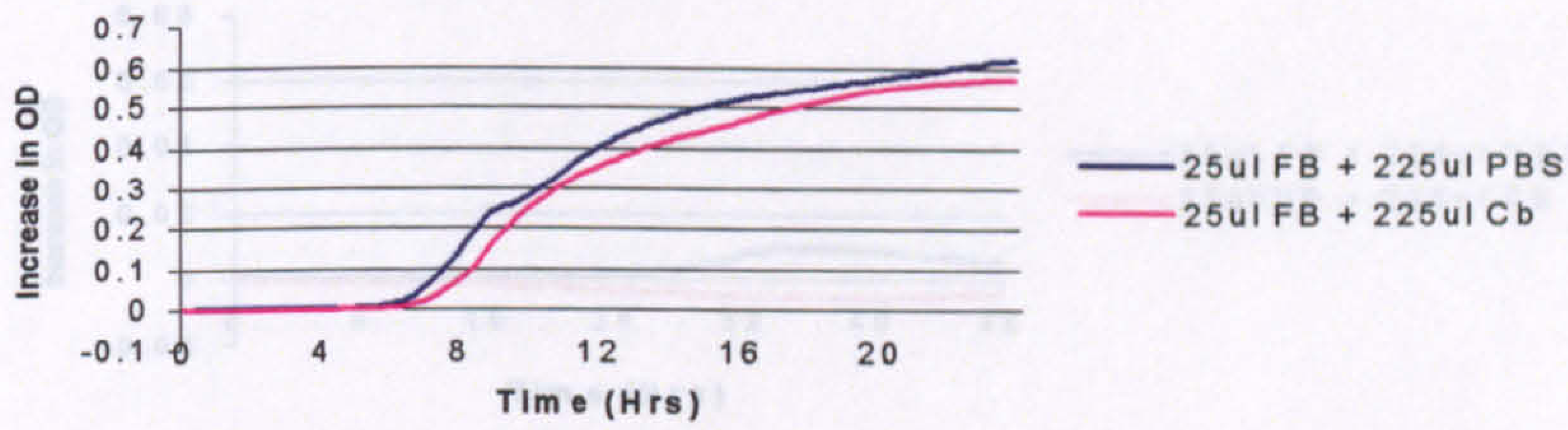


25ul FB + 225ul PBS
25ul FB + 225ul Cb

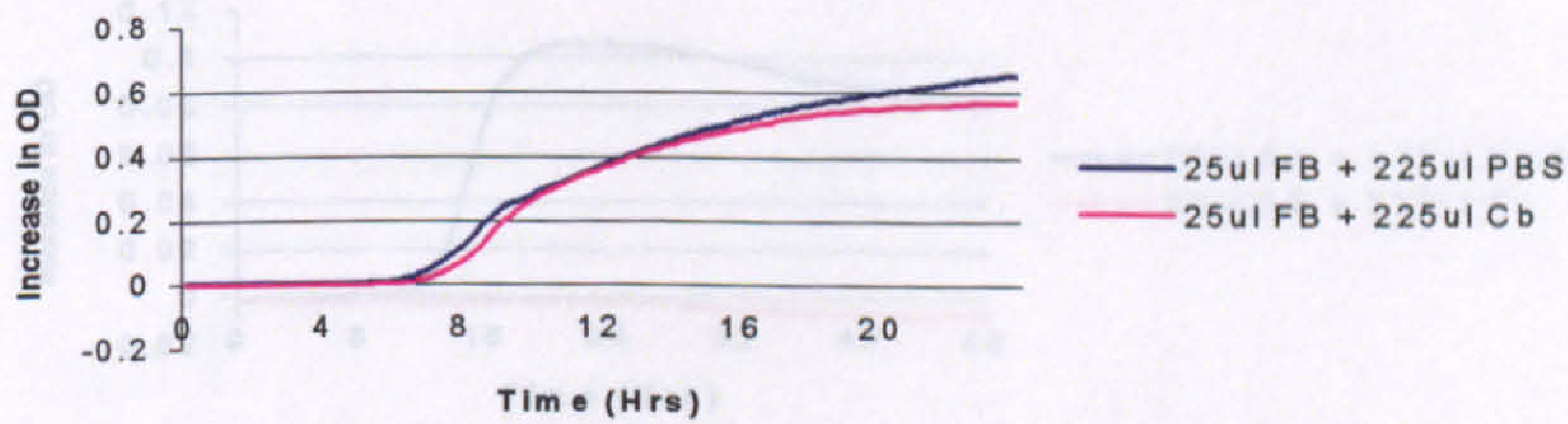
S. aureus 453M

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

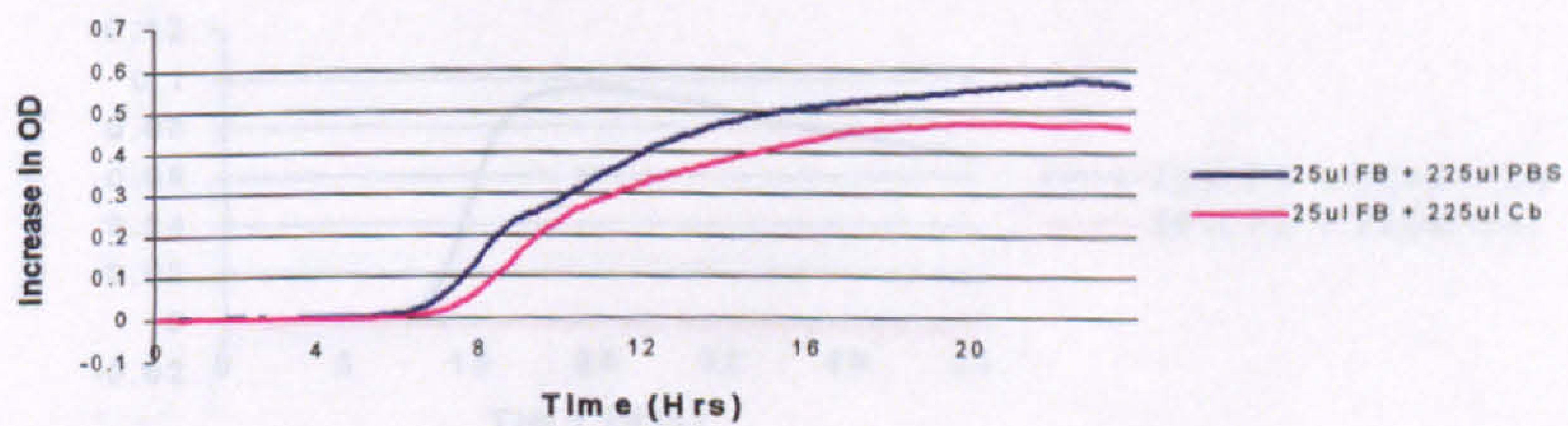
Figure 7.8: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of Six *E. coli* Isolates



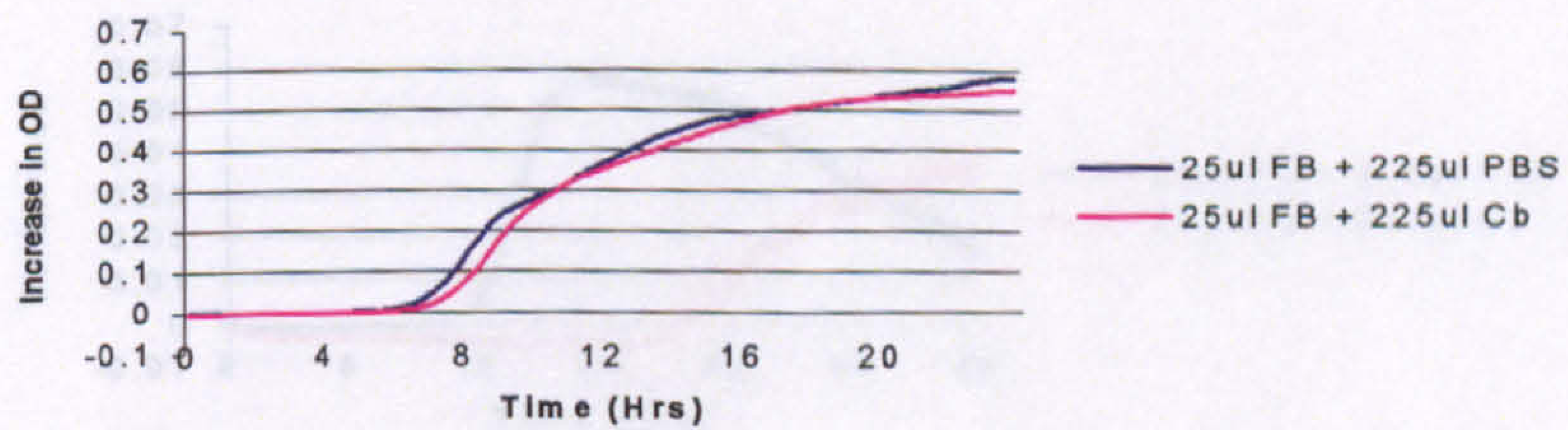
E. coli 1599



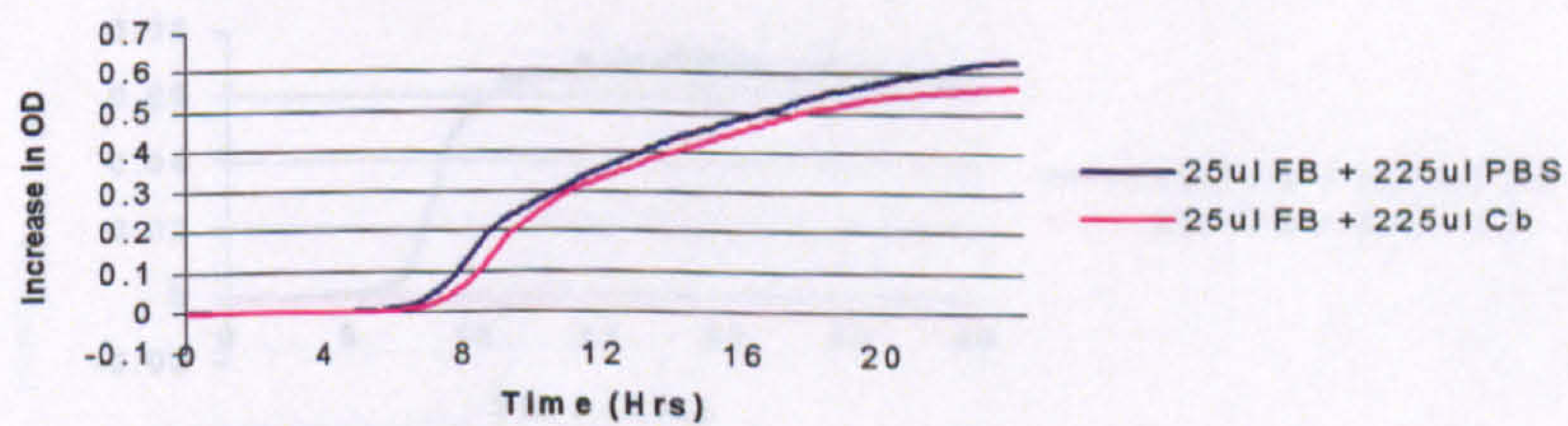
E. coli 2945



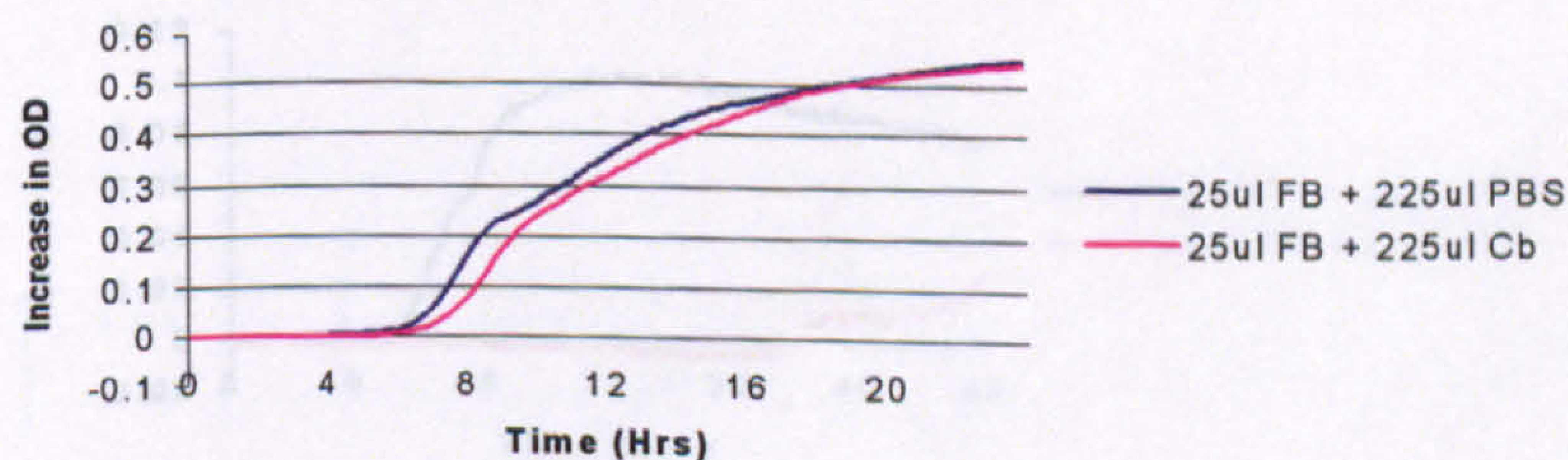
E. coli 5377



E. coli 197M



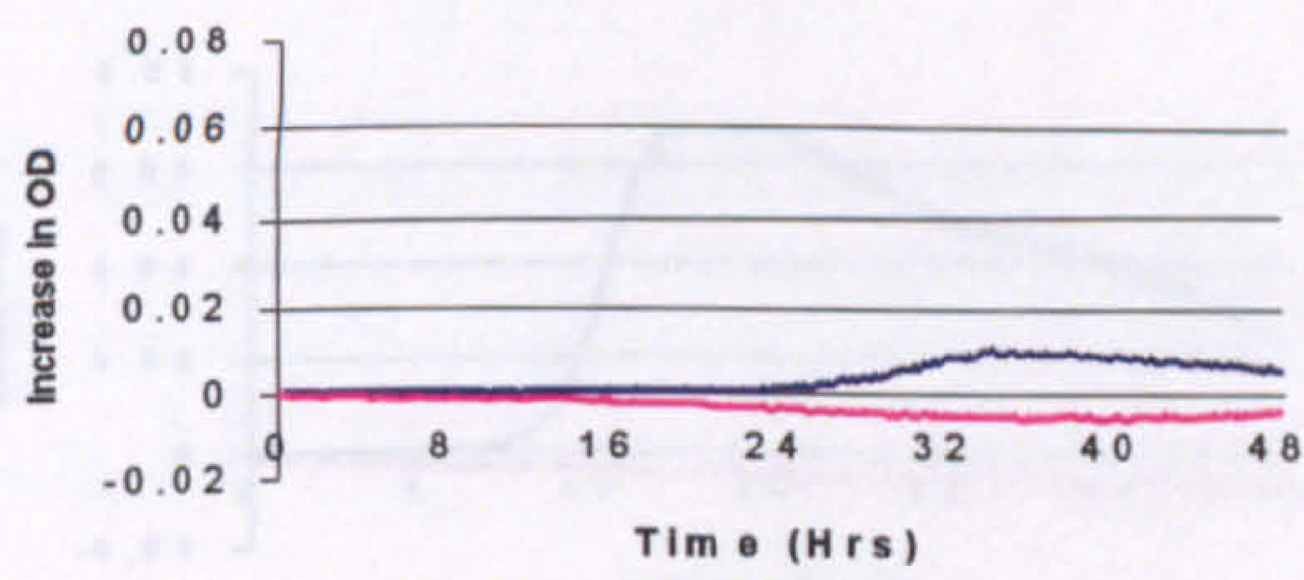
E. coli 298M



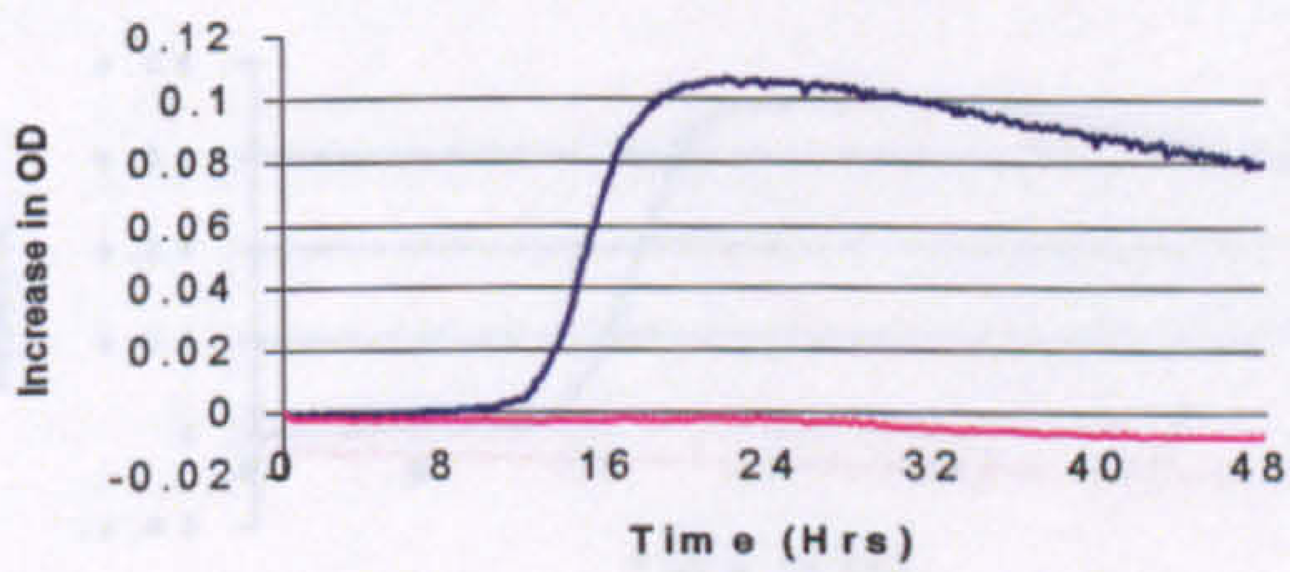
E. coli 466M

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

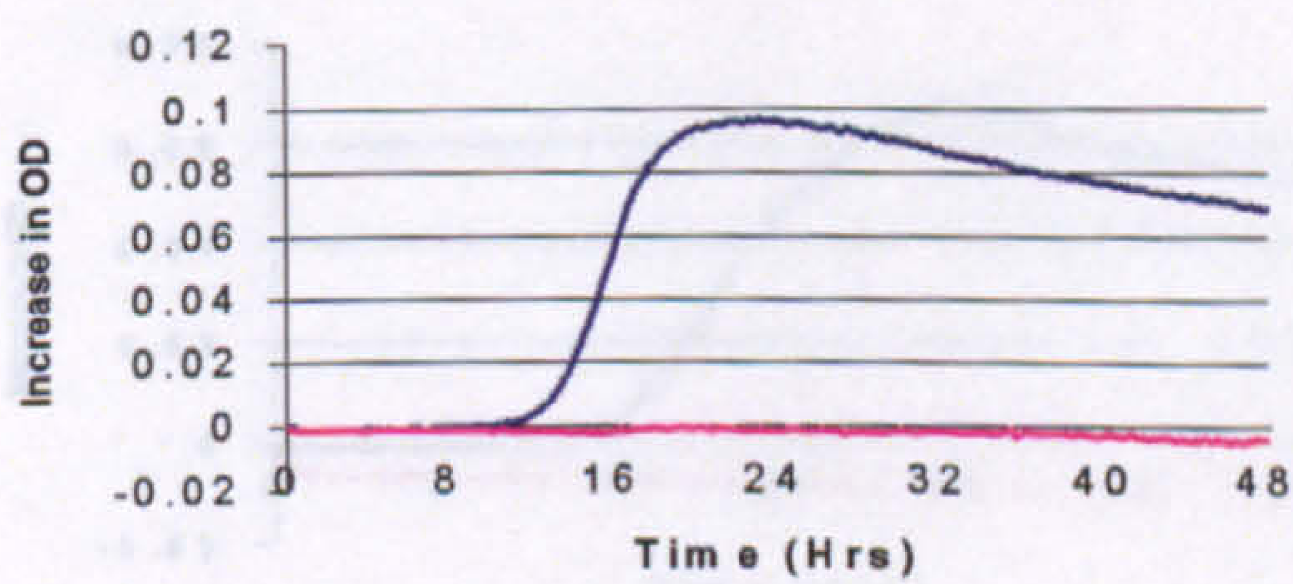
Figure 7.9: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of Six *S. agalactiae* Isolates



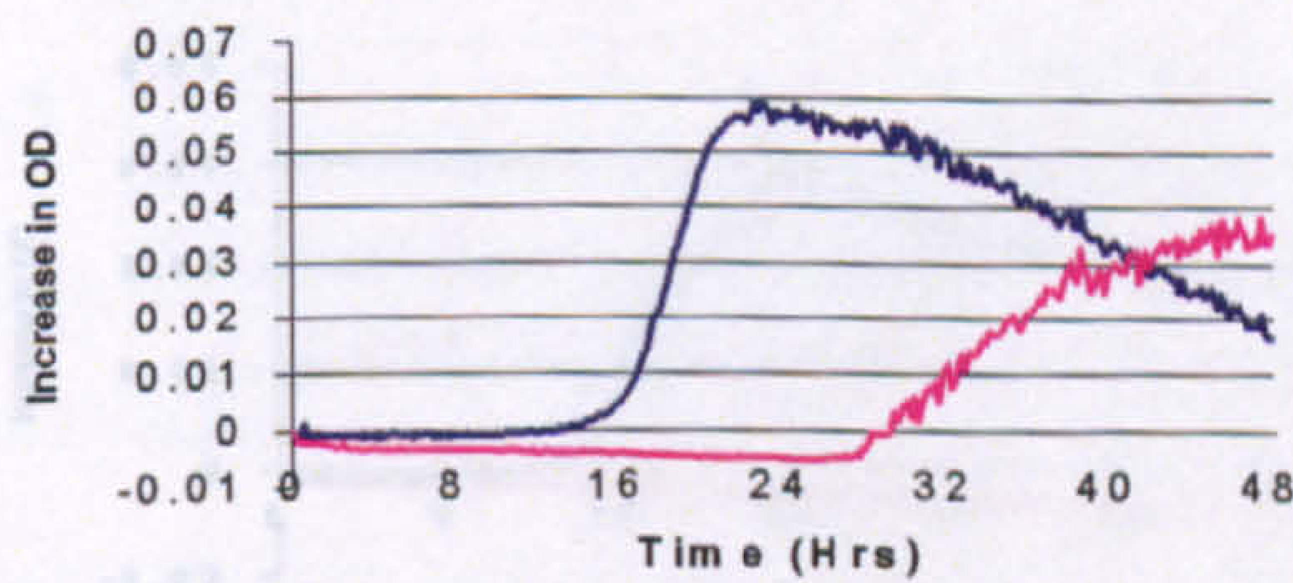
S. agalactiae 184



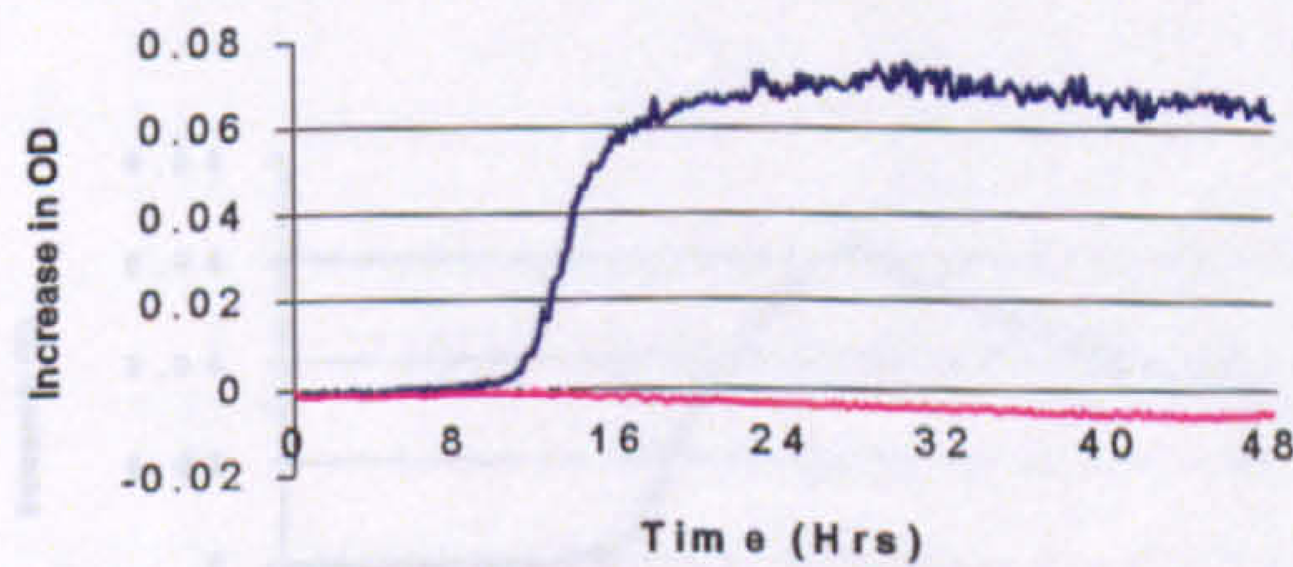
S. agalactiae 185



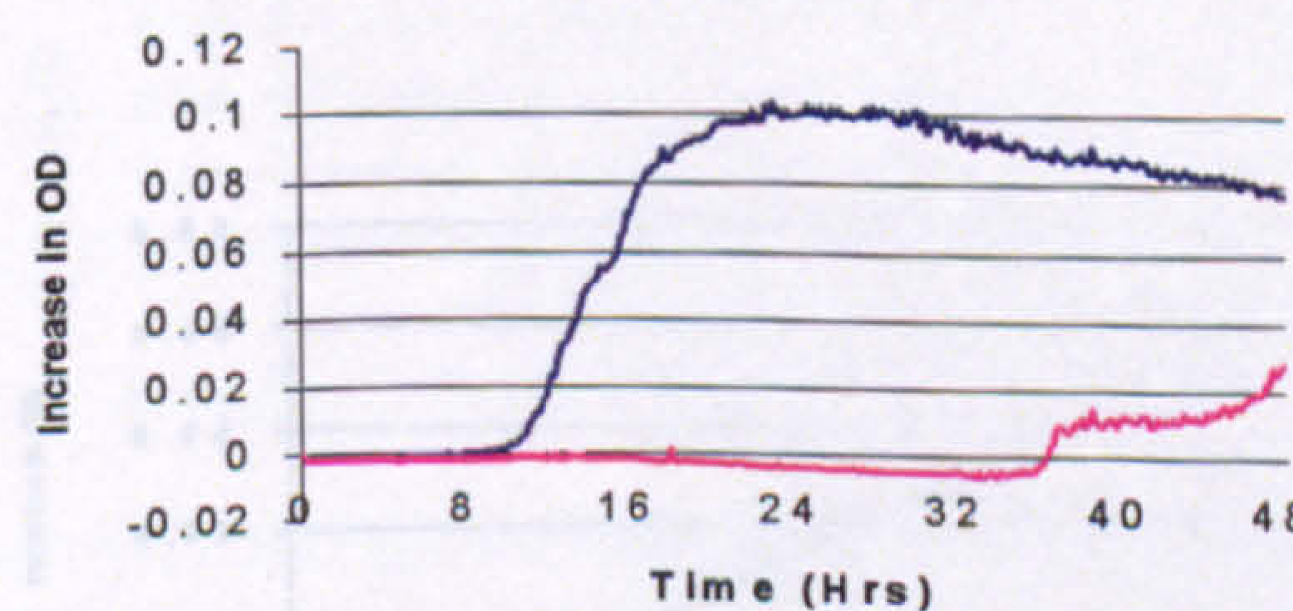
S. agalactiae 186



S. agalactiae No1



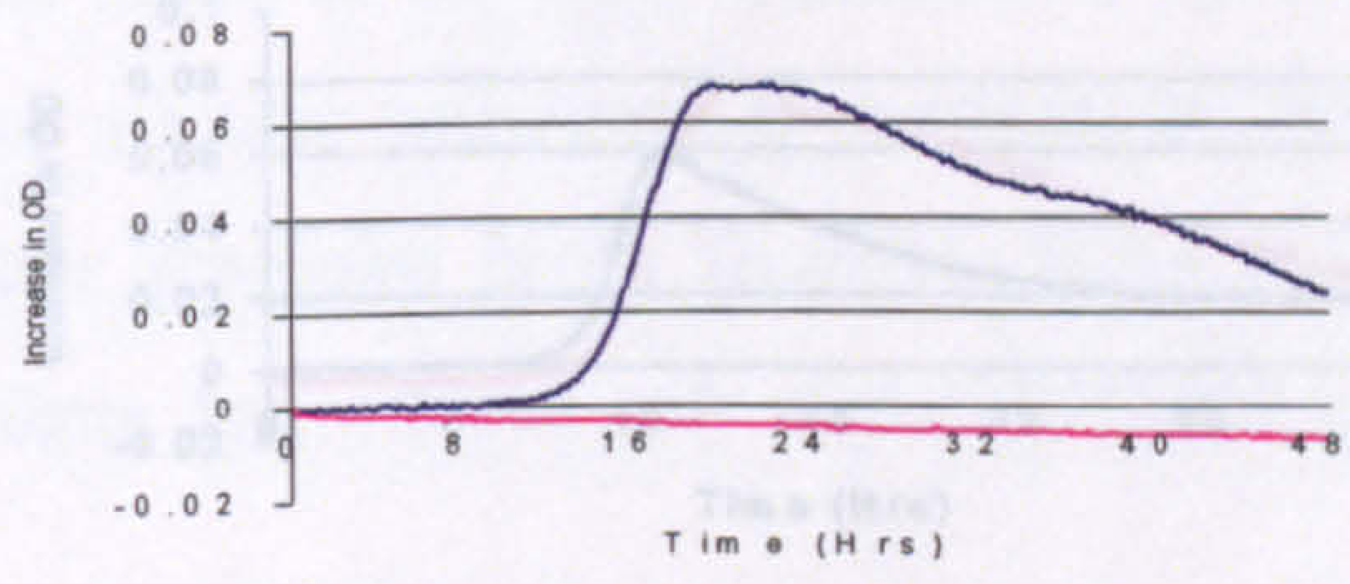
S. agalactiae No2



S. agalactiae No3

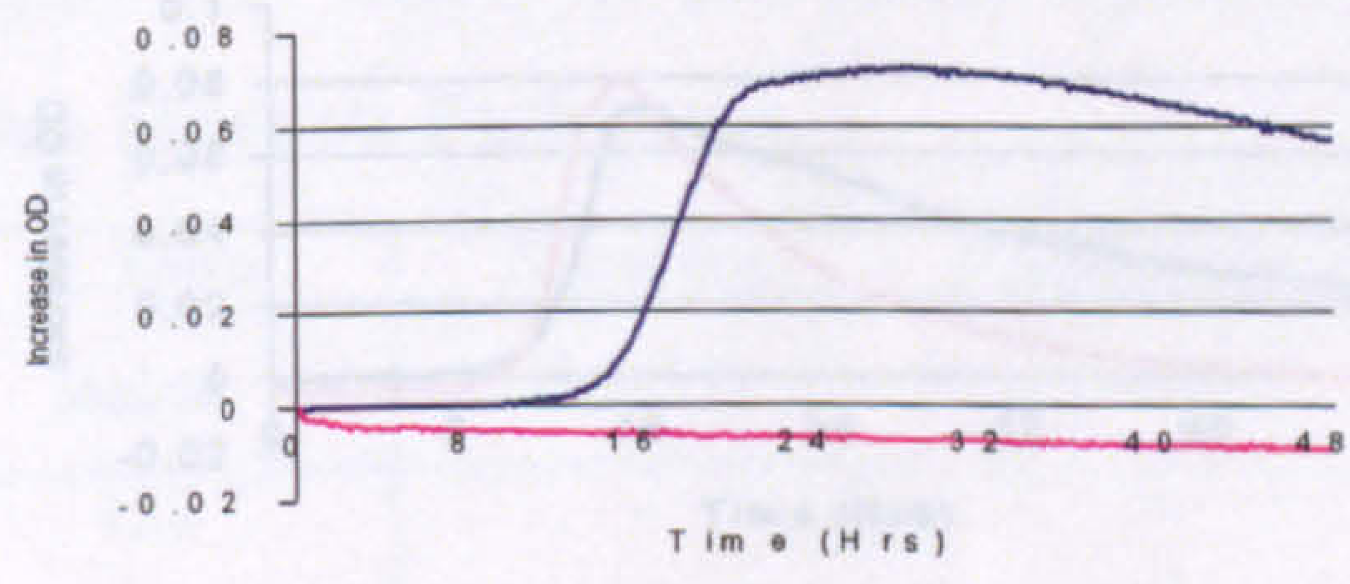
(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

Figure 7.10: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of Six *S. dysgalactiae* Isolates



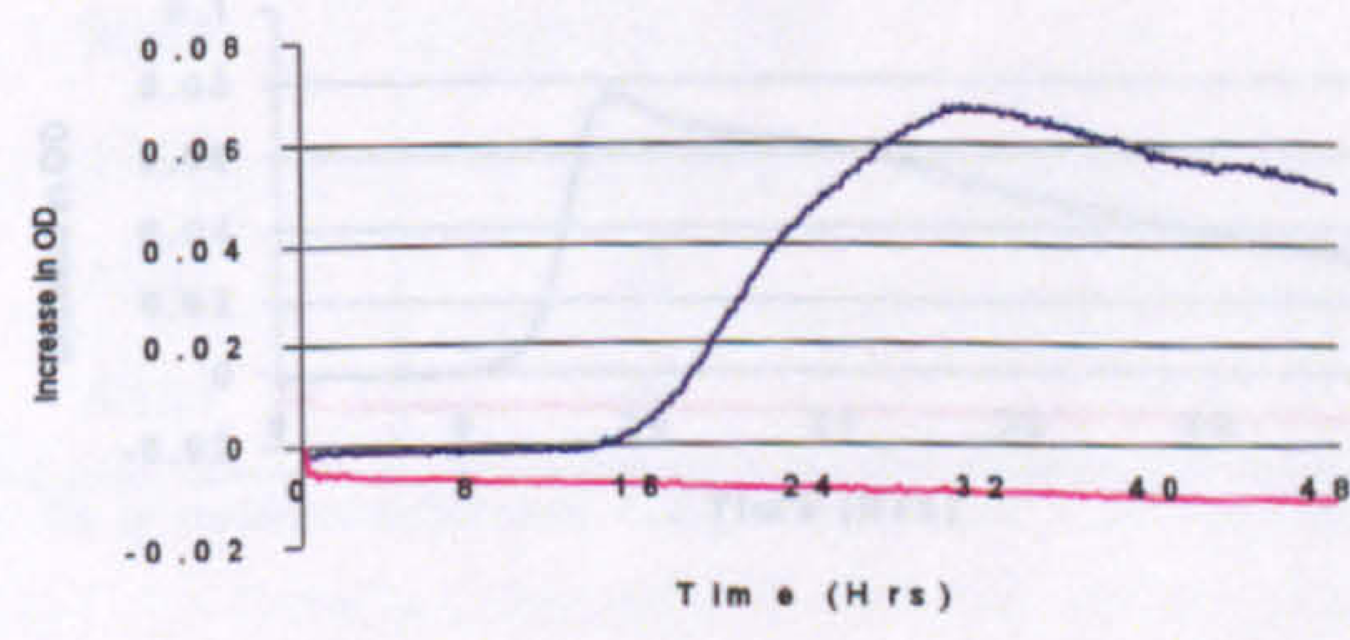
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 3616



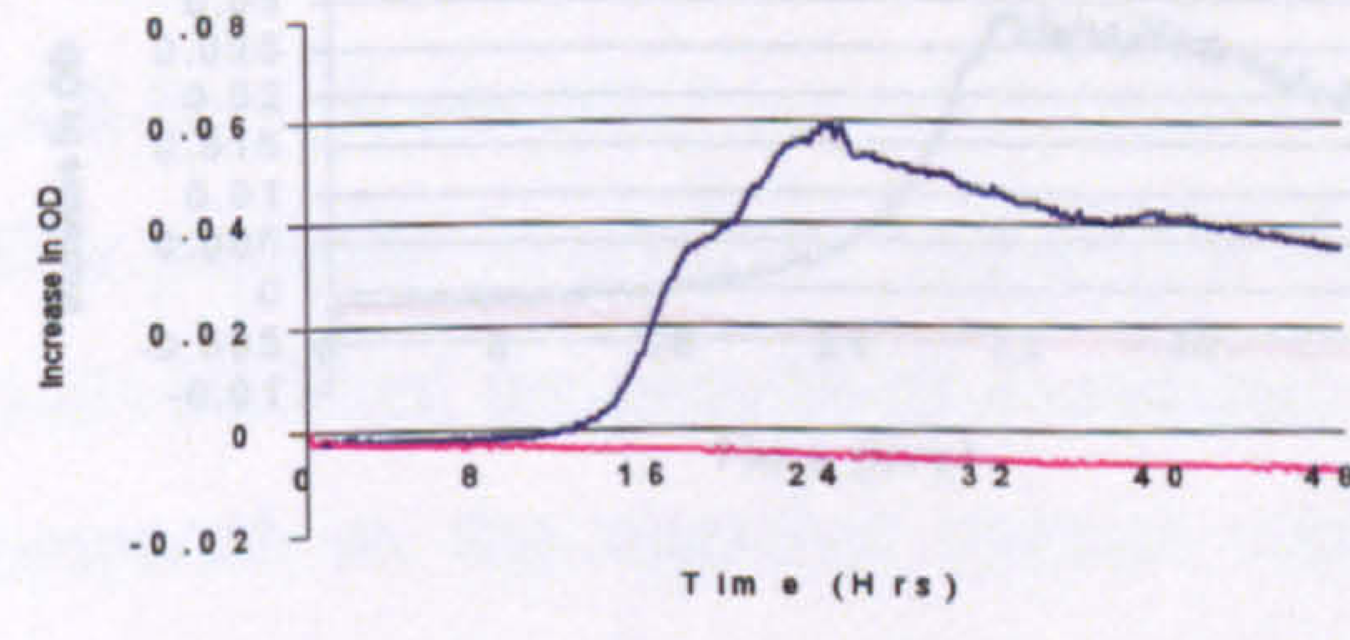
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 4092D



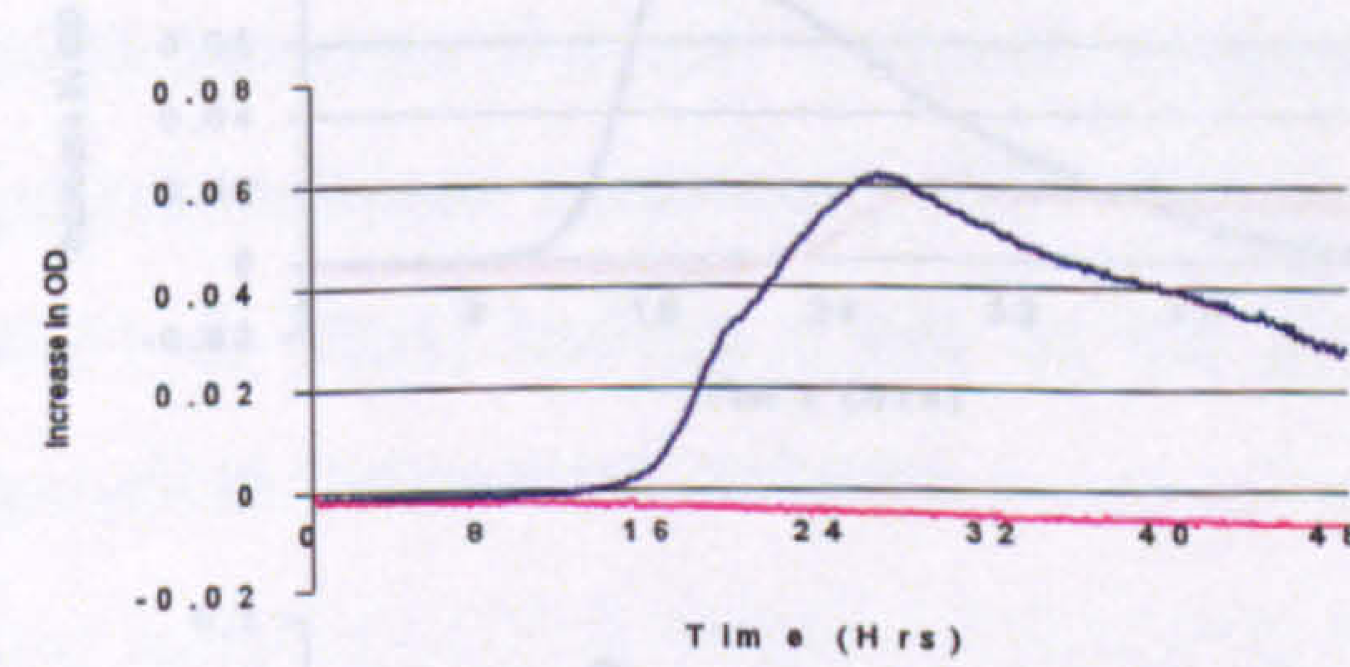
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 5861B



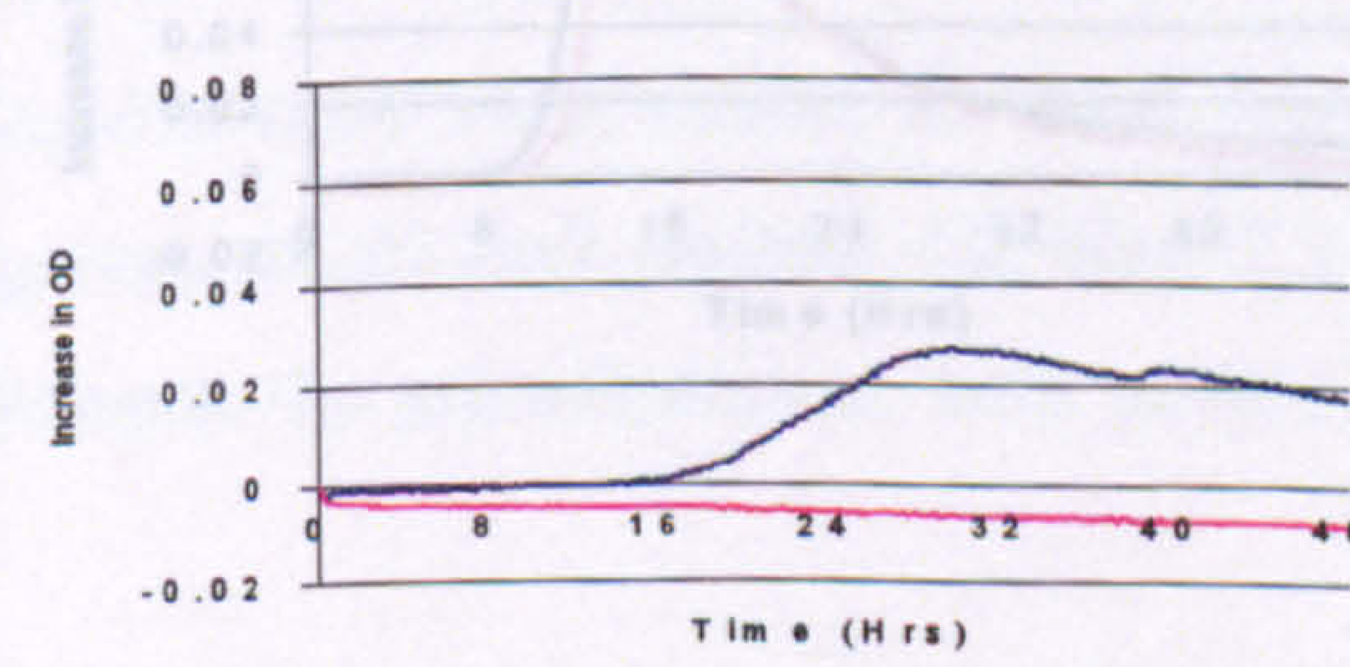
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 55M



25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 108M

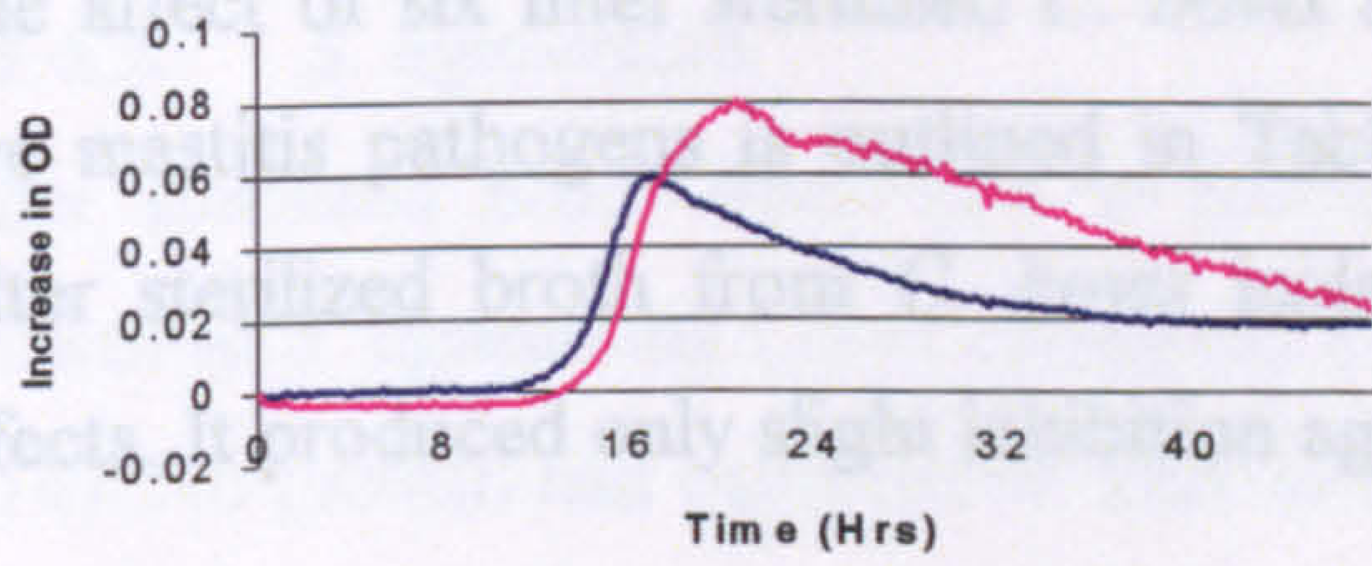


25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. dysgalactiae 421M

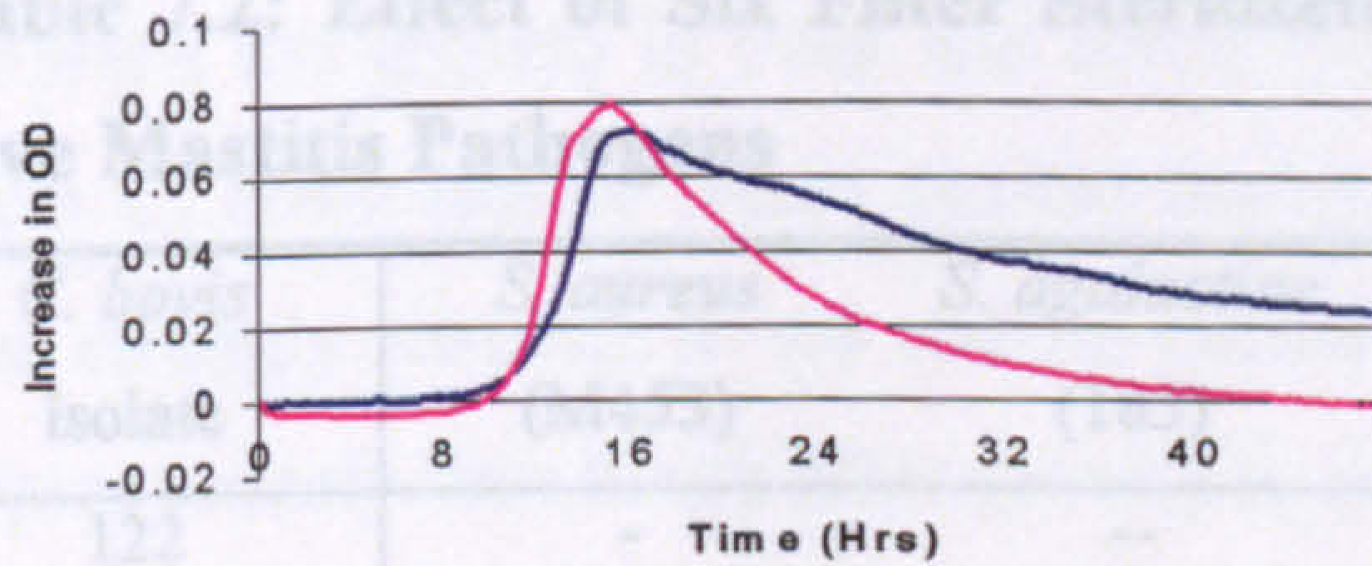
(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

Figure 7.11: Inhibitory Effects of Filter Sterilized *C. bovis* (No. 122) Broth on the Growth of Six *S. uberis* Isolates



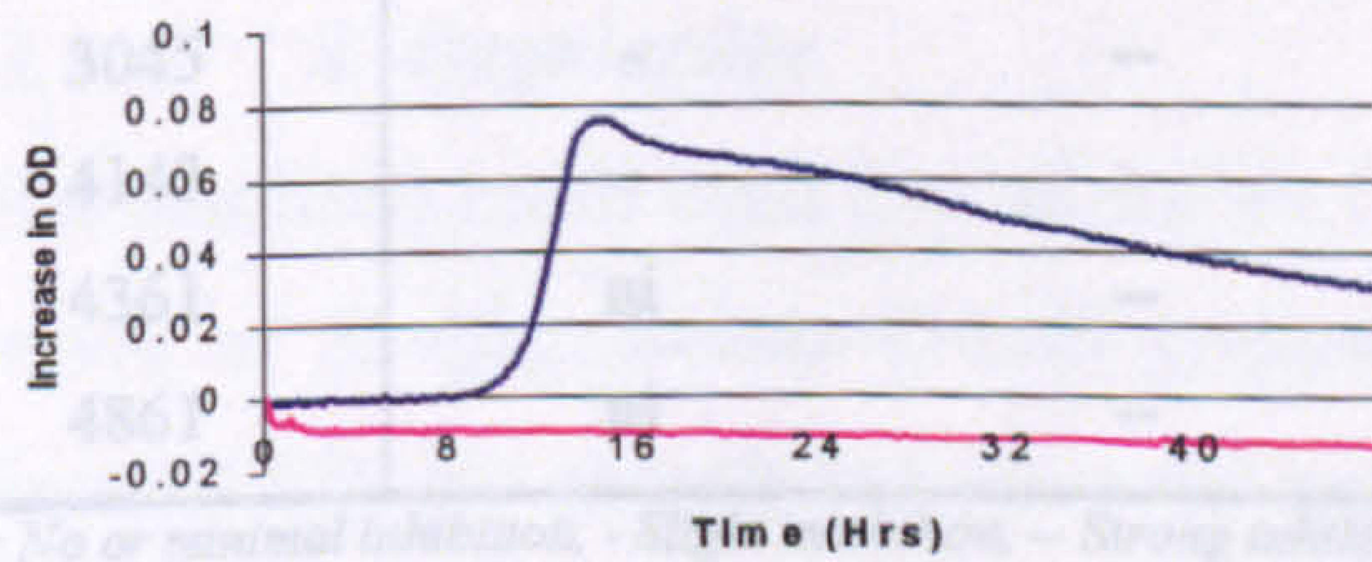
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 1086



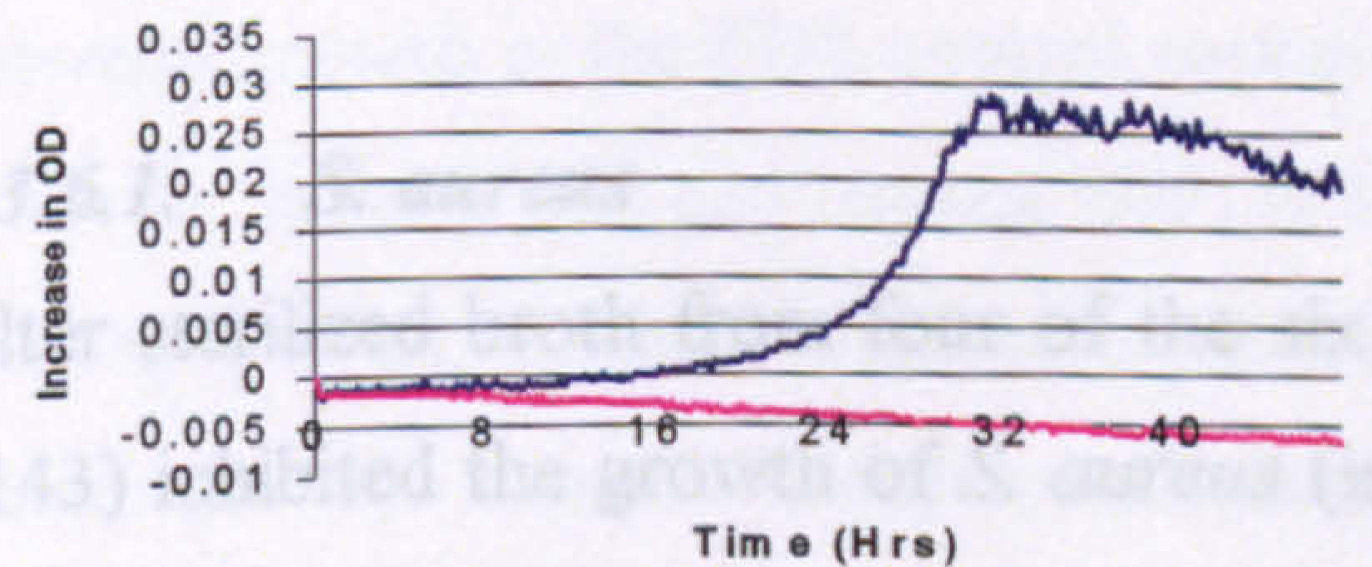
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 2201



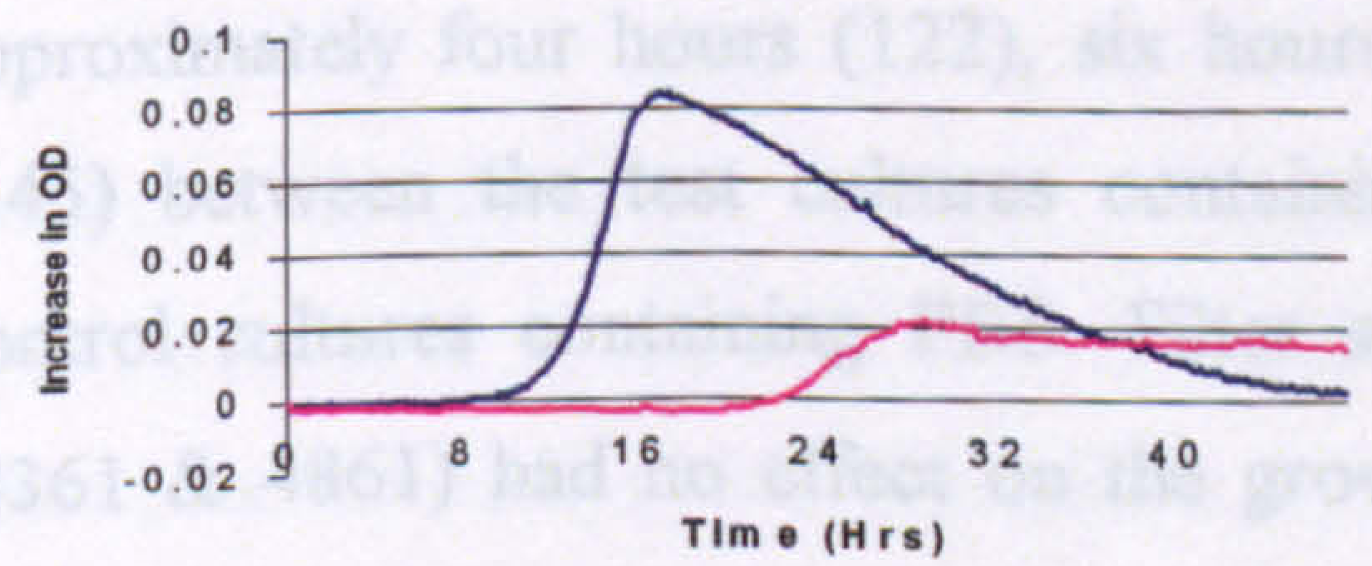
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 4190



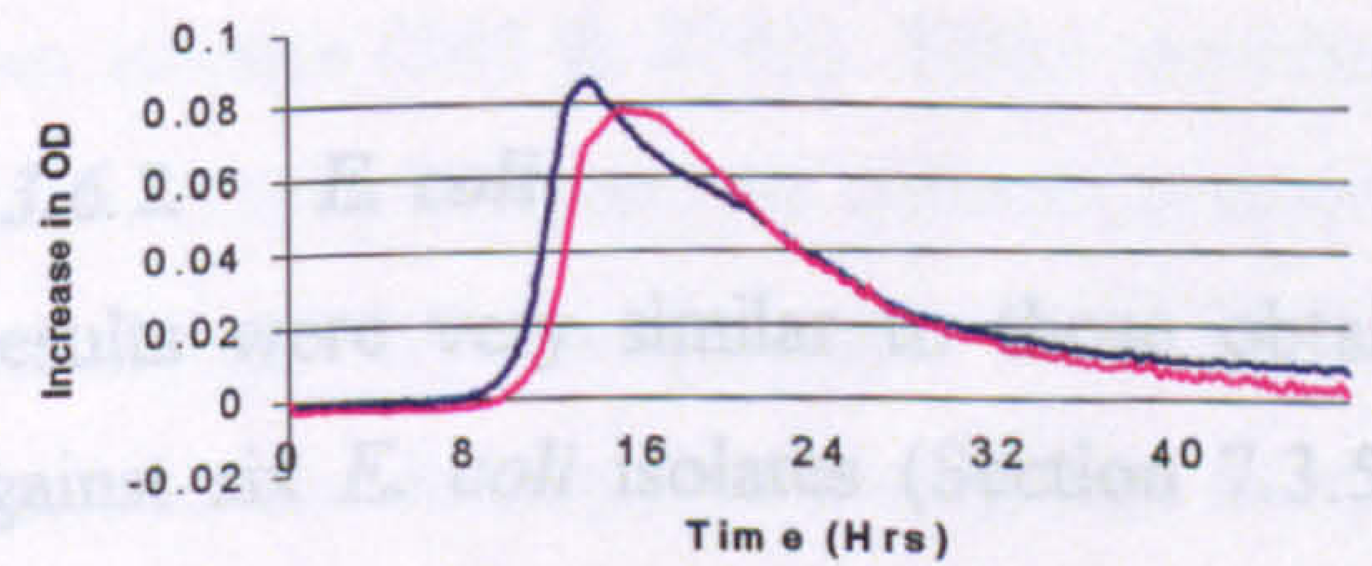
25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 71M



25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 300M



25ul FB + 225ul PBS
25ul FB + 225ul Cb

S. uberis 399M

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

7.3.6. Effect of Six Filter Sterilized *C. bovis* Broths on the Growth Rate of One Isolate of Five Mastitis Pathogens

The affect of six filter sterilized *C. bovis* broths on the growth rate of one isolate of five mastitis pathogens is outlined in Table 7.2 and described in more detail below. Filter sterilized broth from *C. bovis* isolate 4143 demonstrated the least inhibitory effects. It produced only slight inhibition against all three *Streptococcus* species.

Table 7.2: Effect of Six Filter Sterilized *C. bovis* Broths on the Growth Rate of Five Mastitis Pathogens

C. bovis isolate	*S. aureus* (M453)	*S. agalactiae* (185)	*S. dysgalactiae* (3616)	*S. uberis* (4190)	*E. coli* (1599)
122	-	--	--	-	ni
345	-	--	--	--	ni
3045	-	--	--	--	ni
4143	-	-	-	-	ni
4361	ni	--	--	--	ni
4861	ni	--	--	--	ni

(ni: No or minimal inhibition, - Slight inhibition, -- Strong inhibition)

7.3.6.1. *S. aureus*

Filter sterilized broth from four of the six *C. bovis* isolates tested (122, 345, 3045 & 4143) inhibited the growth of *S. aureus* (isolate M453) in broth culture (Figure 7.12), compared to the negative control culture containing PBS. There was a lag of approximately four hours (122), six hours (4143), eight hours (3045) and ten hours (345) between the test cultures containing filter sterilized *C. bovis* broth and the control cultures containing PBS. Filter sterilized broth from two *C. bovis* isolates (4361 & 4861) had no effect on the growth rate of *S. aureus* (M453) compared to growth in the control cultures.

7.3.6.2. *E. coli*

Results were very similar to those obtained previously for *C. bovis* (isolate 122) against six *E. coli* isolates (Section 7.3.5.2.). There was a small but consistent lag between the growth curves, with growth in cultures containing all six *C. bovis* isolate

broths occurring approximately one to two hours after that in the PBS control (Figure 7.13).

7.3.6.3. S. agalactiae

Filter sterilized broth from five of the six *C. bovis* isolates strongly inhibited the growth of *S. agalactiae* (185) compared to the control culture containing PBS (Figure 7.14). No growth had occurred in filter sterilized *C. bovis* broth from isolates 122, 345 and 3045 after 48 hours incubation. There was a lag of approximately 24 hours between the growth in PBS and broth from isolates 4361 and 4861. Broth from the sixth *C. bovis* isolate tested (4143) inhibited the growth of *S. agalactiae* (185) much less strongly. Growth in the culture containing filter sterilized *C. bovis* broth occurred approximately four hours after growth in the control culture containing PBS.

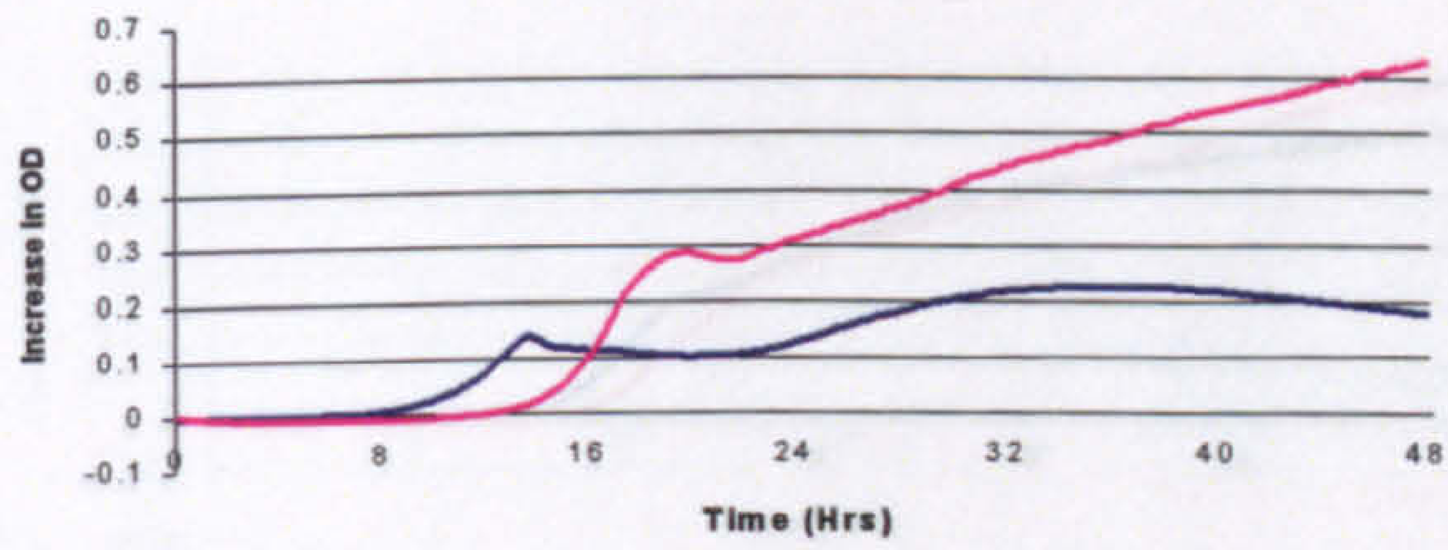
7.3.6.4. S. dysgalactiae

Filter sterilized broth from five of the six *C. bovis* isolates tested strongly inhibited the growth of *S. dysgalactiae* (3616) compared to the control culture containing PBS (Figure 7.15). No growth had occurred in the broth culture from isolates 345 and 3045 after 48 hours incubation. There was a lag ranging from approximately 24 to 32 hours between growth in the PBS control culture and that containing filter sterilized *C. bovis* broth for isolates 122 (24 hours), 4861 (28 hours) and 4361 (32 hours). The growth of *S. dysgalactiae* was not inhibited to the same extent by broth from the sixth *C. bovis* isolate (4143). Growth in the culture containing the broth from this isolate occurred approximately eight hours after growth in the control culture.

7.3.6.5. S. uberis

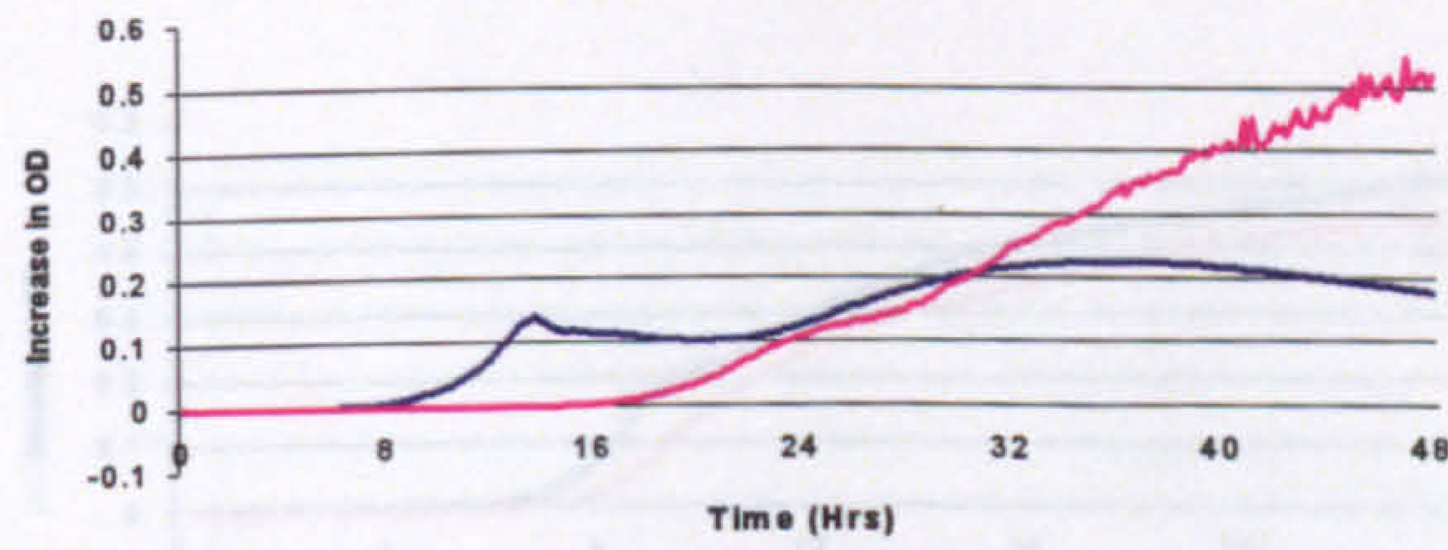
Broth from four of the six *C. bovis* isolates tested strongly inhibited the growth of *S. uberis*, isolate 4192 (345, 3045, 4361 & 4861; Figure 7.16). No growth had occurred in the filter sterilized *C. bovis* broth culture after 48 hours incubation for two of these four isolates (345 & 3045). There were lags of approximately 32 hours (4361) and 28 hours (4861) between the cultures containing filter sterilized *C. bovis* broth and those containing PBS for the other two. The remaining two isolates (122 & 4143) were much less inhibitory, producing lags of approximately eight hours in growth between test and control cultures.

Figure 7.12: Inhibitory Effects of Six Filter Sterilized *C. bovis* Broths on the Growth of a *S. aureus* (M453) Isolate



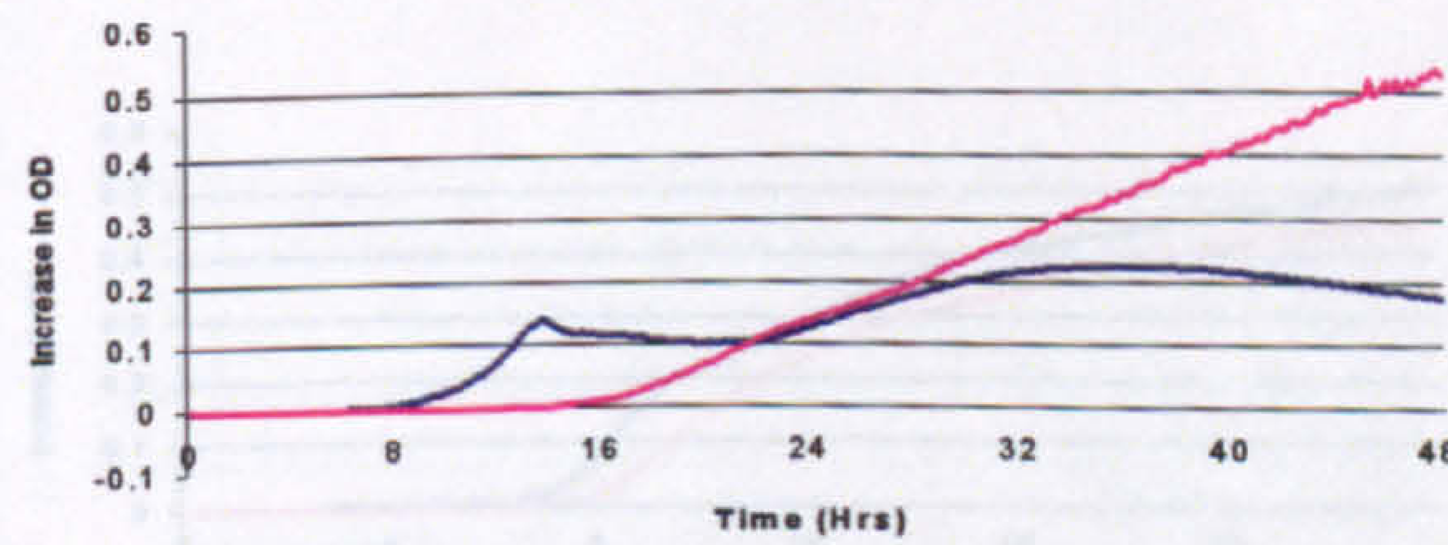
25ul FB + 225ul PBS
25ul FP + 225ul Cb (122)

C. bovis 122



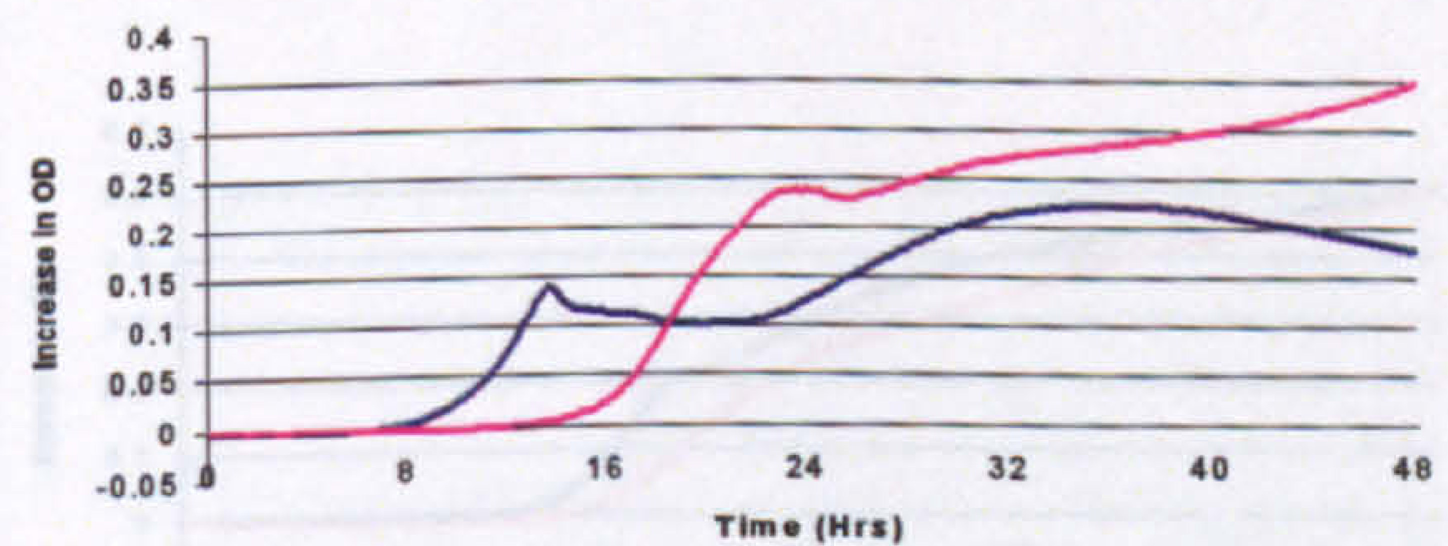
25ul FB + 225ul PBS
25ul FP + 225ul Cb (345)

C. bovis 345



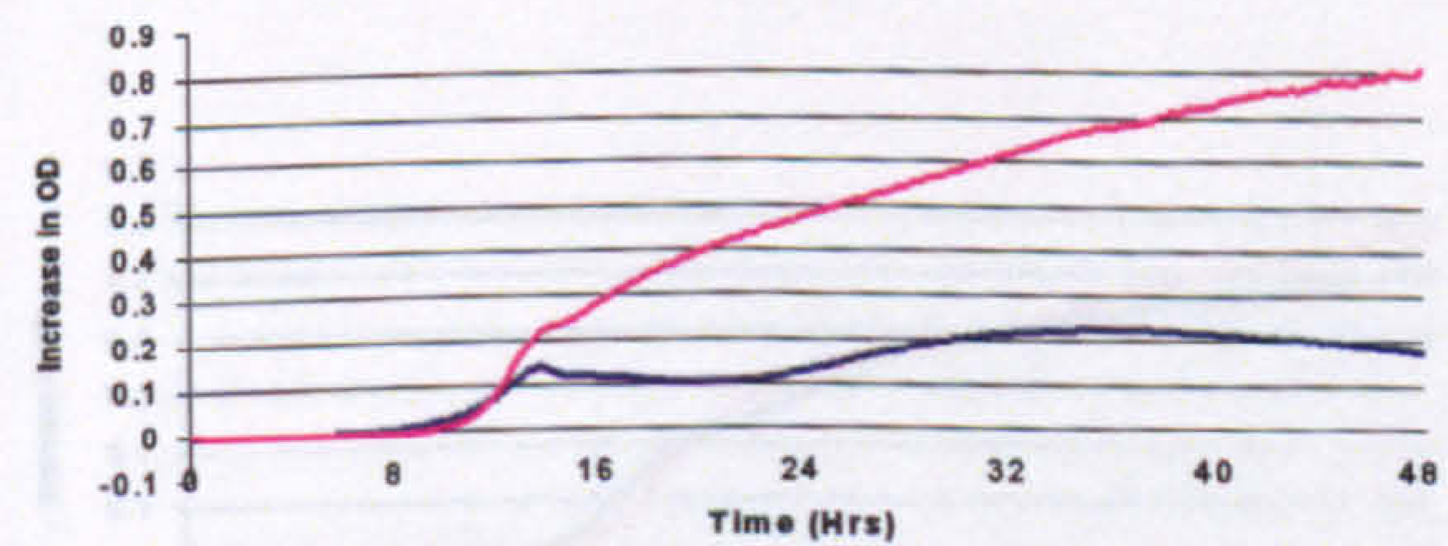
25ul FB + 225ul PBS
25ul FP + 225ul Cb (3045)

C. bovis 3045



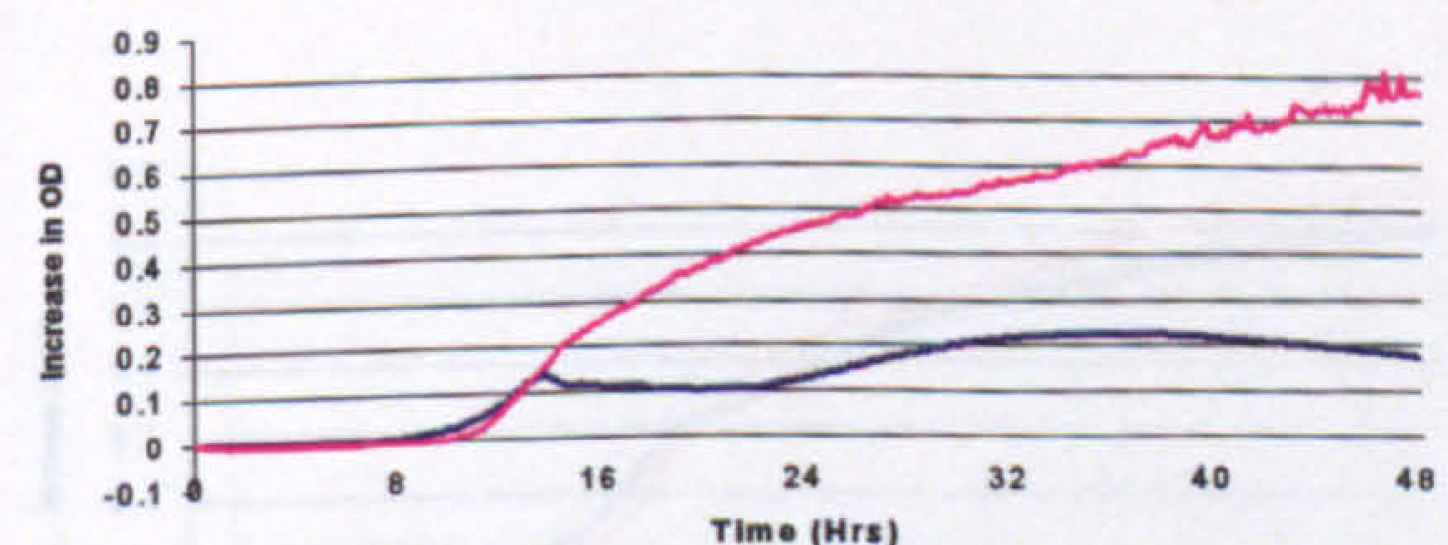
25ul FB + 225ul PBS
25ul FP + 225ul Cb (4143)

C. bovis 4143



25ul FB + 225ul PBS
25ul FP + 225ul Cb (4361)

C. bovis 4361

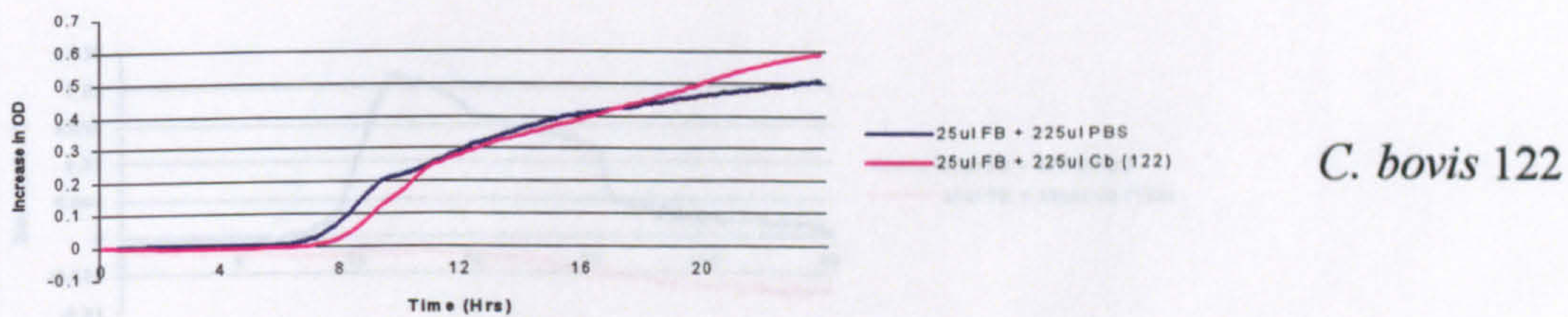


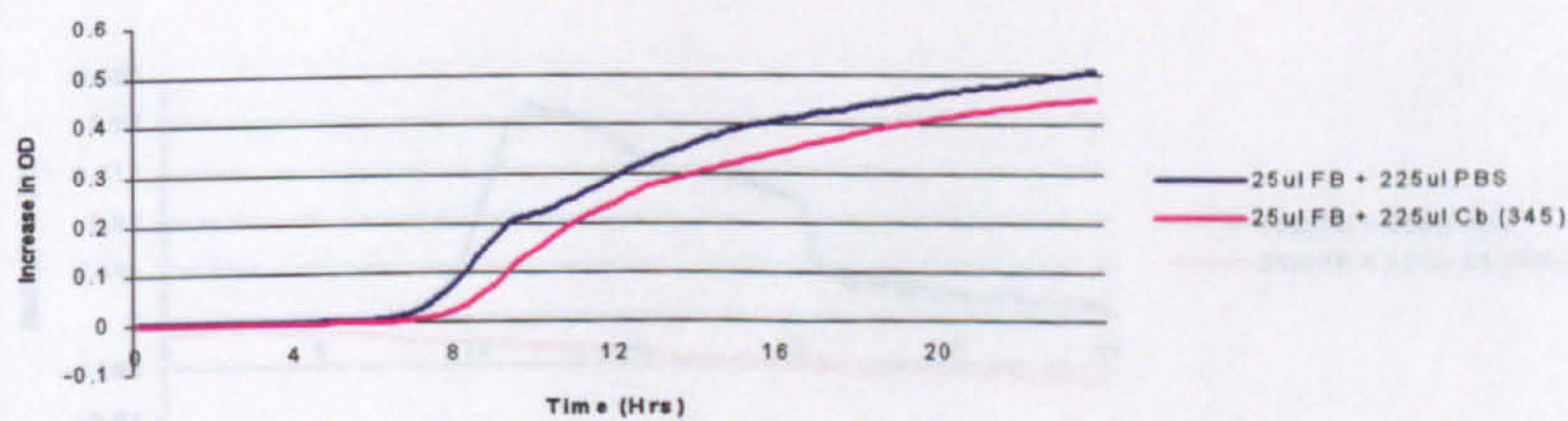
25ul FB + 225ul PBS
25ul FP + 225ul Cb (4861)

C. bovis 4861

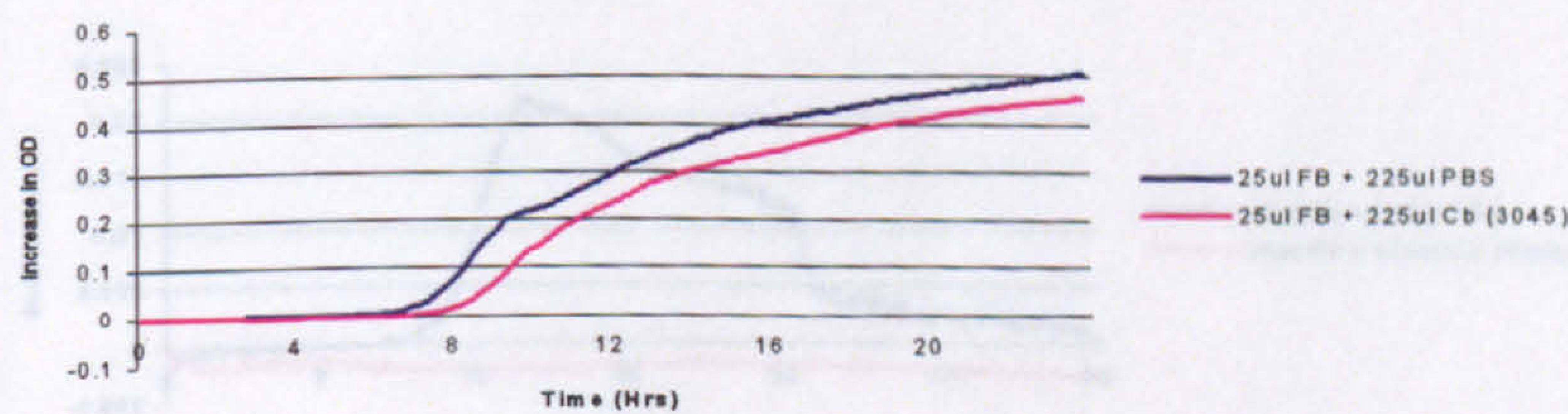
(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

Figure 7.13: Inhibitory Effects of Six Filter Sterilized *C. bovis* Broths on the Growth of an *E. coli* (1599) Isolate

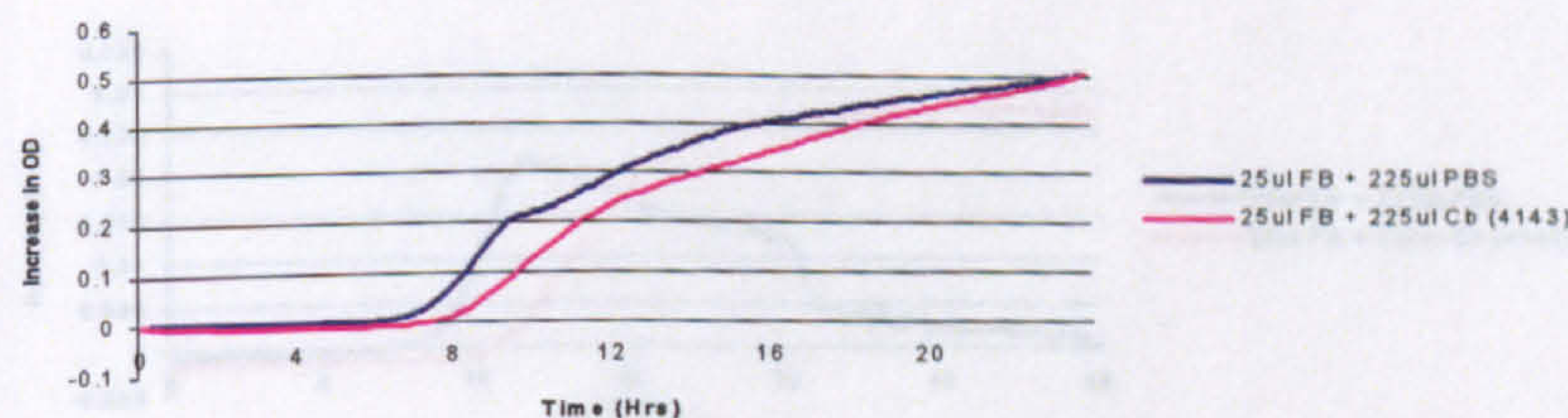




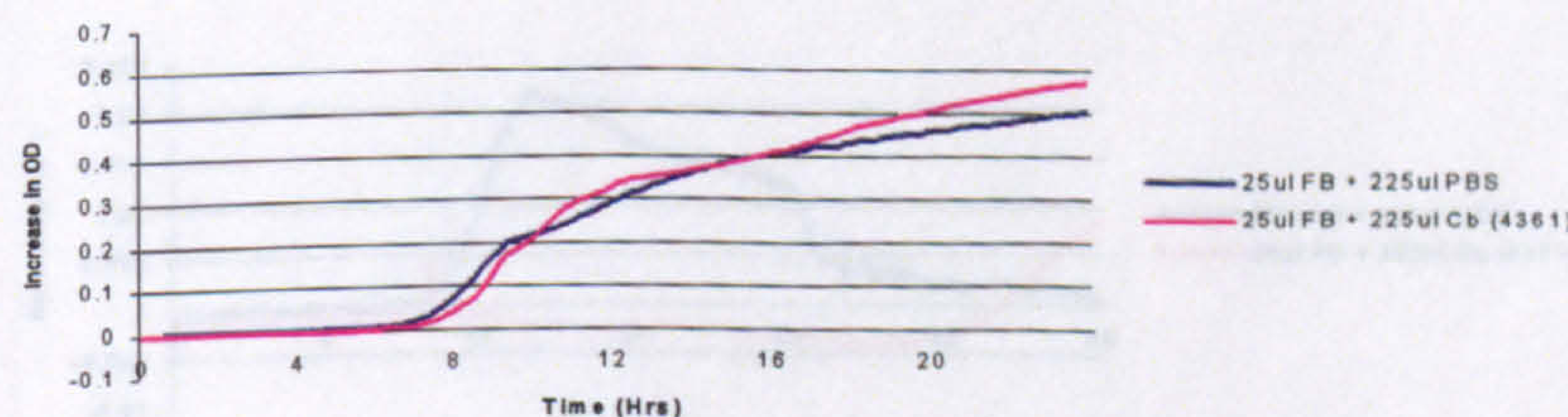
C. bovis 345



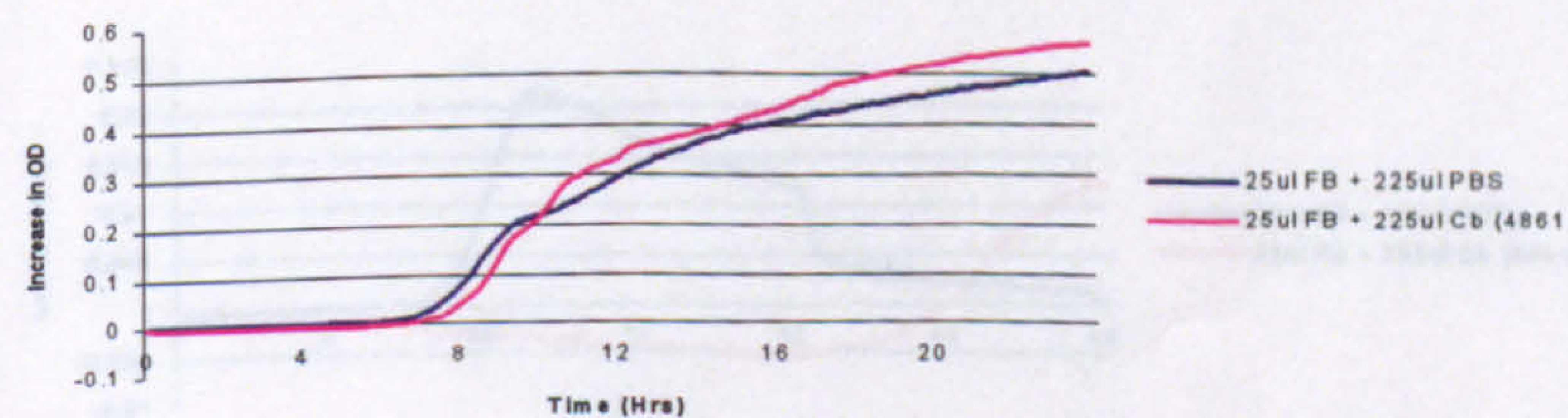
C. bovis 3045



C. bovis 4143



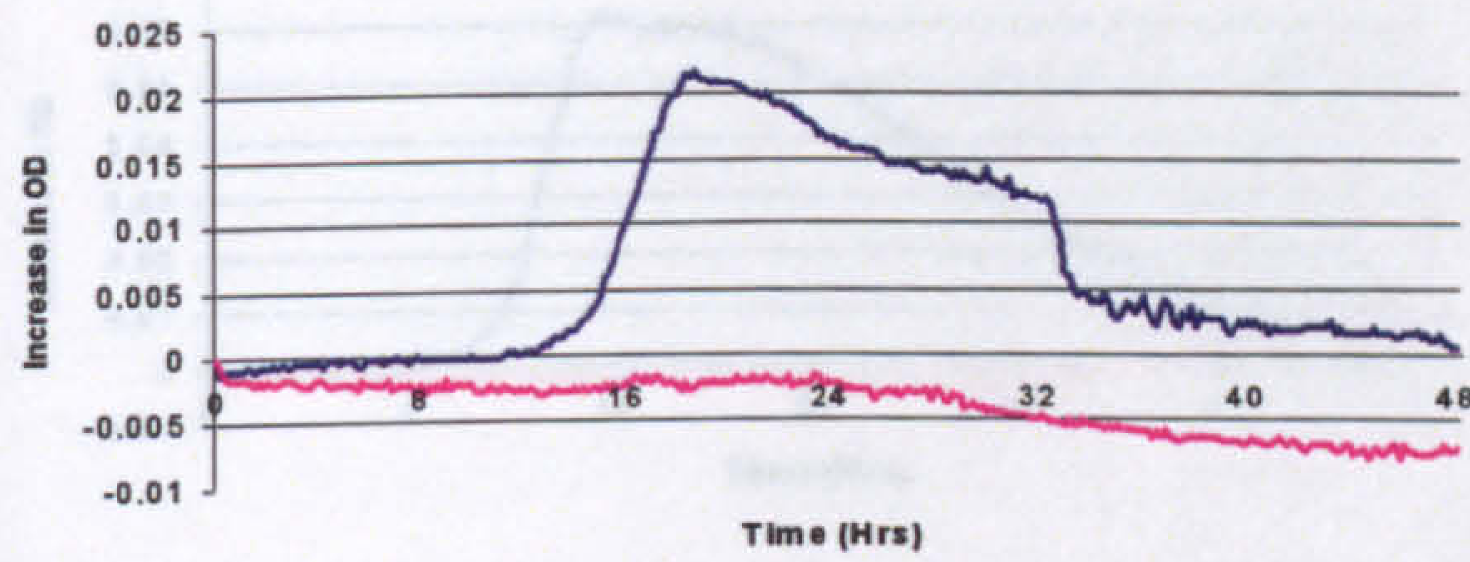
C. bovis 4361



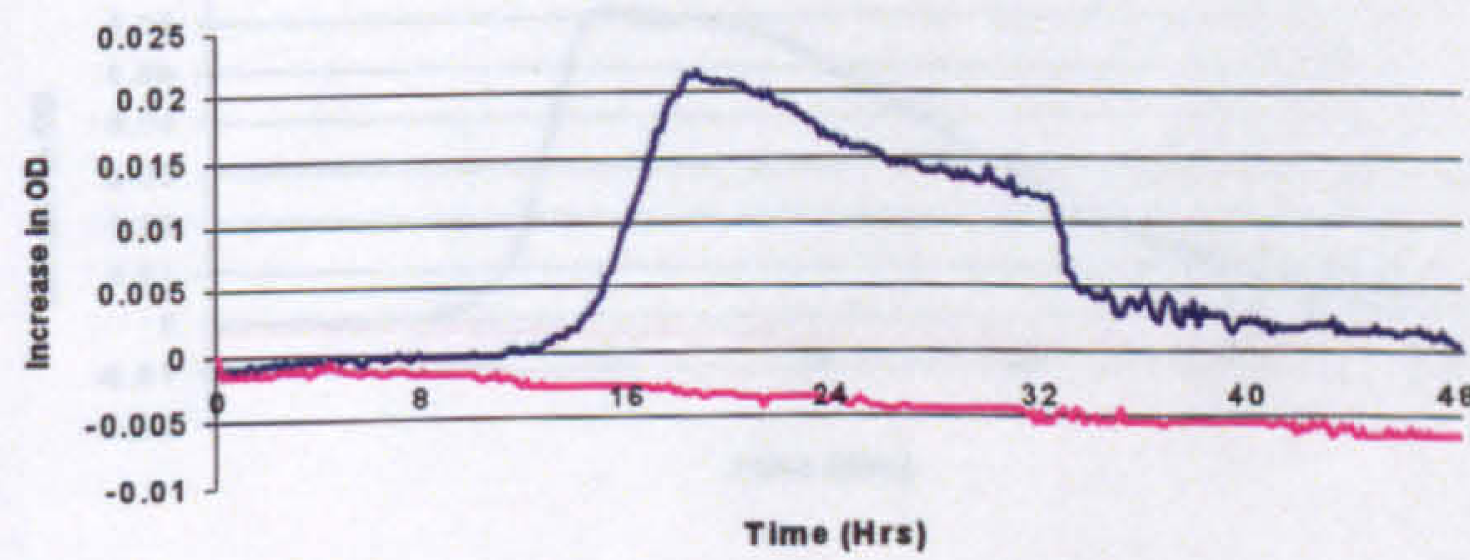
C. bovis 4861

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

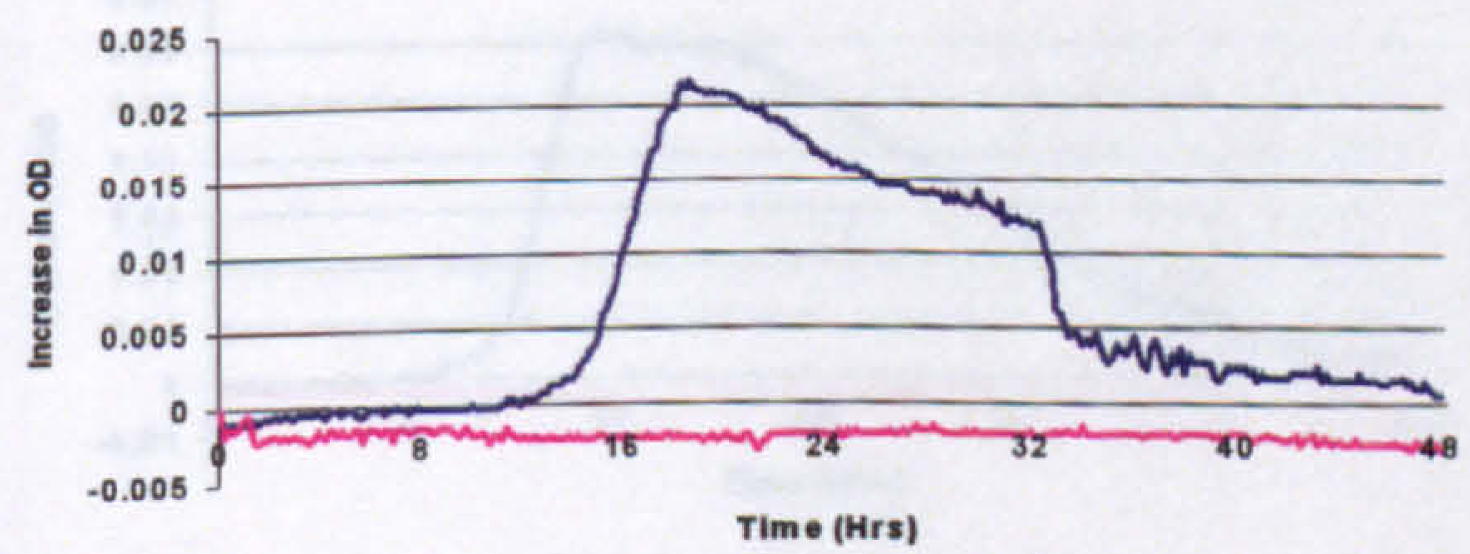
Figure 7.14: Inhibitory Effects of Six filter Sterilized *C. bovis* Broths on the Growth of a *S. agalactiae* (185) Isolate



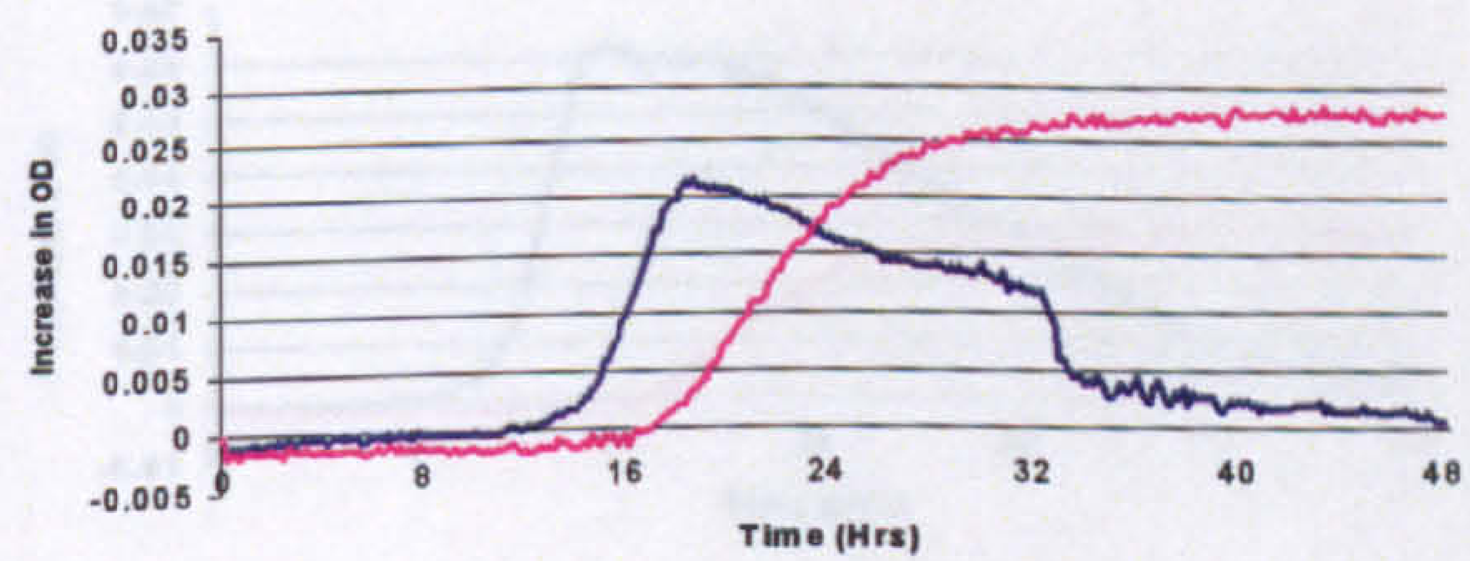
C. bovis 122



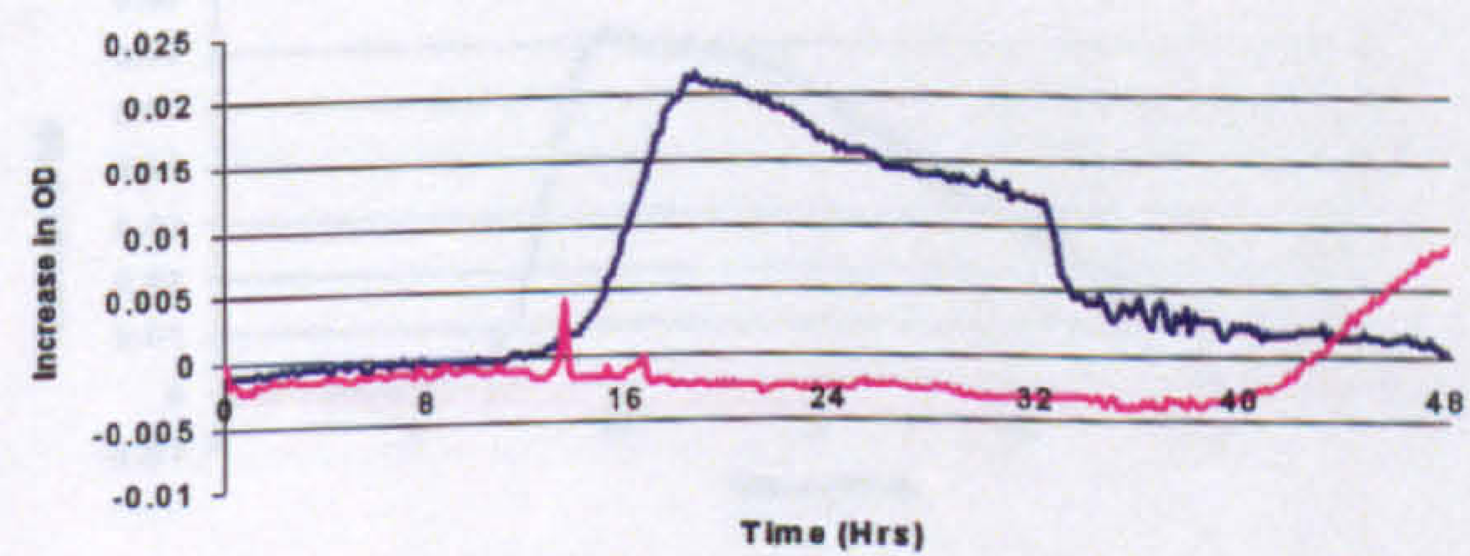
C. bovis 345



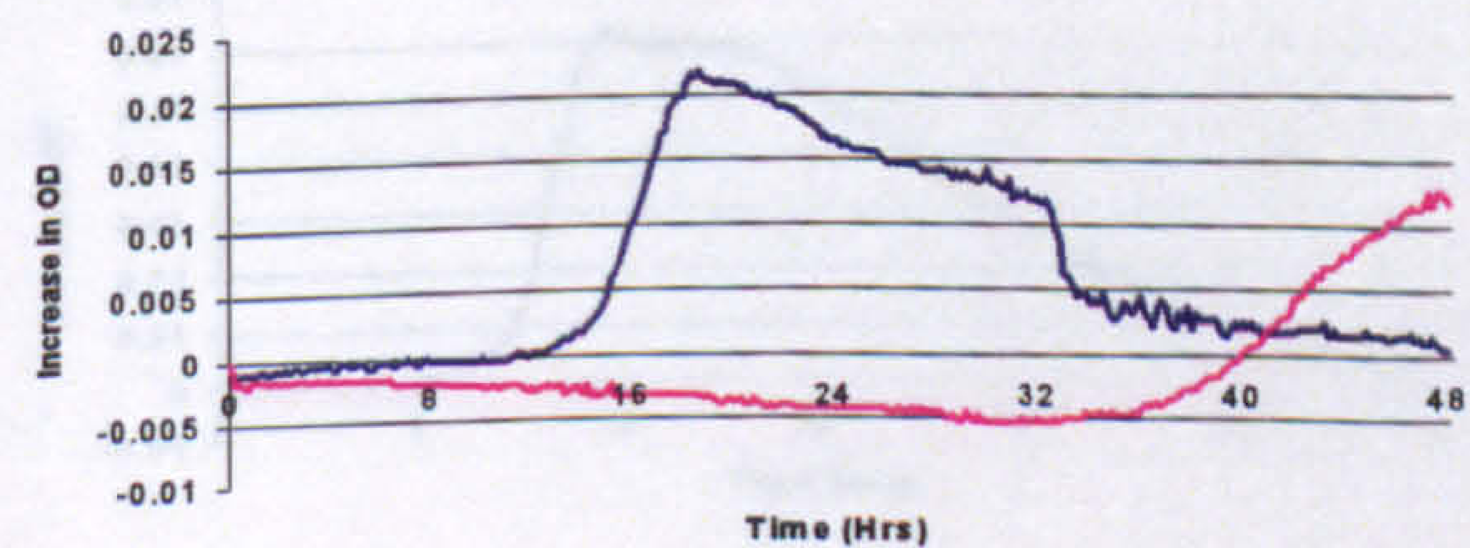
C. bovis 3045



C. bovis 4143



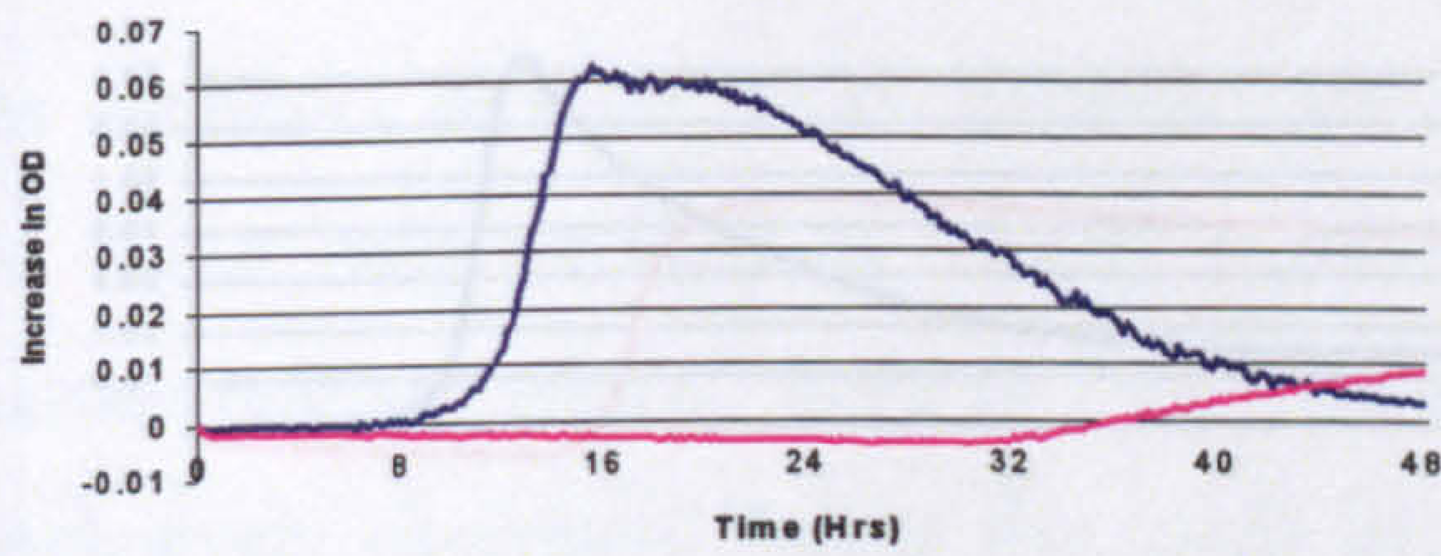
C. bovis 4361



C. bovis 4861

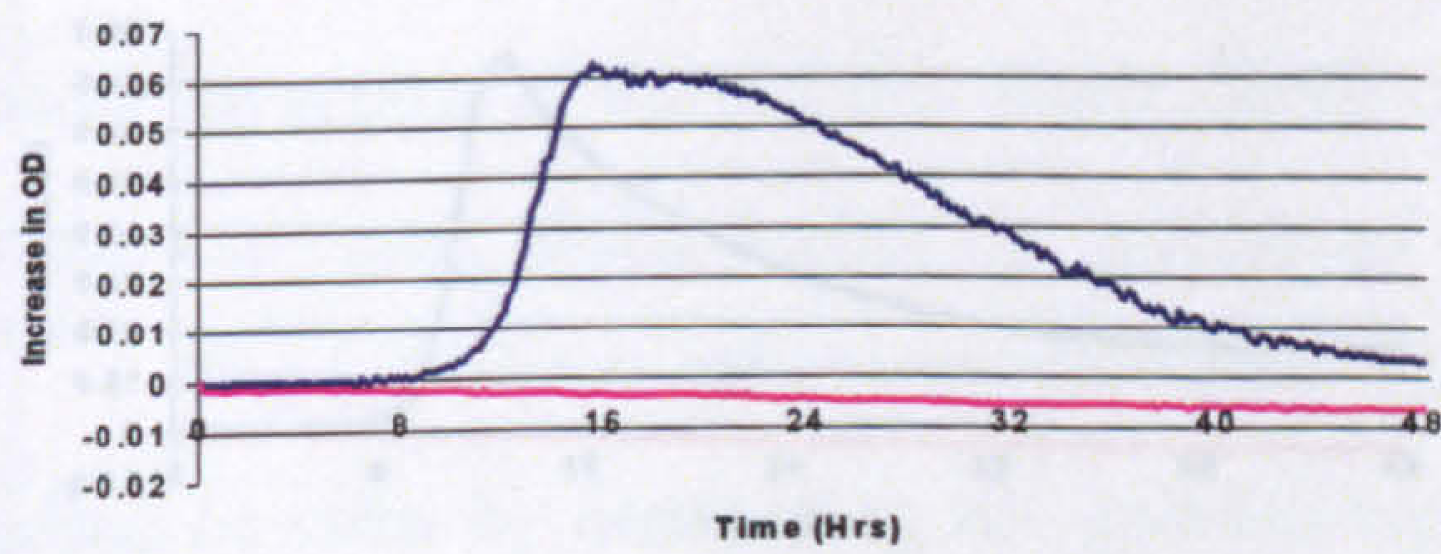
(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

Figure 7.15: Inhibitory Effects of Six Filter Sterilized *C. bovis* Broths on the Growth of a *S. dysgalactiae* (3616) Isolate



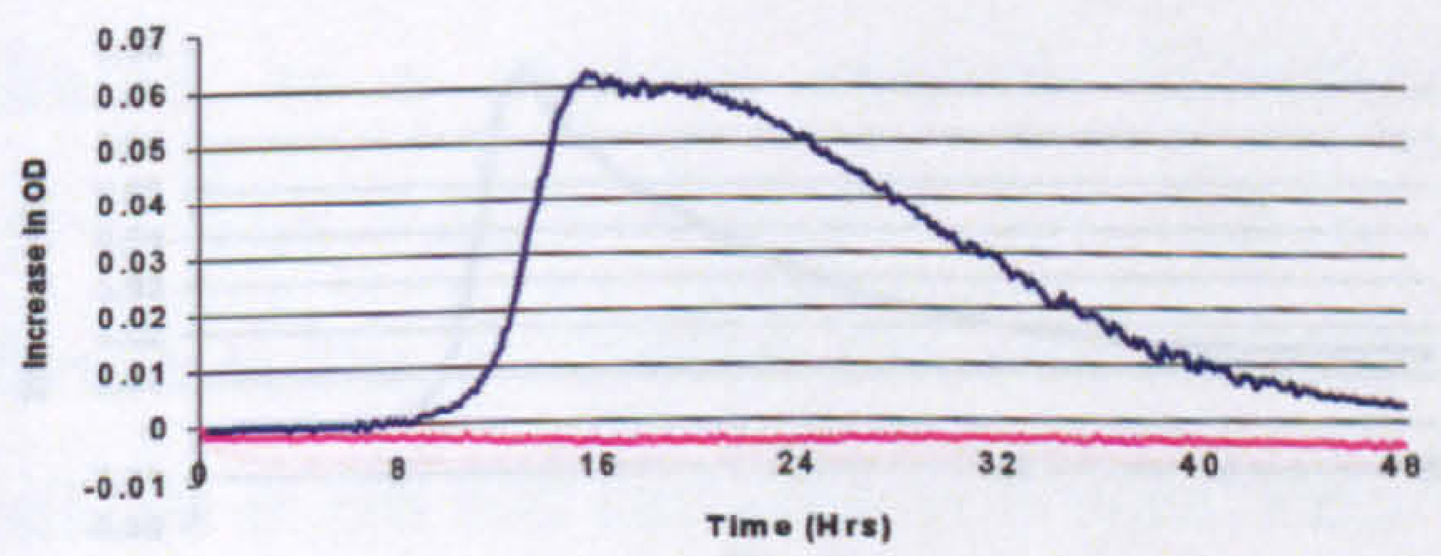
25ul FB + 225ul PBS
25ul FB + 225ul Cb (122)

C. bovis 122



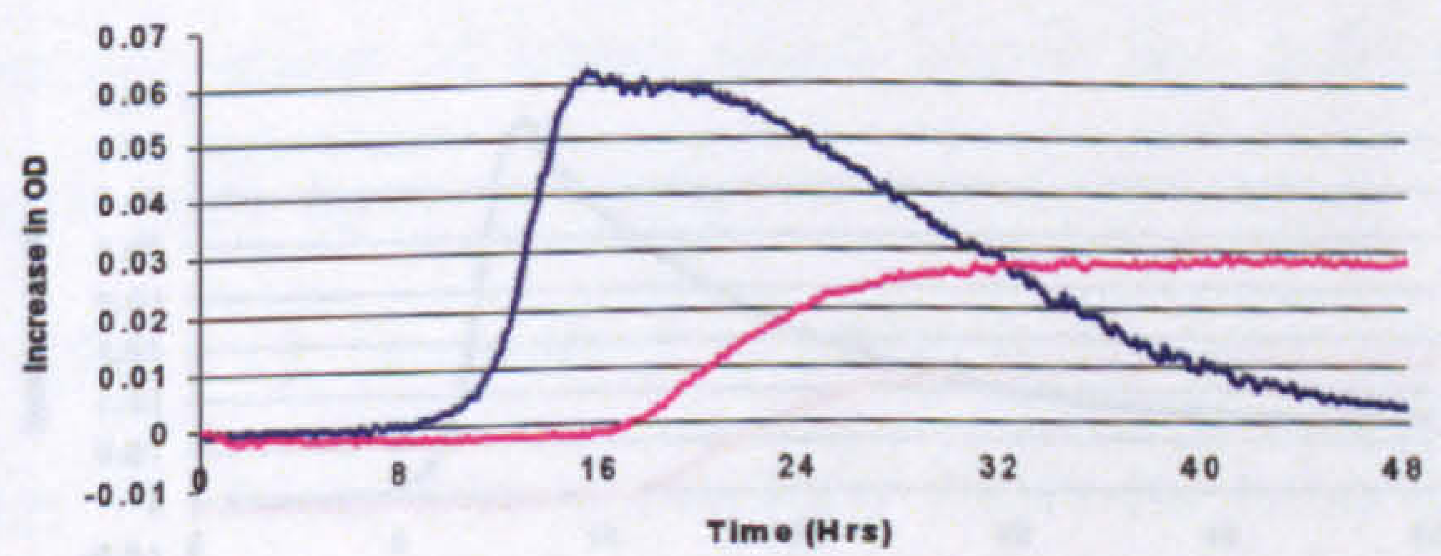
25ul FB + 225ul PBS
25ul FB + 225ul Cb (345)

C. bovis 345



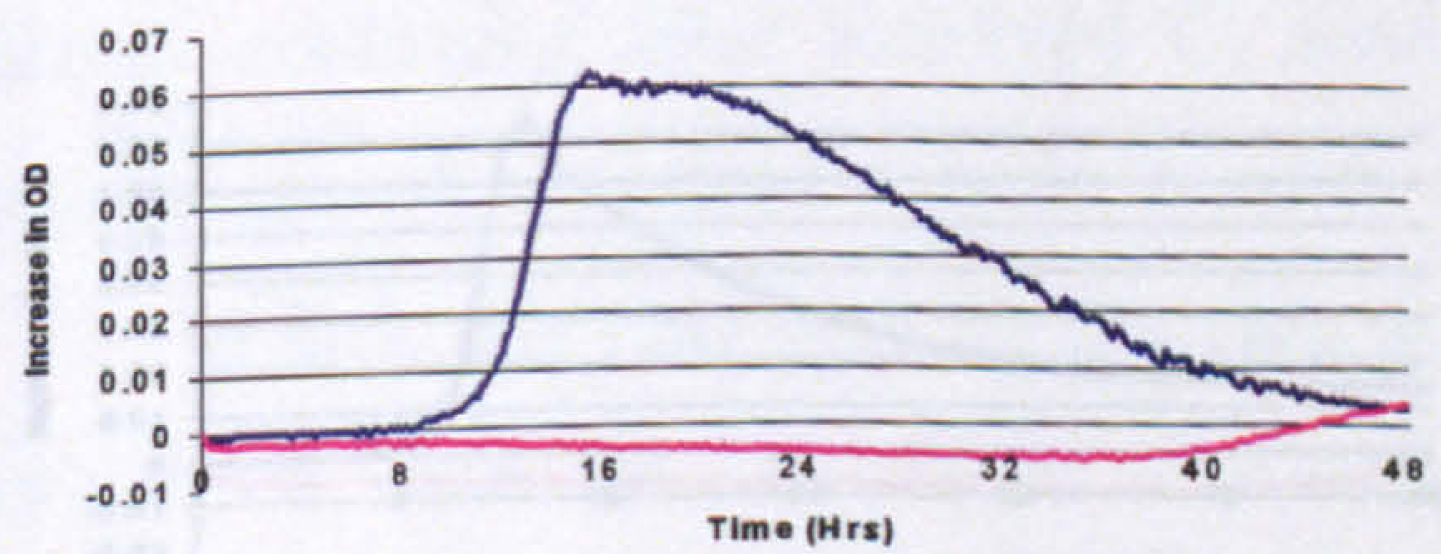
25ul FB + 225ul PBS
25ul FB + 225ul Cb (3045)

C. bovis 3045



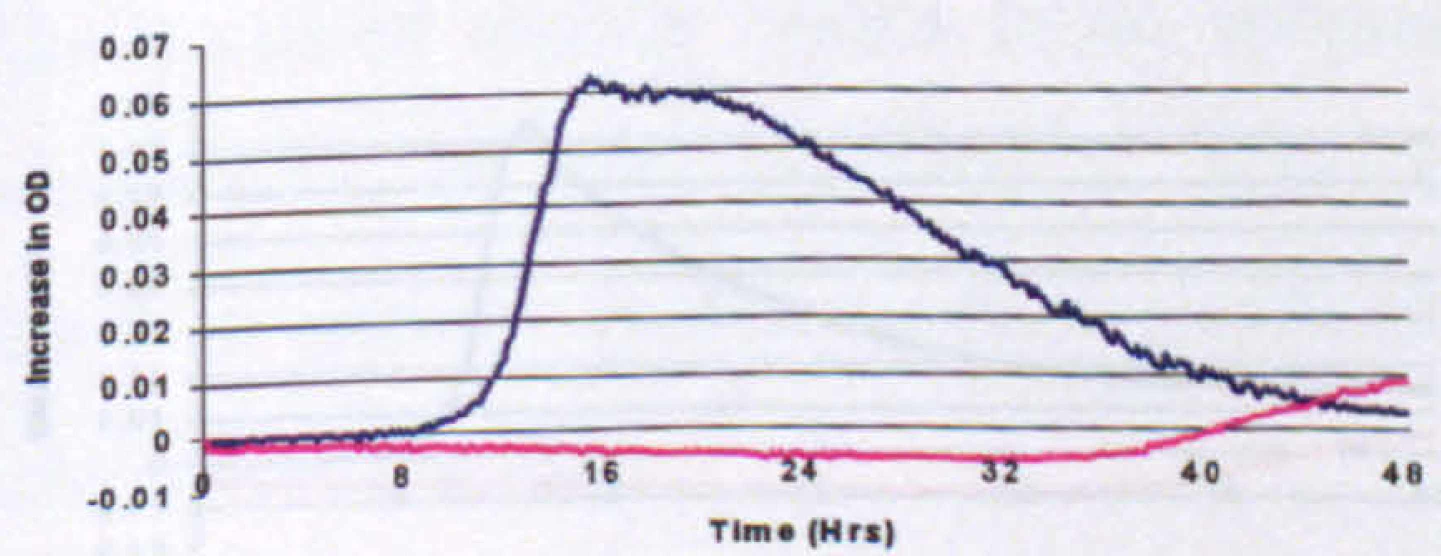
25ul FB + 225ul PBS
25ul FB + 225ul Cb (4143)

C. bovis 4143



25ul FB + 225ul PBS
25ul FB + 225ul Cb (4361)

C. bovis 4361



25ul FB + 225ul PBS
25ul FB + 225ul Cb (4861)

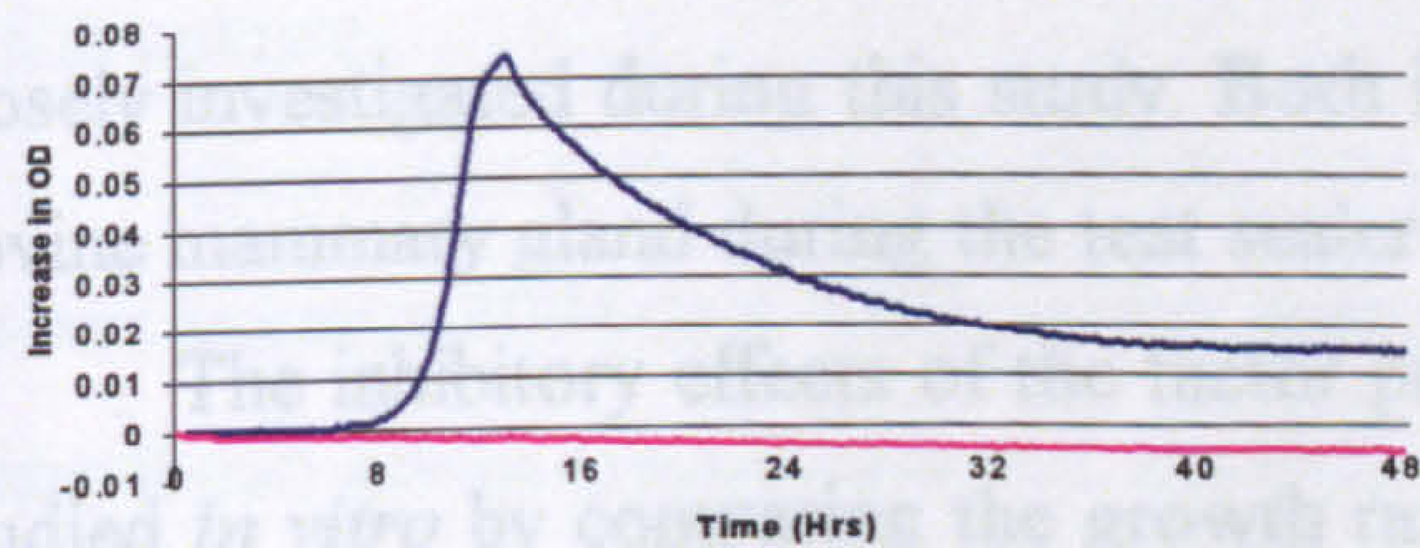
C. bovis 4861

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

Figure 7.16: Inhibitory Effects of Six Filter Sterilized *C. bovis* Broths on the Growth of a *S. uberis* (4190) Isolate

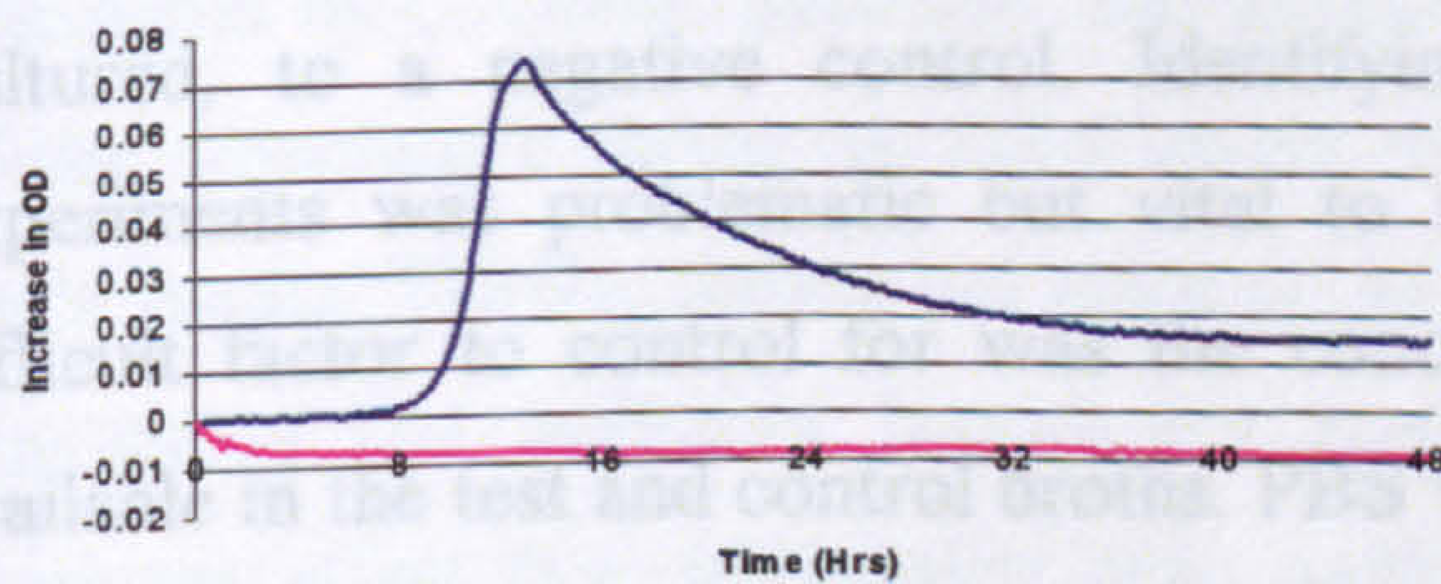
25ul FB + 225ul PBS
25ul FB + 225ul Cb (122)

C. bovis 122



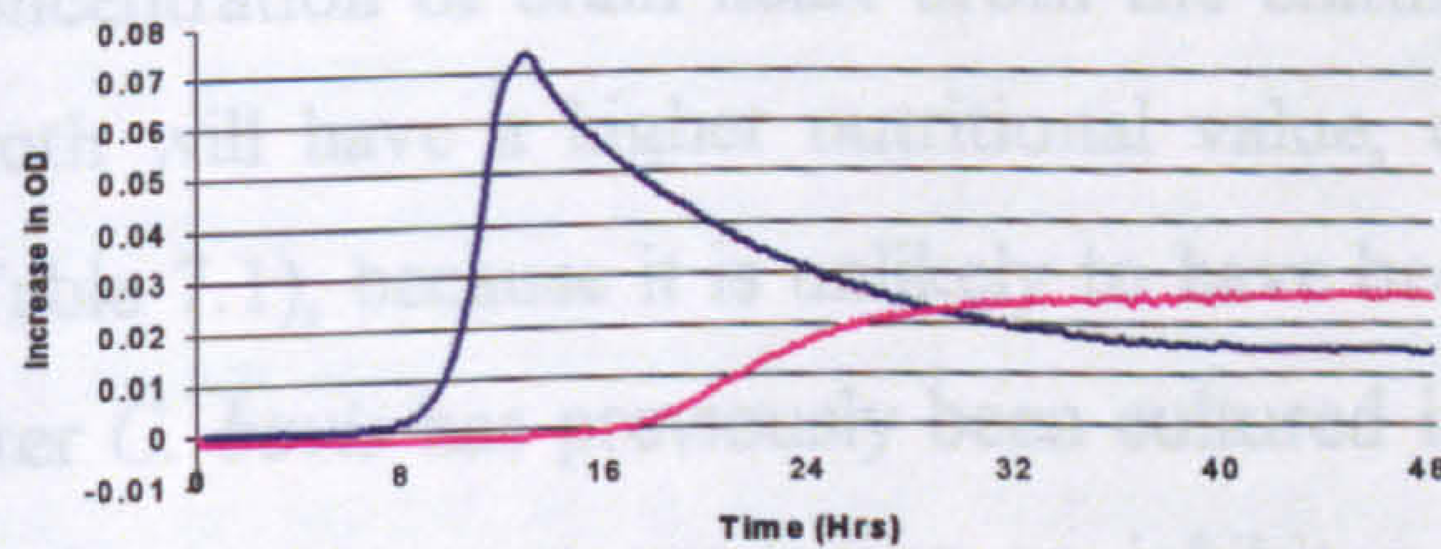
25ul FB + 225ul PBS
25ul FB + 225ul Cb (345)

C. bovis 345



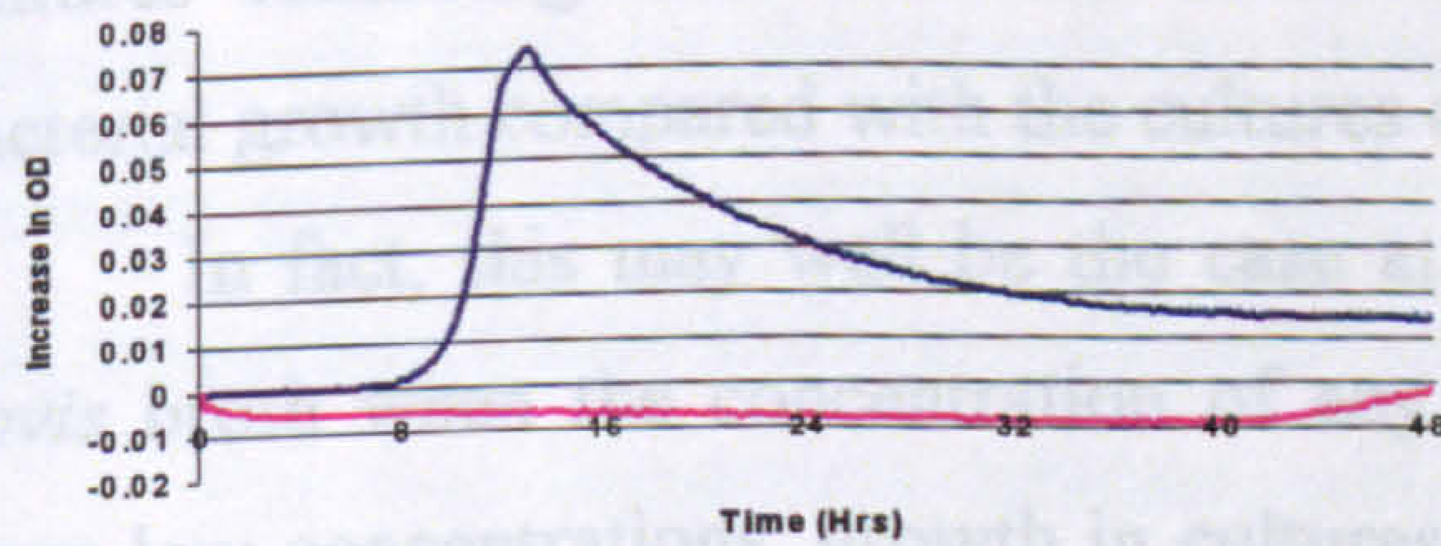
25ul FB + 225ul PBS
25ul FB + 225ul Cb (3045)

C. bovis 3045



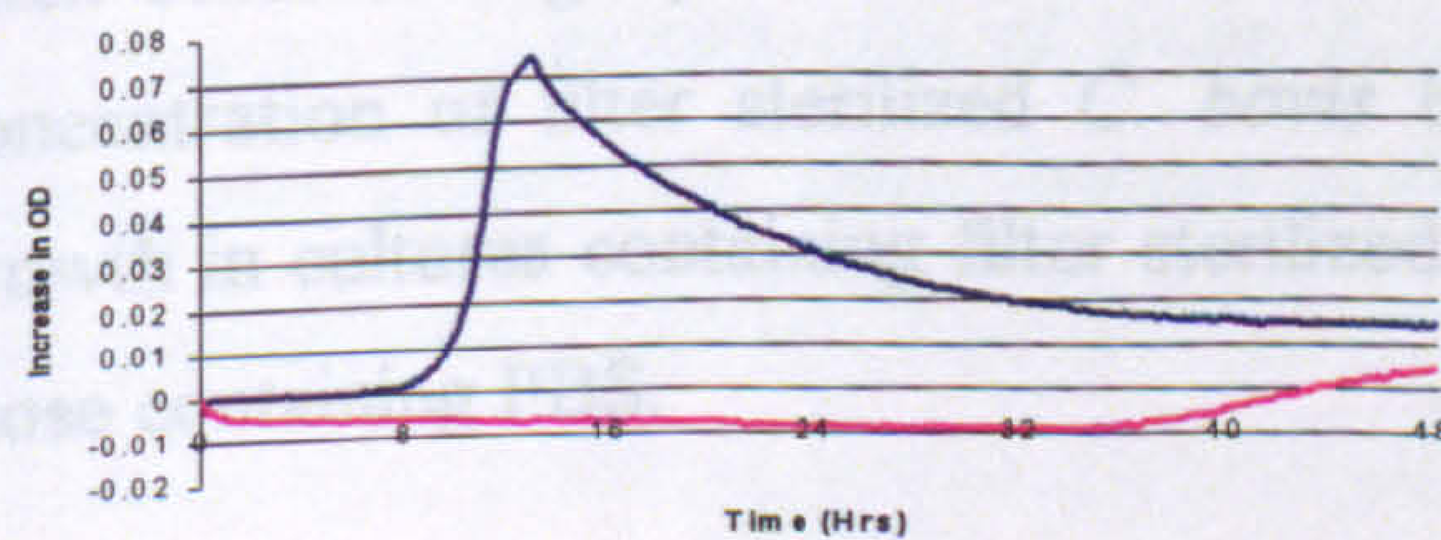
25ul FB + 225ul PBS
25ul FB + 225ul Cb (4143)

C. bovis 4143



25ul FB + 225ul PBS
25ul FB + 225ul Cb (4361)

C. bovis 4361



25ul FB + 225ul PBS
25ul FB + 225ul Cb (4861)

C. bovis 4861

(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilised *C. bovis* broth)

7.4. DISCUSSION

Some of the *C. bovis* isolates tested in this series of experiments produced a factor that was inhibitory to other major mastitis pathogens in broth culture. The production of this factor may partially or wholly explain the protective effect demonstrated for quarters infected with *C. bovis in vivo*. The nature of the inhibitory factor has not been conclusively elucidated during this study although it has been partially characterized. The inhibitory effects of *C. bovis* isolate 122 against *S. aureus* isolate 3094 were most closely investigated during this study. Both isolates were originally identified from the bovine mammary gland during the teat sealer study (Chapter 2).

The inhibitory effects of the factor produced by *C. bovis* in broth culture were studied *in vitro* by comparing the growth rates of the test pathogen (*i.e. S. aureus*) in brain heart broth plus filter sterilized broth in which *C. bovis* had previously been cultured, to a negative control. Identifying a suitable control culture for these experiments was problematic but vital to their integrity. The most important and difficult factor to control for was the concentration and total quantity of nutrients available in the test and control broths. PBS was selected as a negative control to filter sterilized *C. bovis* broth because it has no nutritional value. At any given concentration of brain heart broth the culture containing the filter sterilized *C. bovis* broth will have a higher nutritional value, compared to the culture containing PBS (Table 7.1), because it is unlikely to have been completely nutritionally depleted (even after *C. bovis* has previously been cultured in it for 48 hours). That being the case, if *C. bovis* were not producing an inhibitory product we would actually expect the cultures containing filter sterilized *C. bovis* broth to support a greater degree of bacterial growth compared with the cultures containing PBS.

In fact, this may well be the case at low concentrations of filter sterilized *C. bovis* broth when the concentration of any inhibitory factor is likely to be low. At these low concentrations, growth in cultures containing filter sterilized *C. bovis* broth often occurred slightly before those containing PBS *e.g.* Figures 7.1 & 7.2. As the concentration of filter sterilized *C. bovis* broth in the cultures increased however, growth in cultures containing filter sterilized *C. bovis* broth was delayed compared to those containing PBS.

Another potential explanation for the observations that in cultures containing low concentrations of filter sterilized *C. bovis* broth growth occurred before that in the negative control culture, is the presence of a factor in the used *C. bovis* broth that is stimulating the growth of the test pathogen. During the investigations into the affects of *C. bovis* metabolites on the growth rate of mastitis pathogens on solid media (Chapter 6), it appeared that *C. bovis* produced a factor that stimulated the growth of *S. aureus*. A stimulatory effect was much less apparent in studies involving liquid media, except when low concentration of filter sterilized *C. bovis* broth were included in the cultures.

There are a number of reasons why this may have been the case. Firstly, *S. aureus* isolates 3094 and 5527, which formed the basis of the initial work performed using liquid media (much data not shown), were selected after the solid media study, specifically because they appeared to be predominantly inhibited rather than stimulated by *C. bovis*. Secondly, the growth conditions necessary for the production of the stimulatory factor on solid media may not have been met during culture in liquid media. Thirdly, during the work on solid media it was postulated that where the stimulatory and inhibitory factors occurred together the inhibitory factor had the dominant effect (*i.e.* stimulation only occurred when the inhibitory factor was not present *e.g.* at the edges of the *C. bovis* band). In liquid media it may not be possible for the stimulatory factor to "escape" the effects of the inhibitory factor, therefore the predominant effect seen is inhibition. This does not explain stimulation in cultures containing low concentrations of filter sterilized *C. bovis* broth unless the stimulatory factor is active at a lower concentration than the inhibitory factor. Lastly, the inhibitory and stimulatory factors may be produced at different times during the *C. bovis* growth phase *i.e.* the stimulatory factor may be produced early (possibly to self promote growth whilst nutrients are freely available) and the inhibitory factor may only be produced later in the growth phase or in the plateau phase of growth.

The time constraints inherent within a thesis such as this necessitated a decision to concentrate on the inhibitory effects of *C. bovis*. The stimulatory effect of *C. bovis* on the growth rate of some *S. aureus* isolates has not been extensively investigated during this study. Further work is needed to explore this interesting *in vitro* interaction.

The length of time *C. bovis* was incubated for prior to filter sterilization affected the degree of inhibition produced. Maximum inhibition of *S. aureus* isolate 3094 was produced if *C. bovis* cultures were filter sterilized after 36 to 48 hours incubation at 37°C in an orbital shaker. Filter sterilized broths incubated for longer or shorter times than this demonstrated less inhibitory effects.

A high concentration of the inhibitory factor after defined periods of culture (36 - 48 hours) implies that it is being actively produced during a distinct phase of the growth cycle. If the inhibitory factor were a waste product, it would be expected to accumulate over time. If it were an internal cell component, concentration would be expected to increase following cell death in cultures incubated for extended periods of time. Neither of these scenarios appeared to be the case; in fact the concentration or activity of the inhibitory factor declined from its peak after around 36 to 48 hours incubation. The reduction in concentration of inhibitory factor following extended periods of incubation (greater than 48 hours) implies it is either unstable and breaks down over time or is in some way neutralized, metabolized or utilized by bacteria present in the culture.

Heat-treating filter sterilized *C. bovis* broth to 60 and 80°C for 30 minutes had no effect on the degree of inhibition it produced against *S. aureus*. However heating to 100°C for a similar length of time substantially decreased the level of inhibition demonstrated. This data would suggest that the inhibitory factor produced by *C. bovis* is resistant to temperatures of 80°C but partially inactivated by temperatures of 100°C. Alternatively heating to 100°C could make nutritional factors more freely available to *S. aureus* thus encouraging growth. This would seem unlikely (since the broth was originally heated to temperatures in excess of 130°C during sterilization prior to being used for *C. bovis* culture), unless heating was liberating nutritional factors added or altered by *C. bovis* during culture.

Treating filter sterilized *C. bovis* broth with Proteinase K almost entirely eliminated its inhibitory effects against *S. aureus*. Proteinase K is a broad-spectrum protease that cleaves peptide bonds at the carboxylic sides of aliphatic and aromatic amino acids (Ebeling *et al.* 1974). Proteinase K is rapidly inactivated by temperatures above 65°C (Anon 2002). Removal of the inhibitory effect implies that the factor produced by *C. bovis* is proteinacious in nature or has a protein component essential to its activity *e.g.* a glycoprotein.

Bacteriocins are proteinacious in nature and the majority are small, heat stable cationic peptides (Jack *et al.* 1995). Bacteriocins produced by *Corynebacterium* species have previously been shown to be resistant to temperatures of 100°C (Karabekov *et al.* 1984; Patek *et al.* 1986) and 80°C (Gross and Vidaver 1978), although temperatures between 80 and 100°C progressively inactivated a bacteriocin produced by *C. ulcerans.* Proteinase K has previously been used to investigate the protein nature of bacteriocins (Ryan *et al.* 1996); bacteriocins produced by members of the *Corynebacterium* genus have been shown to be sensitive to its effects (Gross and Vidaver 1978). The duration of initial incubation (of the bacteriocin producing strain) has been shown to effect the level of bacteriocin production; production was maximal between 24 and 48 hours incubation. Levels of bacteriocin activity declined if cultures were incubated for prolonged periods (Dajani and Wannamaker 1969).

This is the first time that *C. bovis* has been shown to produce an inhibitory factor active against major mastitis pathogens. The inhibitory factor appeared to inhibit a range of common mastitis pathogens. Three of six *S. aureus* and *S. uberis* and six of six *S. agalactiae* and *S. dysgalactiae* isolates were markedly inhibited. Variations in the susceptibility of members of the same species to bacteriocins have been demonstrated previously (Abrehem and Zamiri 1983).

Similarly a range of *C. bovis* isolates appeared to inhibit a representative isolate of a range of mastitis pathogens. Five, five, four and four of six *C. bovis* isolates inhibited the growth of *S. agalactiae, S. dysgalactiae, S. aureus* and *S. uberis* respectively. Inhibition of *E. coli* was much less marked during both series of experiments. These results suggest that the quantity or activity of the inhibitory factor produced varies between different strains of *C. bovis* and that the susceptibility of mastitis pathogens within the same species to the inhibitory factor varies. The level of bacteriocin production varies between members of the same species (Nascimento *et al.* 2002); some isolates do not produce them, only one of two *C. bovis* strains investigated for the production of a bacteriocin active against *Listeria monocytogenes* demonstrated inhibition (Carnio *et al.* 1999).

Bacteriocins produced by Gram-positive bacteria tend to be active against a wide range of Gram-positive bacteria (Jack *et al.* 1995). Although members of only two Gram-positive genera were investigated (*Staphylococcus* and *Streptococcus*)

during these experiments, this would appear to be the case for the inhibitory factor identified here.

Inhibitory activity against *E. coli* was consistently much less marked throughout the course of this study. The inhibitory factor identified here may have limited activity against *E. coli*.

Alternatively, the apparent inhibition of *E. coli* may be a function of the rate of bacterial growth in the test and negative control cultures. In the range of control cultures (fresh broth plus PBS) used, as the proportion of PBS in the culture increased, *E. coli* growth began marginally earlier *i.e.* at higher concentration of PBS the growth curves were shifted slightly to the left (Figure AV.I, graph a., page 212). Growth of *E. coli* therefore begins earlier in dilute broth (possibly because the culture conditions are less hypertonic). The converse was true for broths containing filter sterilized *C. bovis* broth. As the proportion of filter sterilized *C. bovis* broth increases the growth curves are shifted slightly to the right (Figure AV.I, graph b, page 212). The result of these two effects is that at low concentration of fresh broth, growth of *E. coli* in the control culture containing PBS occurs earlier than in other control broths, whereas in the test culture containing filter sterilized *C. bovis* broth, growth occurs slightly later than other test cultures. The net result is an apparent lag between the growth curves in test and control cultures (at low concentrations of fresh broth), which may not be due to the production of an inhibitory factor active against *E. coli*.

This same small difference between growth in test and control cultures (at low concentrations of fresh broth) may also be present for some of the other mastitis pathogens investigated during this study. It does not, however, explain the much larger time differences between growth in the test and control cultures that were seen for these organisms. A very small part of the difference may be caused by the effect described here, but most must be caused by other reasons *i.e.* the production of an inhibitory factor.

Repeatability of broth inhibitory characteristics was difficult to achieve during the course of these experiments. Variations in culture conditions can profoundly affect the yield of bacteriocins; for many strains ill-defined factors appear to influence the level of production (Tagg *et al.* 1976). Despite employing seemingly identical incubation conditions throughout, the degree of inhibition produced by some batches of filter

sterilized *C. bovis* broth varied. Examples of this can be seen by comparing the inhibitory effects of two batches of filter sterilized *C. bovis* broth (isolate 122) on *S. uberis* (isolate 4190) (Figures 7.11 & 7.16) and *S. dysgalactiae* (isolate 3616) (Figures 7.10 & 7.15) prepared on separate occasions. This phenomenon may explain some of the apparent variations in level of inhibition between strains of *C. bovis* and mastitis pathogens described above *i.e.* some of this variation may be caused by inter-assay variation. That is certainly not to say that the inhibition itself is not genuine, merely that some of the apparent variation in inhibitory effect may be due to differences in the level of production of the inhibitory factor (in any given batch) and not to real variations in the ability of any given strain of *C. bovis* to produce the factor. Further work is necessary to characterize and purify the factor from broth culture, which would allow the relative level of production by different *C. bovis* isolates and within different cultural batches to be established.

The results described here are not in agreement with those of Hogan *et al* (1987), which failed to demonstrate that *C. bovis* metabolites could inhibit the growth of *S. aureus* and *S. agalactiae* in broth culture. The highest concentration of filter sterilized *C. bovis* broth in test cultures was four percent during this previous work. It was demonstrated during the present study that concentrations approaching 50 percent filter sterilized *C. bovis* broth were necessary before inhibitory effects became apparent. Previous authors may have found inhibitory effects if the concentration of filter sterilized *C. bovis* broth in test cultures had been increased.

Filter sterilization of bacteriocins may result in a loss of activity in the filtrate because of protein binding to the filter membrane (Tagg *et al.* 1976). The syringe driven filters employed during these experiments are designed to minimize protein binding and were chosen accordingly to minimize losses for this reason.

During the course of these experiments a factor produced by *C. bovis* has been identified which inhibits the growth of *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* in broth culture. The level of production of the factor is dependant on the length of time *C. bovis* is cultured for; production is maximal between 36 and 48 hours incubation, after which time concentration of the factor declines. The factor is resistant to heat treatment at 80°C but partially inactivated by heating to 100°C. It is

almost completely inactivated by treatment with the broad-spectrum protease, Proteinase K. All of these finding are consistent with describing the factor as a bacteriocin. However, inactivation of effects by proteolytic enzymes is not considered adequate to predict protein nature (Jack *et al.* 1995). Further work is necessary to identify and then isolate the inhibitory factor before an accurate assessment of its characteristics can be made. Until then it is not possible to definitively describe the inhibitory factor as a bacteriocin.

Whatever the exact nature of the inhibitory factor its potential biological significance *in vivo* is even more difficult to assess based on currently available data. Other authors have previously concluded that the protective effect afforded by *C. bovis* infected quarters is at least partially independent of the elevation in SCC (Lam *et al.* 1997; Schukken *et al.* 1999). Production of the inhibitory factor identified here could form part of the mechanism by which quarters infected with *C. bovis* are less likely to become infected with other more pathogenic organisms. Further work is needed firstly as previously stated to characterize the inhibitory factor and secondly to try and assess its importance *in vivo*. How this could be achieved will depend upon the nature of any factor isolated, but identifying its presence in either the milk or teat canal contents of *C. bovis* infected quarters would provide strong circumstantial evidence of its potential importance.

If production of the factor does prove to be biologically significant, it is possible that the importance of the two separate mechanisms may vary depending on the invading pathogen *i.e.* for one pathogen the protective effect is mediated via the elevation in SCC where as for another it is the production of the inhibitory factor. The inhibitory factor identified here is most active against Gram-positive organisms. *In vivo* the elevation in SCC may be critical in protecting the quarter against IMI with Gram-negative organisms where as the production of an inhibitory factor is protective against Gram-positive organisms. Perhaps more likely is that both methods are responsible but their relative importance and the degree of protection afforded varies depending on the pathogen. It is of course also possible that other as yet unidentified mechanisms, may also play important roles.

The inhibitory effects demonstrated may be due to more than one inhibitory product *i.e. C. bovis* may be producing multiple active factors. Some *Corynebacterium* species produce multiple bacteriocins with differing spectrums of

activity (Gross and Vidaver 1978). Therefore, the inhibitory effects demonstrated against different species may not be mediated via the same factor. If this is the case susceptibility of these factors to heat, Proteinase K and other agents may vary.

The effects demonstrated here could be purely an *in vitro* effect with no relevance to the situation in the field, either because the factor is not produced because of the growth conditions present within the udder or is not present in biologically significant concentrations because of the large volume of distribution within the mammary gland. Alternatively, the factor may be produced at much higher levels *in vivo* because conditions necessary for its production are met.

It is interesting to note that *C. bovis* IMI predominates within the teat canal. The teat canal is a "closed" environment with a very small volume, which may facilitate the local accumulation of significant concentrations of an inhibitory factor. The factor would only get diluted / removed two or three times daily during the milking process. However, the milking process is also the time of greatest risk of IMI from Gram-positive (contagious) organisms (the organisms that the inhibitory factor was most active against *in vitro*). The concentration of an inhibitory factor would be lowest at this time, unless the factor was strongly "bound" *in situ,* and thus able to resist the flushing action of milk ejection. Further work is necessary to investigate this potentially very interesting and biologically important interaction.

CHAPTER 8: DISCUSSION.

8.1. THESIS REVIEW

The first parts of this thesis describe the first positive control study to demonstrate the efficacy of an internal bismuth subnitrate teat sealer in protecting quarters against new dry period IMI's caused by major mastitis pathogens, particularly environmental organisms, under UK field conditions. Whilst undoubtedly some (or most) of the efficacy of the teat sealer is based on its ability to seal the teat and prevent the ingress of pathogens during the dry period it was interesting to note that the *C. bovis* cure rate was significantly lower in the group which received the teat sealer, compared to cows that received antibiotic DCT.

Quarters infected with *C. bovis* have previously been shown to be protected from subsequent infection with other more pathogenic organisms. However characterization of *C. bovis* in many of these papers has been poor. In this thesis lipophilic and non-lipophilic coryneform species were differentiated based on their differential growth on brain heart agar with and without the presence of Tween 80 (a detergent which contains the fatty acids that fulfill the specific growth requirements of lipophilic species). *C. bovis* was then differentiated from other lipophilic coryneform species using a novel speciation system based on endonuclease restriction analysis of the 16S rRNA gene sequence. The method successfully differentiated 781 *C. bovis* isolates from 23 isolates that represented other lipophilic species.

Of these 23 isolates, 20 appeared to represent the same species and were later identified as a previously unrecognized species by sequencing the 16S rRNA gene. The new species was most closely related to *C. auriscanis* an organism first isolated from dogs with otitis. The novel species has been temporarily named "*C. langfordii*" whilst final characterization is completed.

Reanalysis of the database produced during the teat sealer study demonstrated that quarters that retained a *C. bovis* infection during the dry period were significantly less likely to acquire a major pathogen infection during the dry period. This is the first study to demonstrate that *C. bovis* can protect quarters during the dry period, previous work has only demonstrated protection during lactation. Significantly fewer *C. bovis*

IMI were cured during the dry period in the cows that received the teat sealer compared to those that received antibiotic DCT. The lower *C. bovis* cure rate in animals that received the teat sealer may be partially responsible for the efficacy of the teat sealer product described in Chapter 2.

Many authors have suggested that the protective effect afforded by *C. bovis* is mediated by a small rise in the SCC of infected quarters, although recently other authors have concluded that the protective effect is at least partially independent of this SCC elevation. In the final parts of this thesis the hypothesis that *C. bovis* produces a factor that inhibits the growth of other mastitis pathogens was investigated on solid and in liquid media. On solid media an area in which *C. bovis* had previously been cultured inhibited the growth of some strains of *S. aureus* (although others appeared to be stimulated), only mild inhibition of some strains of *E. coli* was noted.

In liquid media the growth of *S. aureus*, *S. dysgalactiae*, *S. agalactiae* and *S. uberis* was inhibited in cultures containing brain heart broth and filter sterilized broth in which *C. bovis* had previously been cultured, compared to a negative control culture. The inhibitory effect could be partially eliminated if the filter sterilized *C. bovis* broth was heated to 100°C and almost completely eliminated if the broth was treated with the broad-spectrum protease, Proteinase K.

These results suggest that *C. bovis* produces a factor containing a protein which can inhibit the growth of the UK's four most important Gram positive mastitis pathogens. Many of the characteristics of this factor are consistent with the description of bacteriocins produced by other *Corynebacterium* species.

8.2. PRACTICAL APPLICATIONS AND FUTURE WORK

8.2.1. Internal Teat Sealers

The teat sealer employed during this thesis will become commercially available in the UK in September 2002 (OrbeSeal®, Pfizer Animal Health). Its launch represents the first major advance in dry cow therapy since the widespread introduction of antibiotic dry cow therapy in the 1960s as part of the five point plan. It is the author's belief that the rational for the continued use of long acting antibiotic DCT in uninfected cows

will become increasingly untenable. The internal teat sealer described here offers the dairy farming industry a real and viable opportunity to reduce prophylactic antibiotic DCT usage.

The ability to use teat sealers (such as the one described) in the field relies on accurately distinguishing uninfected and infected cows and/or quarters reliably and cheaply. Further work is needed to identify the most appropriate methods for selecting uninfected cows suitable for treatment. When considering the use of teat sealers, selection methods/criteria need both high sensitivity and high specificity so that uninfected cows are not excluded unnecessarily and the number of infected cows selected inappropriately is minimized.

Work from this study suggests that mastitis (no cases) and cow level SCC data (all counts < 200,000 cells/ml) during the previous lactation can be used for this purpose. Using these criteria 3.2% of quarters were incorrectly identified as uninfected. Further work is needed to refine these criteria in an attempt to decrease the number of quarters incorrectly identified as uninfected or to relax the criteria further (to decrease the number of false positive quarters excluded) providing the number of false negative quarters included does not increase.

Many dairy farmers in the UK do not currently routinely measure individual cow SCC's. Either dairy farmers need convincing of their worth via education programs or alternative cow side measurements, which accurately reflect SCC (and/or sub-clinical infection status), need to be identified. In collaboration with Dr. Andrew Bradley the author will shortly be undertaking a study to investigate the value of hand held conductivity meters, cow side test strips and the California mastitis test for this purpose. Other methods such as milk enzyme *e.g.* NAGase and acute phase protein whose levels increase in sub-clinically infected quarters may be of value for this purpose in the future.

8.2.2. 16S rRNA Gene Sequence Restriction Analysis

Endonuclease restriction analysis of the 16S rRNA gene sequence proved to be an excellent method for differentiating *C. bovis* from other lipophilic *Corynebacterium* species and allowed the identification of a novel species. However 18.3% (171 isolates) of all coryneform isolates were non-lipophilic. Non-lipophilic *Corynebacterium* species have previously been described as mastitis pathogens (Watts

et al. 2000), although their identity, prevalence and pathogenic significance is poorly understood. The method described for differentiating lipophilic species could be extended to differentiate non-lipophilic species. The method will undoubtedly be more complex (because of the larger number of non-lipophilic species) and probably involve increased numbers of different enzymes. Accurate identification of all its members will allow the prevalence and significance of this potentially very interesting genus to be further investigated and clarified.

8.2.3. Protective Affect of *C. bovis In Vivo*

Previous authors have concluded that *C. bovis* may be significantly involved in intramammary infection dynamics: "*C. bovis* may play an important role as a biological control mechanism in the bovine udder but has received little attention" (Brooks *et al.* 1983); "Colonization of the teat ends with normal flora which inhibit pathogens *in vitro* may therefore increase non-specific resistance to mastitis" (Woodward *et al.* 1988). Some have even concluded that its classification as a pathogen should be reassessed, "... *C. bovis* should more correctly by referred to as a commensal of the bovine udder" (Brooks and Barnum 1984)

The work outlined in Chapter 5 of this thesis is the fourth study investigating the role of infection with *C. bovis* on the subsequent rate of infection with other pathogens which has been analyzed using multivariate statistics. All have demonstrated that *C. bovis* infected quarters are protected against subsequent infection with other pathogens (Schukken *et al.* 1999; Zadoks *et al.* 2001; Green *et al.* 2002).

Based on the results of the work presented here, it is tempting to suggest that milk producers and veterinarians working in the field should deliberately attempt to infect cows with *C. bovis* in order to protect quarters against subsequent infection with other more pathogenic organisms. Despite the conclusive results demonstrated during this thesis, the author is very sceptical as to whether this is a sensible approach. Strain differences in the pathogenicity of *C. bovis* have been demonstrated in the mouse mammary gland (Anderson *et al.* 1985) and strain differences in the level of production of an inhibitory factor have been demonstrated during this thesis. There will always be the danger that an inappropriate strain of *C. bovis* would be selected for artificially infection purposes. If a particularly pathogenic strain were selected there is a danger that it may cause clinical mastitis. If a strain that was only weakly protective

were selected, infected quarters may suffer a rise in SCC but no (or limited) protection against major pathogens.

Perhaps a more sensible approach would be to moderate the control measures that reduce the prevalence of *C. bovis* IMI in the field; these principally include antibiotic DCT and PMTD. With the launch of the internal teat sealer described here, producers now have a real opportunity to reduce antibiotic DCT usage (in cows not infected with major pathogens at drying off) without sacrificing protection from major pathogens during the dry period. Previous workers have shown that in low BMSCC herds (<150,000 cells/ml) stopping PMTD led to a reduction in clinical mastitis (particularly *E. coli* mastitis) (Lam *et al.* 1997), they concluded that this effect may be due to the increased prevalence of *C. bovis* in quarters on which PMTD was discontinued. However PMTD and antibiotic DCT are two of the key measures that also control the prevalence of contagious pathogens, particularly *S. aureus*. Antibiotic DCT must be continued in quarters infected with major pathogens at drying off. An increase in the prevalence of *S. aureus* was seen in quarters on which PMTD was discontinued in the study described above (Lam *et al.* 1997). Producers and veterinarians in the field need to be aware that by limiting the implementation of these control measures to increase the prevalence of *C. bovis* they also risk increasing the prevalence of contagious pathogens, particularly *S. aureus*. Ideally management procedures need to be identified or modified to increase the prevalence of *C. bovis* without directly influencing the prevalence of contagious pathogens.

The prevalence and intramammary infection dynamics of *C. bovis* infection in any given herd is undoubtedly a very complex and poorly understood process. It is likely to be affected by the strains of *C. bovis* and the type, strains and prevalence of major pathogen IMI present within the herd, milking machine function, type and use of antibiotic DCT, efficiency of PMTD, implementation of other aspects of the five point plan, herd genetics, environmental conditions, milk yield, quarter susceptibility (*e.g.* teat end condition), age structure of the herd and many other factors. The appropriate level of *C. bovis* infection within a herd will therefore undoubtedly vary depending on the farm situation. In some very well managed herds with low BMSCC's, where environmental, udder, milking machine and other aspects of farm management are excellent, so that the weight of challenge with major pathogens is low, the appropriate level of *C. bovis* IMI may well be zero because in these herds the elevation in SCC is not outweighed by the level of protection infection with *C. bovis*

may afford. However, in other less well-managed herds where the level of challenge with major pathogens is higher an "ideal" prevalence of *C. bovis* may exist where the increase in SCC is acceptable because the number of quarters protected against infection with major pathogens outweighs any disadvantage. Unfortunately this ideal *C. bovis* prevalence will almost certainly vary from herd to herd depending on farm conditions.

Making appropriate straightforward and practical suggestions for producers and veterinarians in the field to utilize this information is therefore extremely difficult because any suggestions will vary depending on some of the factors outlined above. Therefore, perhaps of much more importance is understanding the processes and mechanisms behind the protective effect demonstrated in field studies. By investigating and understanding the processes it may be possible to identify novel prophylactic/therapeutic agents or managements styles that promote the correct "balance" of infection with *C. bovis* in any given herd.

8.2.4. Inhibitory Factor

Some strains of *C. bovis* produce a factor that is inhibitory to Gram-positive mastitis pathogens. Initial characterization work suggests that this factor may be a bacteriocin; however further work is necessary to confirm its identity. Initially this work will involve further characterization of the protein nature of the factor by investigation of the impact of treating filter sterilized broth with other proteinase enzymes. Following on from that a guide to the molecular weight of the factor will be gained by passage initially through a series of syringe driven molecular weight filters and later a molecular weight column. Methods for purification of the factor are more difficult to predict until more is known of its nature. However, if as it appears, the factor is a protein, methods such as ammonium sulphate precipitation (Lyon *et al.* 1993) followed by separation by ion exchange and size exclusion chromatography (Jack *et al.* 1995) have been used previously. Once purified it would then be possible to elucidate the amino acid sequence by N-terminal sequencing or deduction of the structure from the corresponding bacterial DNA sequence (Jack *et al.* 1995).

Initial work on the factor suggests that it is predominantly active against Gram-positive organisms and certainly against *S. aureus* and three *Streptococcus* species (*S. agalactiae, S. dysgalactiae* and *S. uberis*). There has been renewed interest

in bacteriocins recently because of their potential uses as food preservatives and antibacterial compounds (Jack *et al.* 1995). Although far too early to speculate on its prospective applications, the factor identified during this thesis may have widespread potential outwith the veterinary field in the future.

Whatever the exact nature and potential applications of the factor, demonstration of its existence remains an extremely interesting findings, because it has established that some strains of *C. bovis* are capable of producing a factor which is inhibitory to other mastitis pathogens *in vitro*. Whether the factor is solely or partly responsible for the protective effect demonstrated for *C. bovis* against major pathogens *in vivo* has not been conclusively elucidated during this thesis. It remains an area in need of further research.

APPENDIX I: STANDARD OPERATING PROCEDURES EMPLOYED DURING THE TEAT SEALER CLINICAL STUDY.

Figure AI.I: Standard Operating Procedure 1 - Identification of Dairy Cows used in Field Study

i. Each animal will be identified by the following:
   a. a unique freeze-brand or, in the case that the freeze brand is illegible, the animals' individually ear tag number.
   b. farm details (herd owner, address or farm code traceable to herd owner and address).

ii. Each animals' identification number will be recorded on each data capture form relevant to that animal.

Figure AI.II: Standard Operating Procedure 2 - Clinical Examination of Dairy Cows used in Field Study

i. Record the animals' farm and individual identification number on the appropriate data capture form.
ii. Observe the animal prior to handling.
iii. Measure and record rectal temperature.
iv. Auscultate the heart and lungs.
v. Examine the animal for signs of musculoskeletal, integumentary and gastro-intestinal disturbances.
vi. Examine the mammary gland of each animal, recording any evidence of teat or quarter lesions or abnormal milk.
vii. Record all abnormal findings on the appropriate data capture form.

Figure AI.III: Standard Operating Procedure 3 - Observation of Dairy Cows used in Field Study During the Dry Period

i. Observe the animal in its normal environment at rest and moving.
ii. Observe and palpate the udder if mastitis is suspected.

iii. If secretion is stripped from any teat during the dry period, or an animal is treated with an antibiotic or anti-inflammatory drug, please inform the study investigator as soon as possible.

Figure AI.IV: Standard Operating Procedure 4 - Collection of Quarter Milk Samples for Bacteriological and Somatic Cell Count Analysis

i. Wear a new pair of disposable gloves for each animal.
ii. (If necessary, clean grossly contaminated udders and teats with water.)
iii. (Dry completely with paper towel.)
iv. Remove gross contamination from the teat and quarter with dry paper towel.
v. Dip the teat to be sampled with a product containing 2800ppm available chlorine (Agrisept; Upjohn Pharmacia Ltd).
vi. Leave at least 30 seconds.
vii. Wipe dry with a clean paper towel.
viii. Gently scrub whole teat, concentrating on the teat end with surgical spirit soaked swab.
ix. Leave to dry.
x. Strip foremilk from each quarter.
xi. Gently scrub teat end with surgical spirit soaked swab, furthest followed by nearest.
xii. Leave to dry.
xiii. Avoid touching the teat ends between the time of cleaning and sample collection.
xiv. Unscrew cap from a 20 ml sterile universal and hold it, inner surface downwards, in the same hand as the sample vial. Keep the vial as close to horizontal as possible to minimize entry of dust and debris.
xv. Collect the first bacteriology milk sample with a minimum of pressure, preferably by a single squeezing of the teat. Collect from the nearest quarters first, sample all four quarters. The teat end must not touch the lip of the vial.
xvi. Gently scrub the teats with surgical spirit, furthest followed by nearest.
xvii. Leave to dry.
xviii. Collect the duplicate bacteriology milk samples as described above

xix. Collect the samples for somatic cell count analysis into a flip top vial containing a bronopol and natamycin preservative tablet. Fill vial at least two third full.

xx. Administer dry cow therapy (as per SOP 05 or SOP 06).

Figure AI.V: Standard Operating Procedure 5 - Aseptic Administration of Antibiotic Dry Cow Therapy by Intra-mammary Infusion at Drying Off

i. Collect milk samples in accordance with SOP 4.

ii. Gently scrub teat end with surgical spirit soaked swab, furthest followed by nearest.

iii. Leave to dry.

iv. Strip foremilk from each quarter.

v. Avoid touching the teat ends between the time of cleaning and product administration.

vi. Remove the protective cover from the tip of the intra-mammary syringe, taking care not to contaminate the tip.

vii. Gently insert the tip of the intra-mammary syringe into the teat orifice.

viii. Express the contents of the syringe into the teat.

ix. Holding the teat end closed, massage the antibiotic up into the udder.

x. Administer the entire contents of one tube into each quarter.

xi. Dip all 4 teats with a product containing 2800ppm available chlorine (Agrisept; Upjohn Pharmacia Ltd).

xii. Confine to a loafing yard for at least 30 minutes.

xiii. Record product usage on appropriate data capture form.

Figure AI.VI: Standard Operating Procedure 6 - Aseptic Administration of Non-Antibiotic Dry Cow Therapy by Intra-mammary Infusion at Drying Off

i. Collect milk samples in accordance with SOP 4.

ii. Gently scrub teat end with surgical spirit soaked swab, furthest followed by nearest.

iii. Leave to dry.

iv. Strip foremilk from each quarter.

v. Avoid touching the teat ends between the time of cleaning and product administration.
vi. Remove the protective cover from the tip of the intra-mammary syringe, taking care not to contaminate the tip.
vii. Gently insert the tip of the intra-mammary syringe into the teat orifice.
viii. Express the contents of the syringe into the teat. Do not massage the product up into the udder.
ix. Administer the entire contents of one tube into each quarter.
x. Dip all 4 teats with a product containing 2800ppm available chlorine (Agrisept; Upjohn Pharmacia Ltd).
xi. Confine to loafing yard for at least 30 minutes.
xii. Record product usage on appropriate data capture form.

Figure AI.VII: Standard Operating Procedure 7 - Collection of a Milk Sample from a Dairy Cow in the Case of Clinical Mastitis

i. Wear a new set of disposable gloves for each animal.
ii. (If necessary clean grossly contaminated udders and teats with water.)
iii. (Dry completely with paper towel.)
iv. Remove gross contamination from the teat and quarter with dry paper towel.
v. Dip the teat to be sampled with a product containing 2800ppm available chlorine (Agrisept; Upjohn Pharmacia Ltd).
vi. Leave at least 30 seconds.
vii. Wipe dry with a clean paper towel.
viii. Gently scrub whole teat, concentrating on the teat end with surgical spirit soaked swab.
ix. Leave to dry.
x. Strip at least two streams of milk from the quarter.
xi. Gently scrub teat end with surgical spirit soaked swab.
xii. Leave to dry.
xiii. Avoid touching the teat ends between the time of cleaning and sample collection.

xiv. Unscrew cap from a 20 ml sterile universal and hold it, inner surface downwards, in the same hand as the sample vial. Keep the vial as close to horizontal as possible to minimise entry of dust and debris.

xv. Collect the mastitis sample with a minimum of pressure, preferably by a single squeezing of the teat. The teat end must not touch the lip of the vial.

xvi. Dip the teat to be sampled with a product containing 2800ppm available chlorine (Agrisept; Upjohn Pharmacia Ltd).

xvii. Confine to loafing yard for at least 30 minutes.

APPENDIX II: NUMBER OF QUARTER AND COW IMI ACQUIRED DURING THE DRY PERIOD DIAGNOSED AT DIFFERENT SCC THRESHOLDS, BY CAUSATIVE ORGANISM.

Table AII.I: Number of Quarter and Cow IMI's Acquired During the Dry Period, by Causative Organism (Bacteriologically Positive Plus Quarter SCC >125,000 cells/ml)

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	New quarter IMI during dry period (n=928)	New cow IMI during dry period (n=232)	New quarter IMI during dry period (n=940)	New cow IMI during dry period (n=235)
Coagulase +ve Staphylococci	11	10	8	7
S. dysgalactiae	2	2	0	0
S. uberis	10	10	11	11
Streptococcus spp. (Other)	9	9	11	10
Enterococcus spp.	14[e]	14[c]	33[f]	29[d]
E. coli	11[g]	10[g]	38[h]	31[h]
Klebsiella spp.	0	0	1	1
Serratia spp.	0	0	1	1
Citrobacter spp.	1	1	0	0
Morganella spp.	0	0	1	1
Proteus spp.	2	2	7	7
Enterobacter spp.	0	0	1	1
Hafnia spp.	0	0	1	1
All *Enterobacteriaceae* spp.	14[g]	13[g]	49[h]	42[h]
Ochrobacter spp.	1	1	1	1
Chryseomonas spp.	1	1	0	0
Aerococcus spp.	0	0	1	1
Acinetobacter spp.	13	12	11	9
Non fermenters	8	8	6	6
Bacillus spp.	12	10	6	5
Other non speciated organisms	0	0	1	1
Aspergillus spp.	1	1	2	2
Yeast spp.	0	0	1	1
Mucur spp.	0	0	3	3
All Yeast & Fungi	1[a]	1[a]	6[b]	6[b]

All Major Pathogens	90^{c}	70^{a}	126^{d}	90^{b}
Micrococcus spp.	13	12	11	10
Coagulase –ve Staphylococci	139	98	153	106
Corynebacterium spp.	53^{c}	41^{a}	34^{d}	26^{b}
All Minor Pathogens	152	105	153	102

^{a,b} *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.1$)*
^{c,d} *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.05$)*
^{e,f} *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.01$)*
^{g,h} *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)*

Table AII.II: Number of Quarter and Cow IMI's Acquired During the Dry Period, by Causative Organism (Bacteriologically Positive Plus Quarter SCC >250,000 cells/ml)

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	New quarter IMI during dry period (n=928)	New cow IMI during dry period (n=232)	New quarter IMI during dry period (n=940)	New cow IMI during dry period (n=235)
Coagulase +ve Staphylococci	11	10	8	7
S. dysgalactiae	2	2	0	0
S. uberis	10	10	10	10
Streptococcus spp. (Other)	7	7	10	9
Enterococcus spp.	14^{c}	14^{c}	30^{d}	27^{d}
E. coli	8^{g}	7^{g}	35^{h}	28^{h}
Serratia spp.	0	0	1	1
Citrobacter spp.	1	1	0	0
Morganella spp.	0	0	1	1
Proteus spp.	1^{a}	1^{a}	7^{b}	7^{b}
Enterobacter spp.	0	0	1	1
Hafnia spp.	0	0	1	1
All *Enterobacteriaceae* spp.	10^{g}	9^{g}	45^{h}	38^{h}
Ochrobacter spp.	1	1	1	1
Chryseomonas spp.	1	1	0	0
Aerococcus spp.	0	0	1	1
Acinetobacter spp.	13	12	11	9
Non fermenters	6	6	6	6
Bacillus spp.	12^{a}	10^{a}	4^{b}	3^{b}
Other non speciated organisms	0	0	1	1
Aspergillus spp.	1	1	2	2
Mucur spp.	0	0	3	3
All Yeast & Fungi	1	1	5	5
All Major Pathogens	83^{e}	65^{a}	118^{f}	86^{b}
Micrococcus spp.	9	9	9	8
Coagulase –ve Staphylococci	121	89	125	86
Corynebacterium spp.	54^{c}	36^{a}	32^{d}	23^{b}
All Minor Pathogens	145	96	133	86

[a,b] Numbers within rows and between treatment groups with different superscripts differ ($P < 0.1$)
[c,d] Numbers within rows and between treatment groups with different superscripts differ ($P < 0.05$)
[e,f] Numbers within rows and between treatment groups with different superscripts differ ($P < 0.01$)
[g,h] Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)

Table AII.III: Number of Quarter and Cow IMI's Acquired During the Dry Period, by Causative Organism (Bacteriologically Positive Plus Quarter SCC >500,000 cells/ml)

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	New quarter IMI during dry period (n=928)	New cow IMI during dry period (n=232)	New quarter IMI during dry period (n=940)	New cow IMI during dry period (n=235)
Coagulase +ve Staphylococci	13	12	11	9
S. dysgalactiae	2	2	0	0
S. uberis	7	7	10	10
Streptococcus spp. (Other)	4	4	6	5
Enterococcus spp.	9[e]	9[e]	27[f]	25[f]
E. coli	7[g]	6[e]	28[h]	22[f]
Serratia spp.	0	0	1	1
Citrobacter spp.	0	0	1	1
Morganella spp.	0	0	1	1
Proteus spp.	1[a]	1[a]	7[b]	7[b]
Enterobacter spp.	0	0	1	1
Hafnia spp.	0	0	1	1
All *Enterobacteriaceae* spp.	9[g]	8[g]	38[h]	32[h]
Ochrobacter spp.	1	1	1	1
Chryseomonas spp.	1	1	0	0
Aerococcus spp.	0	0	1	1
Acinetobacter spp.	7	7	5	3
Non fermenters	5	4	3	3
Bacillus spp.	12[e]	10[e]	2[f]	1[f]
Other non speciated organisms	0	0	1	1
Aspergillus spp.	1	1	1	1
Mucur spp.	0	0	3	3
All Major Pathogens	68[c]	56	97[d]	71
Micrococcus spp.	8	8	5	4
Coagulase –ve Staphylococci	95	74	104	73
Corynebacterium spp.	56[g]	37[e]	23[h]	17[f]
All Minor Pathogens	136[a]	90	109[b]	76

[a,b] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.1$)*
[c,d] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.05$)*
[e,f] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.01$)*
[g,h] *Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)*

Table AII.IV: Number of Quarter and Cow IMI's Acquired During the Dry Period, by Causative Organism (Bacteriologically Positive Plus Quarter SCC >1000,000 cells/ml)

	Teat Sealer Group		Antibiotic DCT Group	
Diagnosis	New quarter IMI during dry period (n=928)	New cow IMI during dry period (n=232)	New quarter IMI during dry period (n=940)	New cow IMI during dry period (n=235)
Coagulase +ve Staphylococci	15	13	9	8
S. dysgalactiae	1	1	0	0
S. uberis	6	6	6	6
Streptococcus spp. (Other)	4	4	4	3
Enterococcus spp.	5^{e}	5^{e}	20^{f}	20^{f}
E. coli	6^{g}	6^{e}	25^{h}	21^{f}
Serratia spp.	0	0	1	1
Citrobacter spp.	1	1	0	0
Morganella spp.	0	0	1	1
Proteus spp.	1^{a}	1^{a}	7^{b}	7^{b}
Enterobacter spp.	0	0	1	1
Hafnia spp.	0	0	1	1
All *Enterobacteriaceae* spp.	8^{g}	8^{g}	35^{h}	31^{h}
Ochrobacter spp.	1	1	1	1
Chryseomonas spp.	1	1	0	0
Aerococcus spp.	0	0	1	1
Acinetobacter spp.	2	2	2	2
Non fermenters	5	4	2	2
Bacillus spp.	10^{c}	8^{c}	2^{d}	1^{d}
Other non speciated organisms	0	0	1	1
Aspergillus spp.	0	0	1	1
Mucur spp.	0	0	1	1
All Major Pathogens	56^{a}	49	76^{b}	60
Micrococcus spp.	4	4	5	4
Coagulase –ve Staphylococci	72	60	69	49
Corynebacterium spp.	45^{g}	31^{e}	15^{h}	12^{f}
All Minor Pathogens	107^{c}	74^{c}	77^{d}	52^{d}

^{a,b} Numbers within rows and between treatment groups with different superscripts differ ($P < 0.1$)
^{c,d} Numbers within rows and between treatment groups with different superscripts differ ($P < 0.05$)
^{e,f} Numbers within rows and between treatment groups with different superscripts differ ($P < 0.01$)
^{g,h} Numbers within rows and between treatment groups with different superscripts differ ($P < 0.001$)

Appendix III: Predicted Number of Restriction Sites and Fragment Sized After Endonuclease Restriction Analysis of the 16S rRNA Gene Sequence of *C. bovis*.

Table AIII.I: Predicted Number of Restriction Sites and Size of Fragments Produced (Italics), in Base Pairs, After Endonuclease Restriction of *C. bovis* 16S rRNA Gene Sequences With *Hind* III, *Sma* I, *Pst* I, *Mlu* I, *Pvu* II and *Rsa* I

	bovis D38575	*bovis* X82051	*bovis* AF311390	*bovis* AF311391	*bovis* AF311392	*bovis* AF311393	*bovis* AF311394	*bovis* AF311395	*bovis* AF311396	*bovis* AF311397	*bovis* AF311399	*bovis* AF311400	*bovis* AF311401	*bovis* AF311402	*bovis* AF311403
Hind III	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	345	*346*	*346*	*345*	*346*	*346*	*345*	*344*	*346*	*343*	*346*	*346*	*346*	*349*	*346*
	886	*888*	*888*	*888*	*888*	*888*	*888*	*888*	*889*	*890*	*888*	*888*	*889*	*888*	*888*
Sma I	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1
	487	*488*	*488*	*488*	*488*	*488*	*487*	*486*	*488*	*129*	*488*	*488*	*488*	*491*	*488*
	744	*746*	*746*	*746*	*749*	*746*	*746*	*746*	*747*	*356*	*746*	*746*	*747*	*746*	*746*
										748					
Pst I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mlu I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pvu II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rsa I	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	109	*110*	*109*	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*110*
	159	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*	*160*
	201	*201*	*201*	*200*	*201*	*201*	*200*	*199*	*201*	*198*	*201*	*201*	*201*	*204*	*201*
	355	*356*	*356*	*356*	*356*	*356*	*356*	*356*	*356*	*358*	*356*	*356*	*356*	*356*	*356*
	407	*407*	*408*	*407*	*407*	*407*	*407*	*407*	*408*	*407*	*407*	*407*	*408*	*407*	*407*

	bovis AF311404	*bovis* AF311405	*bovis* AF311406	*bovis* AF311407	*bovis* AF311408	*bovis* AF311410	*bovis* AF311411	*bovis* AF311412	*bovis* AF311413	*bovis* AF311414	*bovis* AF311415	*bovis* AF311416	*bovis* AF311417	*bovis* AF311418	*bovis* AF311419
Hind III	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
	346	*348*	*346*	*346*	*346*	*346*	*346*	*346*	*345*	*346*	*346*	*346*	*346*	*347*	*141*
	888	*888*	*888*	*888*	*888*	*888*	*887*	*888*	*886*	*887*	*889*	*888*	*888*	*888*	*206*
															890
Sma I	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	488	*488*	*488*	*488*	*488*	*488*	*487*	*488*	*487*	*488*	*488*	*488*	*488*	*488*	*488*
	746	*746*	*746*	*746*	*746*	*746*	*746*	*746*	*744*	*745*	*747*	*746*	*746*	*746*	*746*
Pst I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mlu I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pvu II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rsa I	4	4	4	4	4	4	3	4	3	4	4	4	4	4	4
	110	*110*	*110*	*110*	*110*	*110*	*110*	*110*	*160*	*110*	*110*	*110*	*110*	*110*	*110*
	160	*160*	*160*	*160*	*160*	*160*	*201*	*160*	*200*	*160*	*160*	*160*	*160*	*160*	*160*
	201	*201*	*201*	*201*	*200*	*201*	*356*	*201*	*407*	*201*	*201*	*201*	*201*	*202*	*202*
	356	*356*	*356*	*356*	*356*	*356*	*566*	*356*	*464*	*356*	*356*	*356*	*356*	*356*	*356*
	407	*407*	*407*	*407*	*407*	*407*		*407*		*406*	*408*	*407*	*407*	*407*	*409*

	bovis AF311420	*bovis* AF311421	*bovis* AF311422	*bovis* AF311423	*bovis* AF311424	*bovis* AF311425	*bovis* AF311426	*bovis* AF311428	*bovis* AF311429	*bovis* AF311430	*bovis* AF311431	*bovis* AF311432	*bovis* AF311433	*bovis* AF311434	*bovis* AF311435	*bovis* AF311436
Hind III	1 346 888	1 346 888	1 347 887	1 346 888	1 345 888	1 346 888	1 346 888	1 347 888	1 346 888	1 346 888	1 346 888	1 346 888	1 346 888	1 347 883	1 346 888	1 346 888
Sma I	1 488 746	1 488 746	1 489 745	1 488 746	1 487 746	1 488 746	1 488 746	1 489 747	1 488 746	1 488 746	1 488 746	1 488 746	1 488 746	1 489 741	1 488 746	1 488 746
Pst I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mlu I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pvu II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rsa I	5 14 110 160 187 356 407	4 110 160 201 356 407	4 110 161 201 357 405	4 110 160 201 356 407	4 110 160 200 356 407	4 110 160 201 356 407	4 110 160 201 356 407	4 110 160 202 356 408	4 110 160 201 356 407	4 110 160 201 356 407	4 110 160 201 356 407	4 110 160 201 356 407	4 110 160 201 356 407	4 110 160 202 356 402	4 110 160 201 356 407	4 110 160 201 356 407

APPENDIX IV: THE PARTIAL 16S RRNA GENE SEQUENCES FOR *C. BOVIS* ISOLATES 2358, 4375, 5330B AND M218.

Figure AIV.I: The Partial (1170 bases) 16S rRNA Gene Sequence for Isolate 2358

5' - TAC TTC GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG ATA GGA CCA ATC TTT AGT GTG GTT GGT
GGA AAG TTT TGT CGG TAC GAG ATG AGC CCG CGG CCT ATC AGC TTG TTG GTG GGG TAA TGG CCT ACC
AAG GCG ACG ACG GGT AGC CGG CCT GAG AGG GTG TAC GGC CAC ATT GGG ACT GAG ACA CGG CCC AGA
CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA CAA TGG GCG GAA GCC TGA TGC AGC GAC GCC GCG
TGA GGG ATG ACG GCC TTC GGG TTG TAA ACC TCT TTC GCT AGG GAA GAA GCC TTT TTG GGT GAC GGT
ACC TGG ATA AGA AGC ACC GGC TAA CTA CGT GCC AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT
GTC CGG AAT TAC TGG GCG TAA AGA GCT CGT AGG TGG TTT GTC GCG TCG TTA GTG AAA GCC CGG GGC
TTA ACT CCG GGT CTG CTG GCG ATA CGG GCA TAA CTT GAG TGC TGT AGG GGA GAC TGG AAT TCC TGG
TGT AGC GGT GGA ATG CGC AGA TAT CAG GAG GAA CAC CGA TGG CGA AGG CAG GTC TCT GGG CAG TAA
CTG ACG CTG AGG AGC GAA AGC ATG GGT AGC GAA CAG GAT TAG ATA CCC TGG TAG TCC ATG CCG TAA
ACG GTG GGC GCT AGG TGT GGG GGT TTT TCA CGA CTT CCG TGC CGT AGC TAA CGC ATT AAG CGC CCC
GCC TGG GGA GTA CGG CCG CAA GGC TAA AAC TCA AAG GAA TTG ACG GGG GCC CGC ACA AGC GGC GGA
GCA TGT GGA TTA ATT CGA TGC AAC GCG AAG AAC CTT ACC TGG GCT TGA CAT GTA CGG GAT CGG CGT
AGA GAT ACG TTT TCC CTT GTG GCT CGT ATA CAG GTG GTG CAT GGT TGT CGT CAG CTC GTG TCG TGA
GAT GTT GGG TTA AGT CCC GCA ACG AGC GCA ACC CTT GTC TTG TGT TGC CAG CAC GTG ATG GTG GGG
ACT CGC GAG AGA CTG CCG GGG TTA ACT CGG AGG AAG GTG GGG ATG ACG TCA AAT CAT CAT GCC CCT
TAT GTC CAG GGC TTC ACA CAT GCT ACA ATG GTC GGT ACA GTG GGT TGC GAT ACC GTG AGG TGG AGC
TAA TCC CTT AAA GCC GGT CTC AGT TCG GAT TTG GAG TCT GCA ACT CGA – 3'

The partial 16S rRNA gene sequence of isolate 2358 has a single base pair insertion compared to that of isolate 4465. At the position marked in bold an extra thymine base was present.

Figure AIV.II: The Partial (1203 bases) 16S rRNA Gene Sequence for Isolate 4375

5' - GTG AGT AAC ACG TGG GTG ATC TGC CTT GTA CTT CGG GAT AAG CCT GGG AAA CTG GGT CTA ATA CCG
GAT AGG ACC AAT CTT TAG TGT GGT TGG TGG AAA GTT TTG TCG GTA CGA GAT GAG CCC GCG GCC TAT
CAG CTT GTT GGT GGG GTA ATG GCC TAC CAA GGC GAC GAC GGG TAG CCG GCC TGA GAG GGT GTA CGG
CCA CAT TGG GAC TGA GAC ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA ATG
GGC GGA AGC CTG ATG CAG CGA CGC CGC GTG AGG GAT GAC GGC CTT CGG GTT GTA AAC CTC TTT CGC
TAG GGA AGA AGC CTT TTT GGG TGA CGG TAC CTG GAT AAG AAG CAC CGG CTA ACT ACG TGC CAG CAG
CCG CGG TAA TAC GTA GGG TGC GAG CGT TGT CCG GAA TTA CTG GGC GTA AAG AGC TCG TAG GTG GTT
TGT CGC GTC GTT AGT GAA AGC CCG GGG CTT AAC TCC GGG TCT GCT GGC GAT ACG GGC ATA ACT TGA
GTG CTG TAG GGG AGA CTG GAA TTC CTG GTG TAG CGG TGG AAT GCG CAG ATA TCA GGA GGA ACA CCG
ATG GCG AAG GCA GGT CTC TGG GCA GTA ACT GAC GCT GAG GAG CGA AAG CAT GGG TAG CGA ACA GGA
TTA GAT ACC CTG GTA GTC CAT GCC GTA AAC GGT GGG CGC TAG GTG TGG GGG TTT TTC ACG ACT TCC
GTG CCG TAG CTA ACG CAT TAA GCG CCC CGC CTG GGG AGT ACG GCC GCA AGG CTA AAA CTC AAA GGA
ATT GAC GGG GGC CCG CAC AAG CGG CGG AGC ATG TGG ATT AAT TCG ATG CAA CGC GAA GAA CCT TAC
CTG GGC TTG ACA TGT ACG GGA TCG GCG TAG AGA TAC GTT TTC CCT TGT GGC TCG TAT ACA GGT GGT
GCA TGG TTG TCG TCA GCT CGT GTC GTG AGA TGT TGG GTT AAG TCC CGC AAC GAG CGC AAC CCT TGT
CTT GTG TTG CCA GCA CGT GRT GGT GGG GAC TCG CGA GAG ACT GCC GGG GTT AAC TCG GAG GAA GGT
GGG GAT GAC GTC AAA TCA TCA TGC CCC TTA TGT CCA GGG CTT CAC ACA TGC TAC AAT GGT CGG TAC
AGT GGG TTG CGA TAC CGT GAG GTG GAG CTA ATC CCT TAA AGC CGG TCT CAG TTC GGA TTG GAG TCT
GCA ACT CGA CTC CAT – 3'

The partial 16S rRNA gene sequence of isolate 4375 may have a single base pair alteration compared to that of isolate 4465. At the position marked in bold sequencing was unable to ascertain whether the base was adenine or guanine.

Figure AIV.III: The Partial (1170 bases) 16S rRNA Gene Sequence for Isolate 5330b

5' - TAC TTC GGG ATA AGC CTG GGA AAC TGG GTC TAA TAC CGG ATA GGA CCA ATC TTT AGT GTG GTT GGT
GGA AAG TTT TGT CGG TAC GAG ATG AGC CCG CGG CCT ATC AGC TTG TTG GTG GGG TAA TGG CCT ACC
AAG GCG ACG ACG GGT AGC CGG CCT GAG AGG GTG TAC GGC CAC ATT GGG ACT GAG ACA CGG CCC AGA
CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA CAA TGG GCG GAA GCC TGA TGC AGC GAC GCC GCG
TGA GGG ATG ACG GCC TTC GGG TTG TAA ACC TCT TTC GCT AGG GAA GAA GCC TTT TTG GGT GAC GGT
ACC TGG ATA AGA AGC ACC GGC TAA CTA CGT GCC AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT
GTC CGG AAT TAC TGG GCG TAA AGA GCT CGT AGG TGG TTT GTC GCG TCG TTA GTG AAA GCC CGG GGC
TTA ACT CCG GGT CTG CTG GCG ATA CGG GCA TAA CTT GAG TGC TGT AGG GGA GAC TGG AAT TCC TGG
TGT AGC GGT GGA ATG CGC AGA TAT CAG GAG GAA CAC CGA TGG CGA AGG CAG GTC TCT GGG CAG TAA
CTG ACG CTG AGG AGC GAA AGC ATG GGT AGC GAA CAG GAT TAG ATA CCC TGG TAG TCC ATG CCG TAA
ACG GTG GGC GCT AGG TGT GGG GGT TTT TCA CGA CTT CCG TGC CGT AGC TAA CGC ATT AAG CGC CCC
GCC TGG GGA GTA CGG CCG CAA GGC TAA AAC TCA AAG GAA TTG ACG GGG GCC CGC ACA AGC GGC GGA
GCA TGT GGA TTA ATT CGA TGC AAC GCG AAG AAC CTT ACC TGG GCT TGA CAT GTA CGG GAT CGG CGT
AGA GAT ACG TTT TCC CTT GTG GCT CGT ATA CAG GTG GTG CAT GGT TGT CGT CAG CTC GTG TCG TGA
GAT GTT GGG TTA AGT CCC GCA ACG AGC GCA ACC CTT GTC TTG TGT TGC CAG CAC GTG GTG GTG GGG
ACT CGC GAG AGA CTG CCG GGG TTA ACT CGG AGG AAG GTG GGG ATG ACG TCA AAT CAT CAT GCC CCT
TAT GTC CAG GGC TTC ACA CAT GCT ACA ATG GTC GGT ACA GTG GGT TGC GAT ACC GTG AGG TGG AGC
TAA TCC CTT AAA GCC GGT CTC AGT TCG GAT TGG AGT CTG CAA CTC GAC – 3'

The partial 16S rRNA gene sequence of isolate 5330b has a single base pair alteration compared to that of isolate 4465. At the position marked in bold an adenine base has been replaced by a guanine base.

Figure AIV.IV: The Partial (1181 bases) 16S rRNA Gene Sequence for Isolate M218

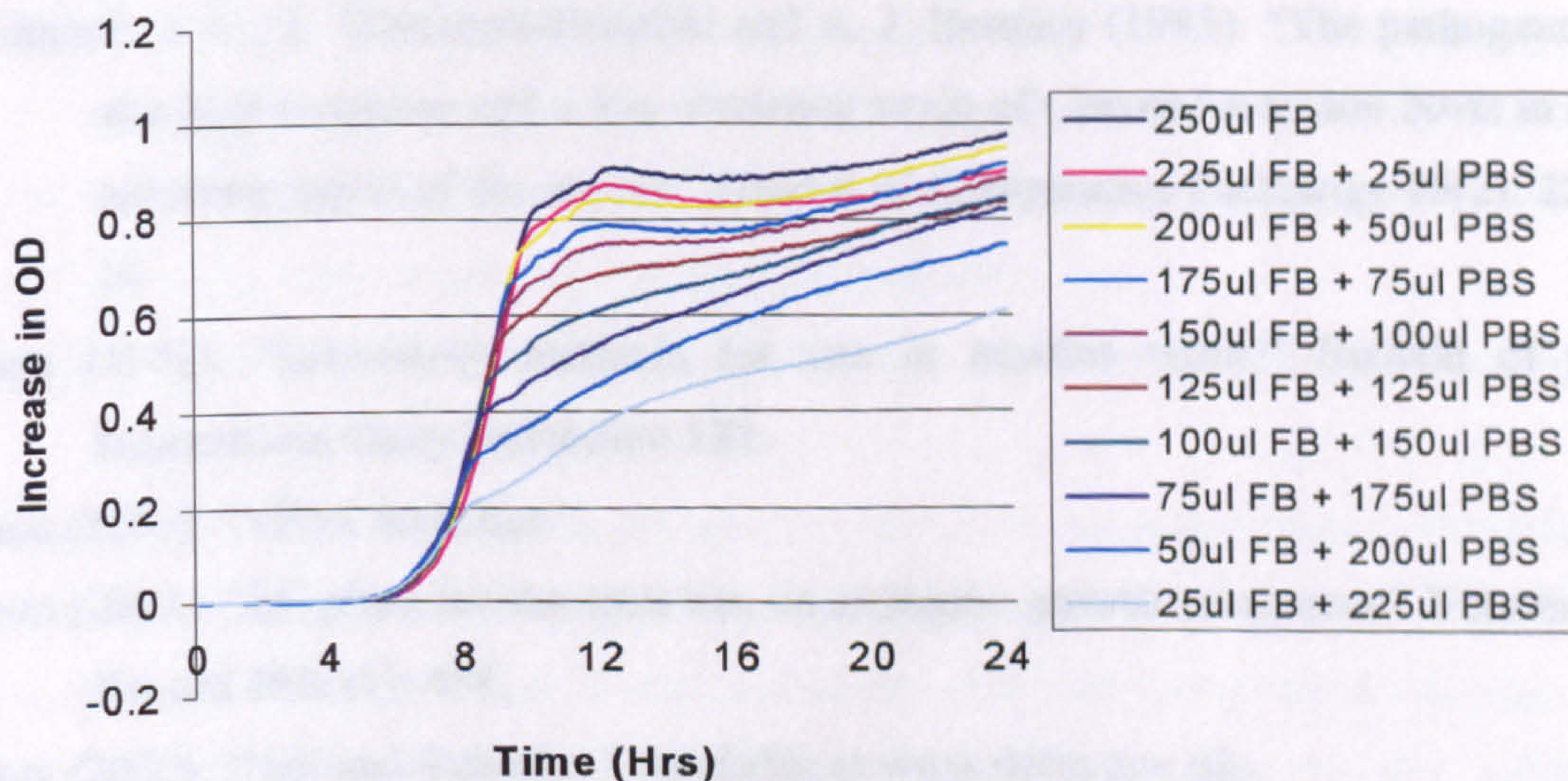
5' - CTG GGA AAC TGG GTC TAA TAC CGG ATA GGA CCA ATC TTT AGT GTG GTT GGT GGA AAG TTT TGT CGG
TAC GAG ATG AGC CCG CGG CCT ATC AGC TTG TTG GTG GGG TAA TGG CCT ACC AAG GCG ACG ACG GGT
AGC CGG CCT GAG AGG GTG TAC GGC CAC ATT GGG ACT GAG ACA CGG CCC AGA CTC CTA CGG GAG GCA
GCA GTG GGG AAT ATT GCA CAA TGG GCG GAA GCC TGA TGC AGC GAC GCC GCG TGA GGG ATG ACG GCC
TTC GGG TTG TAA ACC TCT TTC GCT AGG GAA GAA GCC TTT TTG GGT GAC GGT ACC TGG ATA AGA AGC
ACC GGC TAA CTA CGT GCC AGC AGC CGC GGT AAT ACG TAG GGT GCG AGC GTT GTC CGG AAT TAC TGG
GCG TAA AGA GCT CGT AGG TGG TTT GTC GCG TCG TTA GTG AAA GCC CGG GGC TTA ACT CCG GGT CTG
CTG GCG ATA CGG GCA TAA CTT GAG TGC TGT AGG GGA GAC TGG AAT TCC TGG TGT AGC GGT GGA ATG
CGC AGA TAT CAG GAG GAA CAC CGA TGG CGA AGG CAG GTC TCT GGG CAG TAA CTG ACG CTG AGG AGC
GAA AGC ATG GGT AGC GAA CAG GAT TAG ATA CCC TGG TAG TCC ATG CCG TAA ACG GTG GGC GCT AGG
TGT GGG GGT TTT TCA CGA CTT CCG TGC CGT AGC TAA CGC ATT AAG CGC CCC GCC TGG GGA GTA CGG
CCG CAA GGC TAA AAC TCA AAG GAA TTG ACG GGG GCC CGC ACA AGC GGC GGA GCA TGT GGA TTA ATT
CGA TGC AAC GCG AAG AAC CTT ACC TGG GCT TGA CAT GTA CGG GAT CGG CGT AGA GAT ACG TTT TCC
CTT GTG GCT CGT ATA CAG GTG GTG CAT GGT TGT CGT CAG CTC GTG TCG TGA GAT GTT GGG TTA AGT
CCC GCA ACG AGC GCA ACC CTT GTC TTG TGT TGC CAG CAC GTG ATG GTG GGG ACT CGC GAG AGA CTG
CCG GGG TTA ACT CGG AGG AAG GTG GGG ATG ACG TCA AAT CAT CAT GCC CCT TAT GTC CAG GGC TTC
ACA CAT GCT ACA ATG GTC GGT ACA GTGG GTT GCG ATA CCG TGA GGT GGA GCT AAT CCC TTA AAG CCG
GTC TCA GTT CGG ATT GGA GTC TGC AAC TCG ACT CCA TGA AGT CGG AGT CGC TAG TAA A – 3'

The partial 16S rRNA gene sequence of isolate M218 is identical to that of isolate 4465.

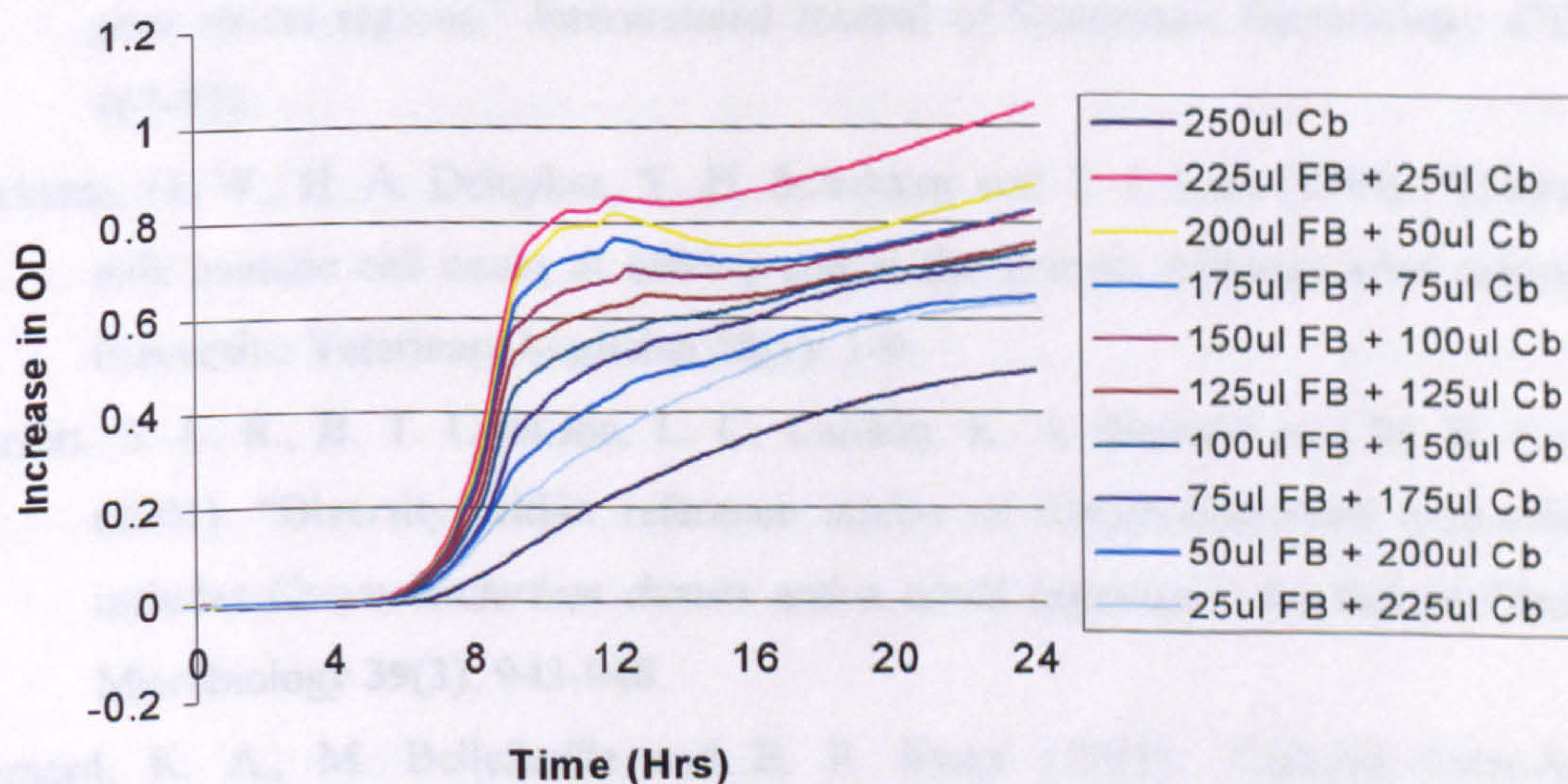
APPENDIX V: EFFECT OF FILTER STERILIZED C. BOVIS BROTH ON THE GROWTH OF E. COLI.

Figure AV.I: Effects of Filter Sterilized *C. bovis* Broth (Isolate 122), on the Growth of *E. coli* (Isolate 1599)

a. Control broth cultures containing varying proportions of PBS



b. Test broth cultures containing varying proportions of filter sterilized *C. bovis* broth



(FB – Fresh broth, PBS – Phosphate buffered saline, Cb – Filter sterilized *C. bovis* broth)

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