Prophylactic Efficacy of Chloramphenicol and Oxytetracycline in Bone Infection in Cattle.

By

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To

MY PARENTS

Certificate - 1

This is to certify that this thesis entitled, *"Prophylactic Efficacy of Chloramphenicol and Oxytetracycline in Bone Infection in Cattle"*, submitted for the degree of Master of Veterinary Sciences, in the subject of Veterinary Surgery and Radiology of Chaudhary Charan Singh Haryana Agricultural University, is a bonafide research carried out by Dr Ramesh Kumar, (93-V-247-M) under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

10/7/96

(A. P. Singh)
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Certificate - 2

This is to certify that the dissertation entitled, *"Prophylactic Efficacy of Chloramphenicol and Oxytetracycline in Bone Infection in Cattle"*, submitted by Dr Ramesh Kumar, (93-V-247-M) to the Chaudhary Charan Singh Haryana Agricultural University, in the partial fulfilment of the requirements for the degree of Master of Veterinary Sciences, in the subject of Veterinary Surgery and Radiology has been approved by the student's advisory committee after an oral examination on the same, in collaboration with an external examiner.

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HISAR

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CONTENTS

INTRODUCTION

Osteomyelitis, inflammation of marrow cavity, adjacent bone and epiphyseal cartilage, is clinically characterised by infectious lesions of the bone with the formation of purulent exudate within the bone. Treatment is aimed at the resolution of the infection by appropriate antibiotic therapy and surgical debridement if sequestrum exists. For the antibiotic therapy to be effective, it is essential that the drug reaches the site of infection in optimum concentration and this concentration is maintained for a duration sufficient to eliminate the infection completely. Information in the available literature concerning disposition of various antibiotics in the bone is inadequate especially for ruminants. While selecting an antibiotic for the treatment of osteomyelitis, factors to be considered include antibacterial spectrum, bactericidal or bacteriostatic action, pharmacokinetic properties, potential toxicity and relative cost (Knifton 1984).

A wound in the bone inevitably contains large haematoma and earlier it was thought that any infection existing in the haematoma would not be affected by antibiotic infused in systemic circulation. Bowers *et al* (1973), however, found that for thirty-six hours after haematoma formation, the clot can effectively be penetrated by high serum concentrations of antibiotics. Selection of an appropriate antibiotic is commonly predicted on the basis of *in vitro* activity of drug against cultured isolates and serum kinetics. Serum kinetics, however, is a poor indicator of antibiotic availability in different tissues including bone. Therefore, better information regarding the concentration of antibiotics in

different bones of various species is a pre requisite to an effective prophylactic and therapeutic regimen for the management of osteomyelitis. In addition, nature of drug, route of administration, volume of distribution, protein binding, metabolism and excretion are likely determinants of drug delivery to the bony tissue (Smilack *et al* 1976).

Prophylactic use of antibiotics in bone infection is controversial. Bowers *et al* (1973) are of the opinion that such practice gives false sense of security, promotes growth of resistant strains and super-infection may develop. On the other side, some controlled studies have shown that appropriate prophylactic use of antibiotics of short duration is effective in reducing the infection rate (Polk & Lopez-Mayor 1969, Fogelberg *et al* 1970).

To successfully treat a clinical case of osteomyelitis or to effectively check infection during orthopaedic surgery, it is imperative that effective dosage of the antibiotic and optimum duration of treatment is known. Considering lack of information in this respect in the bovine, kinetics of gentamicin and kenamycin in serum and metacarpus of normal and infected calves has been investigated in this laboratory (Singh 1992). In view of the fact that metacarpal and metatarsal bones in the bovine are more predisposed to injury and subsequent infection and also to evaluate another group of commonly used antibiotics, the present study in calves was planned with the following objectives:-

1 - To study the minimum inhibitory concentration of oxytetracycline and chloramphenicol *in vitro* against *Staphylococcus aureus*.

2 - To measure serum and bone marrow levels of oxytetracycline and chloramphenicol in normal calves.

3 - To study efficacy of oxytetracycline and chloramphenicol to prevent the experimental bone infection in calves.

REVIEW OF LITERATURE

Oxytetracycline is the most commonly used broad spectrum antibiotic in veterinary practice. Absorption from gut is hindered by calcium and magnesium ions. Plasma binding is 20 to 40 per cent and it diffuses throughout the body but with difficulty into the cerebrospinal fluid. Oxytetracycline is also deposited on active sites of ossification and has a large therapeutic index. Elimination is mainly through glomerular filtration from kidneys and is excreted largely as unchanged.

Chloramphenicol is a nonionised, highly lipid soluble compound which is well absorbed after parental or (except in ruminants) oral administration and distributed in all body tissues and fluids including cerebrospinal and ocular fluids. At the time of peak blood level 50 per cent of drug is bound to albumin. Elimination is mainly via kidneys, unchanged by glomerular filtration and as inactive degradation products by tubular secretion. Yunis & Bloomberg (1964) described two forms of toxicity: a dose dependant reversible suppression of erythropoiesis and an irreversible aplastic anaemia.

MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) of an antimicrobial agent is the minimum concentration of drug which results in complete inhibition of visible growth under specified conditions. One may consider an organism to be susceptible if it is inhibited by a concentration at least one half the mean blood level or one fourth the average peak blood level,

whereas it is considered to be resistant if the MIC is greater than peak blood level (Barry 1976).

A number of factors affect the available concentration of an antimicrobial agent at different sites of the body and the effect of these factors varies with both, the antimicrobial agent and the body tissue. Hence, MIC has inherent limitations as a guide to the concentration of an antimicrobial agent required *in vivo* at the site of infection to produce a favourable clinical response. In experimental and clinical infections, the bacteria are greater in concentration than used in laboratory for determination of *in vitro* activity. When realistic concentrations are used the antibiotic activity is reduced. The optimum selection of antibiotic, therefore, may require a re-examination of laboratory procedures for the determination of sensitivity data (Fry *et al* 1985).

According to Bachmann *et al* (1975), the *in vitro* efficacy of tetracycline (30 µg/ml) and chloramphenicol (30 µg/ml) against *Staphylococcus aureus* isolated from canine, equine, feline and bovine clinical samples was found to be 86 and 96 per cent, respectively. In another study, *Staphylococcus aureus* isolated from infected bones in canine was found to be 44 and 100 per cent sensitive to tetracycline and chloramphenicol, respectively (Hirsh & Smith 1978). The other MIC findings are tabulated as under:

S. No.	Antibiotic	MIC (µg/ml) against *Staphylococcus aureus.*	Investigators
1	Tetracycline	0.25 - 16.0	Hirsh & Smith (1978)
2	Tetracycline	0.7	Washington & Sutter (1980)
3	Chloramphenicol	0.5 - 4.0	Hirsh & Smith (1978)
4	Chloramphenicol	8.0	Washington & Sutter (1980)

DRUG DISPOSITION STUDIES

Disposition studies are of great importance in determining the dosage regimen of any antimicrobial drug. The dosage of antimicrobial drugs is generally computed so as to prevent the drug concentration at the site of infection from falling below the MIC for the causative organism.

It means that effective concentration of drug must be achieved and maintained at the focus of infection. Although blood concentrations of a drug have been used as a rough index of tissue values but the factors, such as, plasma protein and tissue binding, selective sequestration in tissues, pH and pK relationships, lipid solubility and diffusion characteristics guide its concentration in the body tissues. The serum and bone marrow ratios have varied over a ten fold range for many antibiotics. Some of these inconsistencies are due to different elimination kinetics in the two compartments (Norden 1975). Since osteomyelitis involves marrow cavity as well as cortical bone, it is possible that the levels of antibiotics attained in the marrow are better indicator of the possible efficacy of therapy than the ability of the antibiotic to penetrate into the bone itself (Norden 1971).

On administration of tetracycline (50 mg/kg) intravenously in rats, bone concentration of 30, 58 and 30 μg/g of bone were detected at one minute, one hour and 48 hour intervals, respectively (Buyske *et al* 1960). In another study, after an intravenous administration of chloramphenicol (20 mg/kg) in rats, peak serum and bone marrow concentration of 13.5 μg/ml and 6.1 μg/g respectively, were observed at 30 minute post administration interval. Whereas, at 3 hour interval its level declined to 8 μg/ml in serum and 0.6 μg/g in bone marrow (Summersgill *et al* 1982).

Yoder & Packer (1954) found that serum levels of oxytetracycline after intravenous administration rise more rapidly and attained peak value within 30 minutes than the intramuscular administration. Peak oxytetracycline serum level was attained at 2 hour post intramuscular administration interval. Although, with intramuscular administration the peak serum level was lower than with intravenous administration, but the rate of decline in serum was slower and therefore maintaining the serum therapeutic concentration for longer period.

Peak serum levels of oxytetracycline in buffaloes were found at 4 hour period after its intramuscular administration at the rate of 10 mg/kg (Mahashabde *et al* 1992 and Sadekar 1993). The therapeutic effective concentration of 3 μg/ml remained in buffalo blood for 1 to 12 hours (Mahashabde *et al* 1992). Friesian cattle receiving oxytetracycline (20 mg/kg) intramuscularly developed its peak concentration of 5.62 μg/ml in

serum in eight hours (Davy *et al* 1985). However, with similar dose and route Punch *et al* (1985) found its peak plasma concentration within two and half to four hours, in three out of four cows.

Peak plasma level of chloramphenicol were obtained after 4 hours of its intramuscular administration in calves @ 20 mg/kg. But the therapeutic effective concentration (≥ 5.0 μg/ml) in plasma was obtained as early as 2 hours and that remained up to 8 hours (Sisodia *et al* 1973). Studies of Tanner & Wuethrich (1985) in cows show different disposition kinetics; peak serum concentration of 1.7 μg/ml were achieved at 7.3 hours after intramuscular administration of similar dose. In horses, peak serum level of chloramphenicol (1 μg/ml) with similar dose rate and route was reported to reach within one hour; and in pigs with much higher dose rate (50 mg/kg) it took 6 hours to have peak serum level of 1.14 μg/ml (English & Withy 1959 English & Seawright 1961).

In dogs, Baggot *et al* (1977) could obtain oxytetracycline serum levels between 1.25 and 5.00 μg/ml by a prime dose of 10 mg/kg administered intravenously followed by topping-up with 7.5 mg/kg after every 12 hours. Serum oxytetracycline concentration of 1.25 μg/ml or above, is a therapeutic level for most of the susceptible microorganisms.

Verma & Paul (1983) have also worked out oxytetracycline dose regimen for buffalo calves to maintain serum concentration of 0.5, 1.0, 2.0 and 5.0 μg/ml. At serum levels ranging between 2 and 20 μg/ml, nearly 42 per cent of administered oxytetracycline remained bound to plasma proteins. Verma & Paul also proposed that tubular reabsorption plays a small role in excretion in buffalo calves and the main route of excretion is through glomerular filtration.

OSTEOMYELITIS

Various aspects of osteomyelitis are reviewed under following headings:-

MICROBIOLOGY

The bacteriological examination of infected bone over the years has laid open a wide range of organisms, but *Staphylococcus aureus* is the

most ubiquitous one among aerobic bacteria. It has been isolated from animals and man (Beaver 1971, Caywood *et al* 1978, Waldvogel & Vasey 1980 and Walker *et al* 1983-a). Other causative organisms associated with osteomyelitis in cattle include *Corynebacterium, Salmonella, Streptococci, Escherichia coli, Fusiformis necrophorus (Sphaerophorus necrophorus)* and *Actinomyces* (Hickman 1964, Funk 1978, Kessel *et al* 1982 and Firth *et al* 1987). The most common anaerobic bacteria associated with osteomyelitis in animals include *Bacteroids, Fusobacterium, Clostridium* and *Peptostreptococcus anaerobius* (Walker *et al* 1983-b).

EXPERIMENTAL PRODUCTION

Beginning with Rodet in 1884, many investigators have attempted to produce osteomyelitis in animals with different degree of success. Nordan (1970) reported that injection of bacteria alone, either intravenously or directly into the tibia of rabbit, failed to produce osteomyelitis, however, some workers reported success in experimental osteomyelitis with intravenous injection of bacterial suspension in chicken (Emsile & Nade 1983) or injection into the medullary cavity in dog (Varshney *et al* 1989) and in rabbit (Finsterbusch *et al* 1970).

Appraisal of various experimental studies concluded that vascular occlusion secondary to septic thrombosis and consequent bone necrosis are important factors in establishment of osteomyelitis (Trueta 1959 and Waldvogel *et al* 1970). Sodium morrhuate, a sclerotic, when injected along with *Staphylococcus aureus* into the medullary cavity induced osteomyelitis in rabbits (Scheman *et al* 1941 and Nordan 1970). Similarly, in another study in dogs, osteomyelitis was induced by replacing sodium morrhuate with barium sulphate and mixture injected into the nutrient artery (Deysine *et al* 1976). Canine models of experimental osteomyelitis have been developed by inoculating *Staphylococcus aureus* along with acrylic cement or cotton pieces as foreign nidus into the medullary cavity (Fitzgerald 1983, Quinlan *et al* 1983 and Braden *et al* 1987). The bovine model of experimental osteomyelitis was first produced successfully by Singh (1992) in calves using *Staphylococcus aureus* along with saw dust as foreign nidus. Same model was later used by Sangwan (1994) and Singh (1995) for further studies in calves.

Clinical Signs

The clinical signs of osteomyelitis vary widely and depend on its duration and severity. The common clinical signs include lameness, and localised soft tissue swelling, heat, pain and drainage (Caywood *et al* 1978, Cole *et al* 1982 and Varshney *et al* 1989). The additional signs of pyrexia, lethargy and reduced appetite were also noticed in early stages of osteomyelitis in calves (Singh 1992, Sangwan 1994 and Singh 1995). Pyrexia and depression were not common in chronic cases (Harari 1984), moreover, pyrexia was observed to be less frequent in cases of osteomyelitis due to less virulent organism, partially treated or in chronic cases (Waldvogel *et al* 1970). In bovines, the major clinical manifestations of limb bone osteomyelitis are lameness and swelling (Baird 1973).

Radiological Signs

The radiological signs are hardly confirmatory in early stages of osteomyelitis, however, careful attention to soft tissue shadows may reveal diagnosis (Ferguson 1975). Accumulation of purulent exudate within marrow cavity can be massive with minimum radiographic changes (Butt 1973).

The radiological signs of osteomyelitis in the bovine and equine include soft tissue swelling, regular or irregular radiolucent defects usually at metaphysis, increased medullary density, sclerotic zones around radiolucencies, new periosteal bone formation, cortical lysis and sequestration. (Baird 1973, Firth *et al* 1987, McDonald 1989, Singh 1992 and Singh 1995). Radiographic changes in rabbits (Nordan 1970), dogs (Walker *et al* 1975, Caywood *et al* 1978 and Varshney *et al* 1989), goats (Altmaier *et al* 1994) and in man (Nade 1983) are more or less similar.

The classic radiographic changes revealing sequestrum and involucrum are rarely encountered (Caywood et al 1978 and Harari 1984). The increased medullary density is explained by the response of the bone in forming excess intramedullary bone as infection is brought under control (Aegerter & Kirkpatrick 1968).

Osteomedullography

Osteomedullography is a contrast radiographic technique used to demonstrate intra and extraosseous venous circulation. Menegauz *et al*

(1953) were the first to use this technique to assess the fracture healing in man. Since then, several reports have been appearing in the literature about its efficacy in monitoring of delayed union, non-union, coxarthrosis and Perthe's disease (Kaski 1971, Arnoldi *et al* 1972, Puranen & Kaski 1974, Suramo *et al* 1974 and Boobbyer 1978).

The osteomedullography has been used to study the intraosseous venous channels of different long bones and to evaluate bone graft acceptance, fracture healing and cases of delayed union in calves and sheep (Singh & Nigam 1980, Singh *et al* 1982, Chawla *et al* 1983 and Vijaykumar *et al* 1985). Recently the importance of this technique for early diagnosis of experimental osteomyelitis and evaluation of efficacy of different therapeutic regimens has been emphasised by Sangwan (1994) and Singh (1995).

Histopathological Changes

In the bovine model of experimental osteomyelitis, heavy neutrophil infiltration in the marrow and proliferation of periosteum were observed at second post-inoculation week. Abscess and osteolytic changes developed along with periosteal and endosteal new bone formation at fourth week and marked endosteal new bone formation was found at twelfth week (Singh 1992). Working with similar model of experimental osteomyelitis, abscess formation, congestion, haemorrhage, serofibrinous exudation with infiltration of mononuclear and polymorphonuclear cells were observed in acute phase. In subsequent subacute phase at twentieth post-inoculation day, increased infiltration of inflammatory cells and fibrous tissue proliferation were observed (Sangwan 1994).

Almost similar histopathological changes have been reported in man (Bjorksten & Bouquist 1980) and dog (Fitzgerald 1983 and Varshney *et al* 1991).

Prophylaxis

Antibiotic prophylaxis (*Synonym:* antibiotic umbrella) refer to a state where an effective concentration of antibiotic is already present in the circulation when the infecting microorganisms enter the body. It implies that the administration of prophylactic antibiotic must start 24 to 72 hours preoperatively, depending on its pharmacokinetic profile. According

o Clawson & Dunn (1967) prophylactic use of antibiotics appropriately, ensures therapeutic level of antibiotics in any small haematoma, should it develop afterwards in the operative area.

Gustilo (1979) states two basic principles for use of antibiotics for prophylaxis, and they are: one, the antibiotic must be effective against the causative microorganism. Second, it must be administered in appropriate dose, so that its bactericidal or bacteriostatic concentrations can be maintained in the bone. Hierholzer *et al* (1974) are of the opinion that such a prophylactic antibiotic must be bactericidal by virtue of its effect on cell wall synthesis, even in a good proliferating medium, as it is in acute inflammation. An effective antibiotic given before, during and after surgery significantly reduces the postoperative infection rate (Boyd *et al* 1973, Pavel *et al* 1974 and Gustilo & Anderson 1976). However, it has been suggested that prolonged antibiotic use does not appear to reduce infection rate and a period of 3 to 5 days is considered as an appropriate duration (Gustilo & Anderson 1976, Patzakis 1982 and Mader & Cierny 1984). Clinical reports also support this view. Prophylactic use of antibiotics for 3 to 6 days or 7 to 10 days, appear to be similar in prevention of the rate of postoperative wound infection (Altemeier *et al* 1955, Boyd *et al* 1973 and Pavel *et al* 1974). Aforementioned generalisation may not hold similar promises for ruminants. Gentamicin and kenamycin started immediately after inoculation of infection and continued for another 10 days precluded the osteomyelitis in calves (Singh 1992); however, use of kenamycin only for three days failed to prevent bone infection (Singh 1995). This necessitates further detailed studies on pathogenesis and prophylactic antibiotic use in osteomyelitis in ruminants.

MATERIALS & METHODS

Experimental design of the present study was done in four major sections *viz*, *In vitro* efficacy studies, experimental studies, disposition studies and prophylactic trials.

IN VITRO EFFICACY STUDIES

The minimum inhibitory concentration of oxytetracycline and chloramphenicol *in vitro* was estimated against *Staphylococcus aureus* by agar dilution method (Washington & Sutter 1980) with certain modifications as described by Chaudhary (1985).

DILUTIONS OF ANTIBIOTICS

Serial two fold dilutions of oxytetracycline and chloramphenicol (160.00 to 1.25 μg/ml) were prepared in sterilised Muller-Hinton broth[1] by the method described by Washington & Sutter (1980).

PREPARATION OF MULLER-HINTON AGAR PLATES

For each of the above mentioned dilutions of antibiotics, plates were prepared by mixing 1 ml of the drug solution with 9 ml of sterilised Muller-Hinton agar medium[2]. The plates were incubated at 37 °C for 24 hours to check any contamination.

INOCULUM PREPARATION

Inoculum was prepared by suspending a 24 hour growth[3] of *Staphylococcus aureus* in 0.9% saline solution. It was then matched to 0.5

1 = Hi-Media Laboratories Private Limited, Mumbai, India.
2 = Hi-Media Laboratories Private Limited, Mumbai, India.
3 = *Staphylococcus aureus* was cultured on nutrient agar plates for 24 hours.

McFarland Tube No. 1. This McFarland tube was prepared by adding 0.5 ml of 0.048 M (1.175%) $BaCl_2.2H_2O$ into 99.5 ml of 0.36 N (1% *v/v*) H_2SO_4.

Plate Inoculation

The Muller-Hinton agar plates, containing different concentrations of test antibiotics, were inoculated by streaking with 1 mm loop full of the inoculum. Streaking was done immediately after the inoculum was prepared. Finally the inoculated plates were incubated at 37 °C for 24 hours.

Interpretation

After 24 hours of incubation of the Muller-Hinton agar plates, minimum inhibitory concentration (MIC) was recorded as the lowest concentration of test antibiotic that showed complete inhibition of microbial growth.

EXPERIMENTAL STUDIES

Experimental *in vivo* studies were done on 16 male healthy calves of 12 to 18 months and weighing 88 ± 3 kg. All calves were kept under similar husbandry conditions for a month before and during the entire period of experimentation. They did not receive any antimicrobial medication for 30 days before start of the experiment. The 16 calves were than randomly divided in four groups of four animals each for following experiments:-

Group No.	Treatment	Experimental Set-up
I	Uninfected	Disposition studies of oxytetracycline.
II	Uninfected	Disposition studies of chloramphenicol.
III	Infected	Control group – UNTREATED ANIMALS.
IV	Infected	Prophylactic trials of oxytetracycline.

Experimental Osteomyelitis

Staphylococcus aureus isolated from a pus sample of a clinical case was used for inducing experimental osteomyelitis. Routine microbiological test identified this isolate as haemolytic and coagulase positive. Antibiotic

sensitivity test labelled it as sensitive to oxytetracycline, chloramphenicol, ciprofloxacin, amikacin and cephalaxin, but resistant to polymixin-B and penicillin-G.

An optical density measure of trypticase soy broth suspension of bacteria was used to estimate the number of organisms in the inoculum. Each animal received 4 ml of inoculum containing approximately 7×10^9 *Staphylococcus aureus* in one millilitre. Metatarsal bone was used for induced osteomyelitis.

INFECTION OF METATARSUS

Experimental osteomyelitis was produced in calves Group-III and Group-IV. Distal medial metaphysis of the right metatarsal bone was selected for intramedullary injection of inoculum. Each calf was held comfortably in right lateral recumbency and the site was prepared for aseptic minor surgery. Area was anaesthetised by layer-wise infiltration of about 4 ml of lignocaine[4]. A stab incision deep up to the periosteum was made and a hole was drilled to provide access to the medullary cavity. A sterilised polyethylene catheter, with luer mount adapter on free end, was snugged in the hole. About 5 to 6 ml of bone marrow was aspirated through a glass syringe to make space for 4 ml of inoculum to be injected therein. This was followed by intramedullary infusion of a pinch of sterilised saw dust suspended in about 2 ml of 0.9% saline solution (Singh 1992). Stab incision was closed with a horizontal mattress suture.

DISPOSITION STUDIES

Drug disposition studies were done in eight calves of Group-I and Group-II. These calves had not received inoculum. Calves of Group-I received a single intramuscular dose of oxytetracycline[5] @ 20 mg/kg and calves of Group-II received chloramphenicol[6] @ 20 mg/kg. Central pool

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4 = XYLOCAINE™: 2% Lignocaine Hydrochloride IP (20 mg/ml) ASTRA-IDL Ltd., 12th Mile, Bellary Road, Bangalore, India.

5 = OXYSTECLIN™: Oxytetracycline Dihydrate IP (50 mg/ml); Ambalal Sarabhai Ltd., Veterinary Division, Sarabhai Chemicals, Wadi Wadi, Baroda, India.

6 = NEOCHLOR™: Chloramphenicol IP (100 mg/ml), Health Line Pvt. Ltd., Bangalore, India.

serum and bone marrow samples from these calves was collected for assay.

Serum Sampling

About 10 ml of venous blood was collected in sterilised test tubes from jugular vein. Blood samples were drawn before and at 1, 2, 4, 6, 8, 12 and 24 hour interval after administration of the test antibiotic. Blood was allowed to clot and serum harvested. Serum and working standards of antibiotics were stored at 4 °C. They were then assayed within 24 hours.

Bone Marrow Sampling

Bone marrow of metatarsus was approached by aseptic technique as described earlier in *'Infection of Metatarsus'* under section of *'Experimental Studies'*. Bone marrow samples were aspirated before and at 1, 2, 4, 6, 8, 12 and 24 hour interval after the administration of the test antibiotic. Blood or its clot if aspirated with the marrow was excluded and bone marrow stored at 4 °C.

Antimicrobial Assay

Large plate microbial assay technique as described by Bennet *et al* (1966) and modified by Chaudhary (1985) was used for antibiotic assay.

Pure powdered form of oxytetracycline[7] (100% potency) and chloramphenicol[8] (90% potency) was used for preparation of their stock solution. The stock solution of oxytetracycline (1000 μg/ml) was prepared in 0.1 N hydrochloric acid, whereas intermediate and working standards were prepared in 0.1 M monopotassium phosphate buffer[9] with pH of 4.5. The stock solution of chloramphenicol (1000 μg/ml) was prepared in ethanol. The intermediate and working standards were prepared in 1% phosphate buffer[10] with pH of 6.0. The working standards of both the test antibiotics ranged from 64.000 to 0.125 μg/ml that were diluted by serial two fold dilution.

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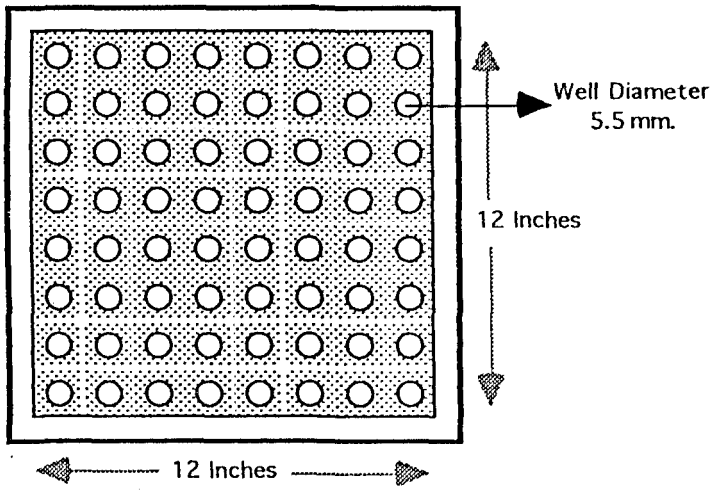
7 = Hi-Media Laboratories Private Limited, Mumbai, India.
8 = Hi-Media Laboratories Private Limited, Mumbai, India.
9 = 0.1M MONOPOTASSIUM PHOSPHATE BUFFER, pH 4.5 ± 0.05: *Composition*: Monobasic potassium phosphate 13.6 g and Distilled water to make 1000 ml.
10 = 1% PHOSPHATE BUFFER, pH 6.0 ± 0.05: *Composition*: Dibasic potassium phosphate 2 g Monobasic potassium phosphate 8 g and Distilled water to make 1000 ml.

For assay of oxytetracycline, Antibiotic Assay Medium[11] (No. 8) was used as medium for culture and *Bacillus cereus* var. *mycoides*[12] (ATCC-11778) as test organism. Spore suspension of the test organism was prepared in accordance with methodology described by Arret *et al* (1971). One millilitre of this suspension was used for every 250 ml of agar. Similarly for assay of chloramphenicol, Antibiotic Assay Medium[13] (No. 1) was used as medium for culture and *Sarcina lutea*[14] (ATCC-9341) as test organism. Bacterial suspension of test organism was prepared as described by Arret *et al* (1971) and 5 ml of this suspension was used for every 250 ml of the medium.

ASSAY PROCEDURE

The prepared test organism suspension was added to the sterilised assay medium at 56 °C and mixed before pouring on sterilised glass plates of 14 x 14 inches (closed inner area of 12 x 12 inches). These glass plates had been carefully levelled and kept in sterile air chambers. After pouring the assay medium with test organism *i.e.* seeded agar, the glass plates were sealed and placed in a refrigerator as 'ready for use within 24 hours'.

At the time of use, 64 wells of 5.5 mm diameter, in 8 rows and columns were punched *(See diagrammatic representation of the Large Plate)* in a



Large Plate for Microbial Assay

11 = ANTIBIOTIC ASSAY MEDIUM NO 8: Hi-Media Laboratories Private Ltd., Mumbai, India.
12 = CULTURE NO. ATCC-11778: Institute of Microbial Technology, Chandigarh, India.
13 = ANTIBIOTIC ASSAY MEDIUM NO 1: Hi-Media Laboratories Private Ltd., Mumbai, India.
14 = CULTURE NO. ATCC-9341: National Chemical Laboratory, Poona, India.

bacteria free environment. In each well 40 μl of unknown or test samples and standard solutions in triplicate was poured with a push button automatic micro-pipette. These large plates were then sealed with glass top to prevent ambient contamination and were kept at room temperature for about 15 minutes to allow proper diffusion of solutions.

Plates seeded with *Sarcina lutea* bacterial suspension for chloramphenicol assay were incubated at 32 to 35 °C, and those seeded with *Bacillus cereus* var. *mycoides* spores for oxytetracycline assay were incubated at 30 °C. For both assays plates were incubated for 18 hours and zones of bacterial growth inhibition were measured with electric zone reader. Standard curve for oxytetracycline and chloramphenicol were plotted from the size of zones of inhibition of reference standards. From these standard curves unknown concentration of these antibiotics in serum and bone marrow at different intervals were determined.

CALCULATION OF DOSE REGIMEN

Based on the observed pharmacokinetic profile of oxytetracycline and also keeping in view the minimum inhibitory concentration against *Staphylococcus aureus*, maintenance dose interval and route of administration, the loading and maintenance doses were computed. This dose regimen was then used in prophylactic trials.

Since chloramphenicol was not detected in the bone marrow, using the described assay procedure, dose regimen for chloramphenicol was not calculated.

PROPHYLACTIC TRIALS

Four infected calves from Group-IV were used for prophylactic trial of oxytetracycline. From the disposition studies the calculated prophylactic regimen of oxytetracycline for bone infection in bovines was as under:-

1 - Loading dose	4.559 mg/kg
2 - Maintenance dose	2.974 mg/kg
3 - Route	Intramuscular
4 - Interval	24 hours

This prophylactic schedule was followed in calves and aforementioned loading dose was administered at the time when bone marrow was

inoculated with *Staphylococcus aureus*. It was followed by maintenance dose administered intramuscularly after every 24 hours for 5 days. These calves did not receive any other medication during the course of study.

Calves of prophylactic and control groups, subjected to osteomyelitis, were monitored for its pathogenesis. Following parameters were considered for monitoring:-

Clinical Signs

Rectal temperature, status of appetite, general spontaneous activity, lameness, local swelling, pain at the site and drainage were monitored at regular intervals for 6 weeks in calves of prophylactic and control groups.

Radiological Examination

Medio-lateral radiographs were taken on second, fourth and sixth post-inoculation week either to confirm the infection or to monitor the progression of osteomyelitis.

Osteomedullography

Osteomedullography was done on sixth post-inoculation week in all calves of control and prophylactic group. Additionally, osteomedullography was done in four calves to establish normal venous circulation of the metatarsal region. For osteomedullography the calves were sedated with intravenous administration of 6% chloral hydrate[15] @ 50 mg/kg. A rubber tourniquet was applied above the tarsal joint. The bone marrow was approached in similar manner as described in *'Infection of Metatarsus'* under *'Experimental Studies'*. A 13 gauge hypodermic needle was introduced in the medullary cavity and 10 ml of sodium iothalamate[16] was injected rapidly. Medio-lateral radiograph was taken immediately after and at 2 minutes interval after completion of the injection. Tourniquet was then released and subsequent radiographs were taken at 5, 10 and 20 minutes post injection. High speed films and high speed intensifying screens were used. Radiographic exposure factors remained between 8 and 10 mAS, 60 and 65 kVP and 90 cm FFD without grid.

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15 = Chloral Hydrate IP: Chemical Centre, Bombay, India.

16 = CONRAY-420™: Sodium Iothalamate (70% w/v), Rhone-Poulenc (INDIA) Ltd., Rhone-Poulenc House, Worli, Bombay, India.

Cultural Examination

Bone marrow samples were also collected from the calves of control and prophylactic groups for bacteriological examination, at six week interval for presence or absence of *Staphylococcus aureus*.

Gross & Histopathological Examination

At the termination of the experiment, all calves of control and prophylactic group were humanely euthanised and test limb was dismembered at tarso-metatarsal joint. The limb was deskinned and made free from all soft tissue material. Metatarsal bone was then longitudinally cut in two halves with electric saw, to record any gross deviations from normalcy.

Bone tissue from area of maximum bony reaction was collected and decalcified in 5% nitric acid (solution were changed after every 12 hours). Decalcification of bone was confirmed by periodic pin-prick and radiographic examination. The decalcified bone specimens were processed in routine fashion and 5 micron thick paraffin embedded sections were cut and stained with Haematoxylin & Eosin.

ANALYSIS OF DATA

Data obtained was subjected to statistical analysis of mean and standard error.

RESULTS

IN VITRO EFFICACY STUDIES

The minimum inhibitory concentrations of oxytetracycline and chloramphenicol *in vitro* against *Staphylococcus aureus*, used to induce bone infection were found to be 2.0 and 4.0 μg/ml respectively, by agar dilution method.

DISPOSITION STUDIES

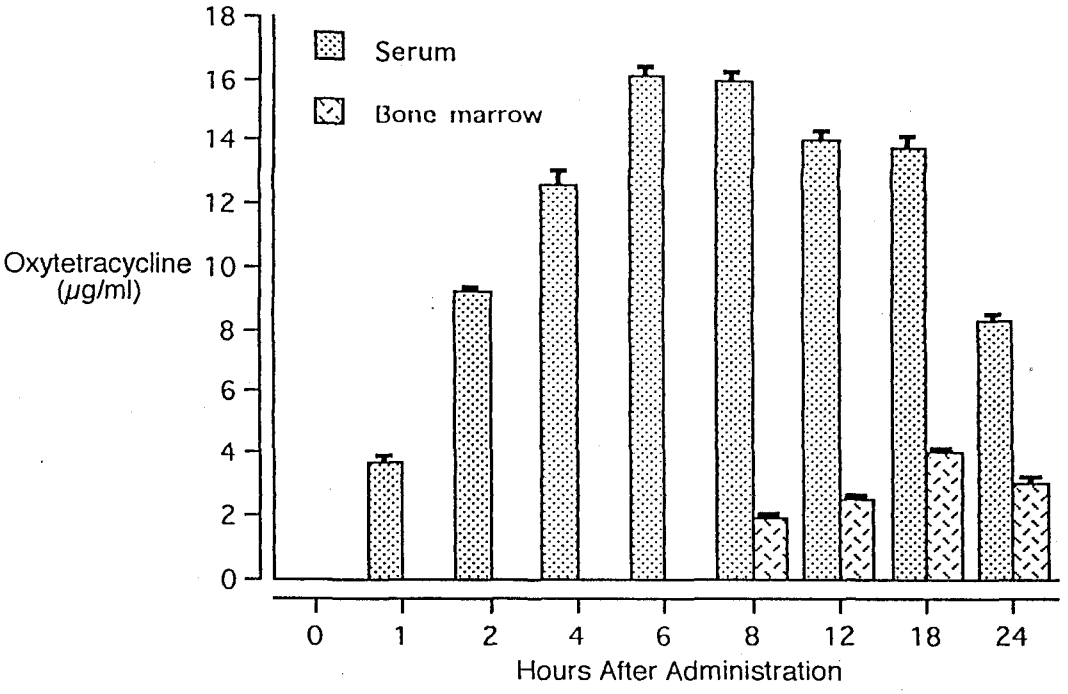
OXYTETRACYCLINE

Serum and bone marrow concentrations of oxytetracycline at different time intervals after its single dose (20 mg/kg) intramuscular administration are presented in table-1 and figure-1.

Table - 1: Oxytetracycline concentration (μg/ml) in serum and bone marrow of uninfected calves before and after its single intramuscular administration @ 20 mg/kg.

Hours after Adminis-tration	Oxytetracycline Concentration (μg/ml)			
	Central Pool Serum		Bone Marrow	
	Mean ± Standard Error	Range	Mean ± Standard Error	Range
Before	Not Detected	Not Detected	Not Detected	Not Detected
1	3.700 ± 0.182	3.3 to 4.1	Not Detected	Not Detected
2	9.200 ± 0.129	8.9 to 9.5	Not Detected	Not Detected
4	12.600 ± 0.454	11.8 to 13.8	Not Detected	Not Detected
6	16.125 ± 0.279	15.4 to 16.6	Not Detected	Not Detected
8	15.975 ± 0.239	15.4 to 16.5	1.875 ± 0.085	1.7 to 2.1
12	14.000 ± 0.316	13.2 to 14.6	2.475 ± 0.125	2.2 to 2.8
18	13.800 ± 0.316	13.0 to 14.8	3.975 ± 0.165	3.6 to 4.3
24	8.300 ± 0.212	7.8 to 8.7	3.025 ± 0.151	2.6 to 3.4

Figure - 1: Oxytetracycline concentration (μg/ml) in serum and bone marrow of uninfected calves before and after its single intramuscular administration @ 20 mg/kg.



A mean serum concentration of 3.7 ± 0.182 μg/ml was recorded at one hour after the administration of drug. Then, the concentrations in serum increased progressively and a peak concentration of 16.125 ± 0.279 μg/ml was observed at 6 hour. Thereafter, a gradual decline in the serum oxytetracycline concentrations was observed and a mean serum oxytetracycline concentration of 8.3 ± 0.212 μg/ml was recorded at 24 hour interval.

Oxytetracycline (1.875 ± 0.085 μg/ml) appeared in the bone marrow samples after eight hours of its intramuscular administration and it reached the peak of 3.975 ± 0.165 μg/ml at 18 hours. However, it declined to 3.025 ± 0.151 μg/ml by 24 hours.

The calculated loading and maintenance doses were 4.559 and 2.974 mg/kg, respectively, to be administered intramuscularly after every 24 hours.

CHLORAMPHENICOL

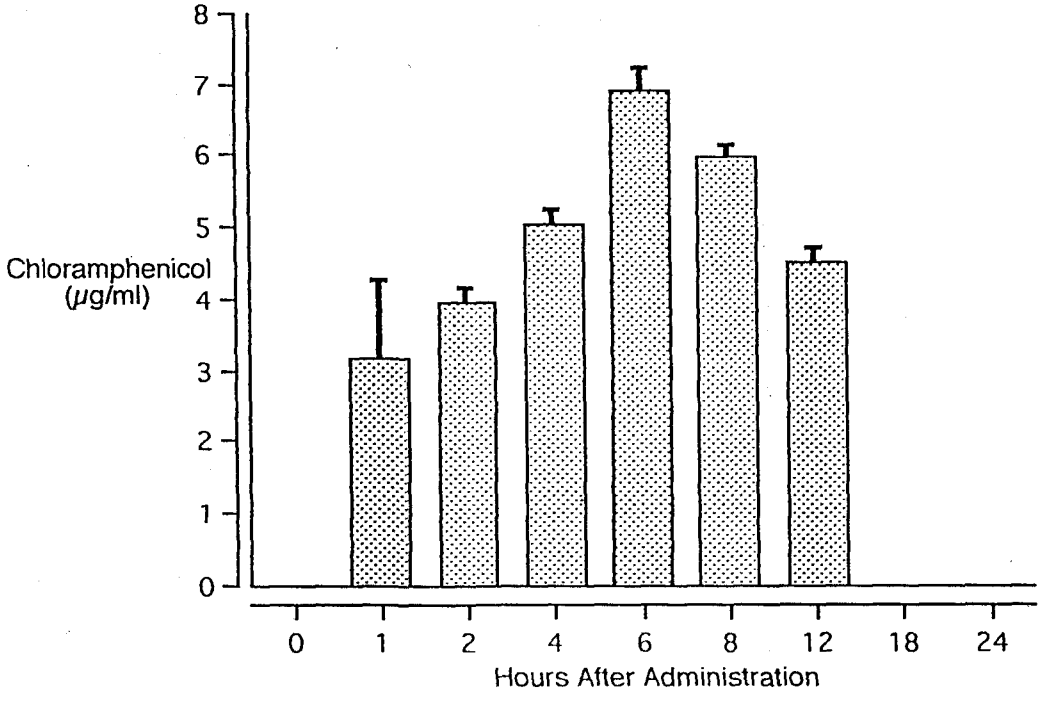
Serum and bone marrow chloramphenicol concentration at different intervals after its single intramuscular administration (20 mg/kg), are shown in table-2 and figure-2.

Table - 2: Chloramphenicol concentration (μg/ml) in serum and bone marrow of uninfected calves before and after its single intramuscular administration @ of 20 mg/kg.

Hours after Adminis-tration	Chloramphenicol Concentration (μg/ml)			
	Central Pool Serum		Bone Marrow	
	Mean ± Standard Error	Range	Mean ± Standard Error	Range
Before	Not Detected	Not Detected	Not Detected	Not Detected
1	3.175 ± 1.1	2.6 to 3.6	Not Detected	Not Detected
2	3.975 ± 0.165	3.6 to 4.3	Not Detected	Not Detected
4	5.025 ± 0.193	4.4 to 5.6	Not Detected	Not Detected
6	6.925 ± 0.271	6.8 to 7.4	Not Detected	Not Detected
8	5.975 ± 0.165	5.4 to 6.4	Not Detected	Not Detected
12	4.525 ± 0.188	4.2 to 4.9	Not Detected	Not Detected
18	Not Detected	Not Detected	Not Detected	Not Detected
24	Not Detected	Not Detected	Not Detected	Not Detected

The mean serum concentration of chloramphenicol at one hour after its intramuscular administration (20 mg/ml) was 3.175 ± 0.11 μg/ml. It reached its peak of 6.925 ± 0.271 μg/ml at 6 hours. At 12 hour it declined to 4.525 ± 0.188 μg/ml. Chloramphenicol was not detected in the serum at 18 and 24 post administration hour.

Figure - 2: Chloramphenicol concentration (μg/ml) in serum of uninfected calves before and after its single intramuscular administration @ 20 mg/kg.



All marrow samples collected before and after intramuscular administration of chloramphenicol at the rate of 20 mg/kg, did not contain chloramphenicol in detectable amount. Since chloramphenicol did not reach the bone marrow in detectable concentration. Chloramphenicol was not detected in bone marrow samples at any interval, before and after its intramuscular administration. Therefore, the dosage regimen was not calculated and the proposed prophylactic trial with this antibiotic was abandoned.

CLINICAL FINDINGS

The post-inoculation observations regarding general activity, lameness, swelling, pain and discharge in the calves of the control group are shown in table-3.

There was no significant change in rectal temperature during the period of investigation. All calves remained lethargic and showed decreased appetite for two weeks after induction of bone infection. All calves suffered from mild to moderate lameness for four weeks and remained lame, though mild, for next two weeks. One calf suffered from severe lameness for two weeks. A diffused swelling along the distal half of

Table - 3: Clinical observations in calves of control group. Figures in parenthesis indicate number of calves manifesting the signs.

WEEK	General Activity	Appetite	Lameness	Diffused Swelling	Pain on Palpation	Discharge
I	Reduced (4)	Reduced (4)	Severe (1) Moderate (3)	Soft (4)	Yes (4)	Absent (4)
II	Reduced (4)	Reduced (4)	Severe (1) Moderate (3)	Soft (2) Hard (2)	Yes (4)	Absent (4)
III	Reduced (2) Normal (2)	Normal (4)	Moderate (4)	Hard (4)	Yes (4)	Absent (4)
IV	Normal (4)	Normal (4)	Moderate (3) Mild (1)	Hard (4)	Yes (4)	Present (1) Absent (3)
V	Normal (4)	Normal (4)	Mild (4)	Hard (4)	Yes (3) No (1)	Present (1) Absent (3)
VI	Normal (4)	Normal (4)	Mild (4)	Hard (4)	Yes (3) No (1)	Present (1) Absent (3)

the metatarsus was observed in all the calves. Initially, that is, up to 2 weeks, the swelling was soft which became firm at the later stage (Fig. 3). Pain on palpation was evident in all calves throughout the observation period except in one calf where it remained only upto 4 weeks. Intermittent discharge was noticed only in one animal from third week onwards.

Calves receiving prophylactic oxytetracycline showed mild lameness and local swelling for only 3 to 4 days after inducing the infection. Thereafter, signs of lameness and swelling were not observed.

RADIOLOGICAL FINDINGS

Mild to moderate soft tissue swelling was a consistent radiographic feature observed at second post inoculation week in all the calves of control group (Fig. 4-A & B). Varying degree of generalised increased medullary density intermingled with radiolucent foci was another feature. Radiolucent foci were observed mostly in the distal half of the test bone around the site of infection - inoculation. In two calves, normally smooth endosteal surface was roughened and mild periosteal reaction with lytic changes were also observed (Fig. 5-A & B).

At fourth week, mild to moderate lamellar periosteal reaction, pronounced endosteal surface irregularities, multiple radiolucent foci, cortical lysis and purulent tracts were the important radiological signs (Fig. 6-A & B). However, severity of these changes was variable. In two animals lysis of epiphyseal plate was also noticed (Fig. 7-A & B).

Radiological signs observed at sixth week were almost similar to those of fourth week, however, in two animals more pronounced lytic changes, subperiosteal and cortical purulent tracts, widespread radiolucent zones and sequestra were observed (Fig. 8, 9-A & B).

Radiographic changes indicating evidence of bone infection were not observed in the animals of prophylactic group at any interval (Fig. 10-A & B), except in one calf where progressive sclerotic and reparative changes were demonstrated at fourth and sixth week (Fig. 11).

OSTEOMEDULLOGRAPHY

The technique used for osteomedullography helped in clear visualisation of the both extraosseous and intraosseous venous circulation of the metatarsal region. Deep sedation induced by intraosseous administration of chloral hydrate (6% w/v) provided good chemical restraint for drilling hole and inserting hypodermic needle into the medullary cavity. The contrast material could be injected without any discomfort to the calf.

NORMAL VENOUS CIRCULATION OF THE METATARSAL REGION

The injected contrast material in the medullary cavity pooled around the site of injection in the distal metaphysis presenting a more or less homogenous radio-opaque appearance that sometimes had spur like projections in ascending direction. Most of the contrast material then passed rapidly in to the venous sinusoids that radiated from the point of injection and drained into small venules. These venules appeared radiographically as a network of interlacing channels of varying sizes extending to varying distances proximally in the medullary cavity. The venular network in the distal metaphyseal region converged and formed the medullary veins that invariably are present on the endosteal surfaces and joined the venular network of the proximal metaphysis (Fig. 12-A & B). In addition to opacification of veins adjacent to endosteal surface, in one animal, the contrast material also radiated from the point of injection to the centre of medullary cavity. Though, it coursed for the most part in the centre of the medullary canal, it had an undulating course and at times approached the endosteal surface of the cortex. The nutrient vein was not clearly marked, however, in one radiograph it appeared to be present in the proximal third of diaphysis draining the medullary veins into the extraosseous veins. In the distal and proximal metaphyseal regions, the numerous venules drained in to large thin walled veins that emerged from the bone opacifying the draining extraosseus veins.

There were four sets of veins constituting the extraosseus venous circulation of the metatarsal region that included three superficial veins, namely, dorsal metatarsal vein, lateral plantar metatarsal vein and medial plantar metatarsal vein and one deep vein *i.e.* deep plantar metatarsal vein (Fig. 13 & 14).

Dorsal metatarsal vein was present on the dorsal surface of metatarsus that was a direct communication of dorsal common digital vein and proximally it continued as recurrent tarsal vein. A large anastomosing vein running obliquely downward and backward in the distal third of the metatarsus joined it to the distal plantar arch. The dorsal metatarsal vein was more clearly discernible on the medio-lateral projections.

Lateral and medial plantar metatarsal veins connected the distal and proximal plantar arches on the plantar surface of the metatarsus. They received the abaxial digital vein of the corresponding side beyond the distal plantar arch and continued proximally into the sphenous vein. From the proximal plantar arch, a perforating tarsal vein ascended towards the tarsal joint. These veins were better visualised on planto-dorsal projections.

The deep plantar metatarsal vein was of relatively smaller diameter that connected to the distal and proximal plantar arches and was more clearly demonstrated on the medio-lateral projections.

Three veins of smaller calibre were noticed in the proximity of metatarsal bone towards the median plane in planto-dorsal radiographs. These veins appeared to be the satellite veins accompanying the dorsal metatarsal artery, as per the anatomical distribution.

Radiographs taken at 5 minute post-injection interval showed almost complete clearance of the contrast material from the both extraosseous and intraosseous venous channels, however, pooling of the contrast material in the distal and proximal metaphyseal region was still evident. After 10 minutes of injection, the contrast material had completely disappeared from the medullary cavity (Fig. 15).

CONTROL GROUP

Osteomedullograms of osteomyelitic metatarsi did not show any evidence of the pooling of the contrast material in the distal or proximal metaphyseal region, however, the radiographic picture was different when compared with the normal one. The veins running close to endosteal surface were not clearly marked in most animals. Wherever, medullary

vessels were visualised, these had irregular and dilated course. In some animals, sacculations of medullary veins were clearly evident (Fig. 16). In most of the osteomedullograms, medullary cavity was comparatively more radio-opaque in appearance than normal.

The extraosseus venous channels were not seen except in two animals in which osteomedullograms demonstrated the enrichment of the extraosseus venous tributaries and tortuous course of the major veins of the region (Fig. 17). Contrast material in the medullary cavity was present even after twenty minutes of the injection (Fig. 18).

PROPHYLACTIC GROUP

Osteomedullograms obtained at sixth week after the prophylactic oxytetracycline treatment showed no evidence of stasis of the contrast material either in distal or proximal metaphyseal region, however, slightly increased opacity of the medullary canal was evident (Fig. 19). In one animal, mild stasis of the contrast material was noticed in the distal metaphyseal region but not in the proximal metaphysis (Fig. 20). In none of the animals, dilatations or sacculations of the medullary vessels were visualised.

The contrast material disappeared almost completely after 10 minutes of injection (Fig. 21).

CULTURAL EXAMINATION

Bacteriological examination of marrow specimen taken at sixth week confirmed the presence of *Staphylococcus aureus* organisms in control animals.

Bone marrow aspirates from the animals of prophylactic group were found sterile on cultural examination.

GROSS OBSERVATIONS

The test bone of the animals of control group appeared thick and irregular. Extensive adhesions were noticed between the surrounding soft tissues and the bone. Longitudinal section of the infected bone revealed haemorrhagic marrow having purulent tracts, necrotic foci and endosteal

and periosteal bone formation (Fig. 22). However, these gross changes were not seen in the animals of prophylactic group (Fig. 23).

HISTOPATHOLOGICAL OBSERVATIONS

Histopathological observations confirmed the presence of osteomyelitic changes in the animals of control group. The periosteum was thickened due to fibrous tissue proliferation. Endosteal and periosteal new bone formation was seen. The osteolytic changes with presence of scattered necrotic foci were observed in most of specimens. Fibrosis around the foci of inflammation was also evident along with infiltration of macrophages in the marrow (Fig. 24 & 25).

In the animals of prophylactic group these histopathological changes were absent. However, in one animal infiltration of macrophages along with fibrosis was evident (Fig. 26).

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DISCUSSION

In the present study, experimental osteomyelitis was produced by injecting *Staphylococcus aureus* along with sterile saw dust directly into the medullary cavity of the metatarsus in calves. The vascular stasis due to septic microembolisms is thought to enhance the development of osteomyelitis (Waldvogel *et al* 1970). A foreign nidus fulfils the requirement of microembolism and produces obliteration of small precapillary arterioles with infected microemboli (Deysine *et al* 1976). Sodium morrhuate (Norden 1970), barium sulphate (Deysine *et al* 1976) and acrylic bone cement or cotton piece (Fitzgerald 1983) have been tried as foreign nidus in producing osteomyelitis with varying degree of success. Saw dust used in the present study appears to fulfil the role of foreign nidus in producing microembolism and obliteration of small vessels. Saw dust along with bacterial inoculum has been used previously to produce experimental osteomyelitis in calves with encouraging results (Singh 1992, Sangwan 1994 and Singh 1995). Norden (1970) failed to produce osteomyelitis by injecting bacteria alone either intravenously or directly in to the tibia of rabbits. However, Emslie & Nade (1983) and Varshney *et al* (1989) were able to produce osteomyelitis by injecting bacteria alone intravenously in chicken and by direct infusion in to bone of dogs, respectively.

In the control animals, clinical signs of reduced general activity, decreased appetite, lameness, swelling, pain and drainage were observed. These clinical signs were severe up to second postinoculation week. Afterwards, the clinical signs subsided and that could be due to host

defence mechanism isolating the infection by reactive new bone formation. During third and fourth week, mild to moderate lameness and swelling at and around the site of inoculation were observed. Afterwards, mild lameness and swelling were the main clinical manifestations up to the sixth week. The different clinical signs observed in the present study have been reported to occur in clinical and experimental osteomyelitis in cattle and dogs (Weaver 1972, Baird 1973, Caywood *et al* 1978, Firth *et al* 1987, Varshney *et al* 1989, Singh 1992 and Singh 1995).

The radiological signs of osteomyelitis in the present study at second post-inoculation week included increased intramedullary density, radiolucent foci, roughening of endosteal surface and periosteal reaction. Similar radiological signs have been reported by the end of second week in rabbit, man and dog (Norden 1970, Nade 1983 and Varshney *et al* 1989). However, Singh (1995) did not find any significant radiographic change in calves until the end of third week after inoculation. This might be due to the variations in the virulence of pathogen and or host defence mechanism. By the fourth week more pronounced radiological signs of osteomyelitis were recorded that included multiple radiolucent foci, periosteal reaction, cortical lysis and purulent tracts. Lytic changes, subperiosteal purulent tracts, cortical purulent tracts and sequestrum were observed at sixth week. Similar radiographic changes have been reported by other authors in different species (Norden 1970, Baird 1973, Walker *et al 1975*, Caywood *et al* 1978, Silverman & Ackerman 1978, Fitzgerald 1983, Nade 1983, Harari 1984, Varshney *et al* 1989, Singh 1992 and Singh 1995). The lytic changes in bone *i.e.* radiolucencies are the result of release of proteolytic enzymes from the lysed neutrophils and activation of osteoclasts to absorb bone salts. The bone, in an attempt to counteract infection, produces new bone either from periosteum or endosteum.

The technique used for osteomedullography demonstrated both extraosseus and intraosseous venous circulation of the metatarsal region. Deep sedation induced by chloral hydrate facilitated smooth performance of medullographic procedure by avoiding any discomfort to the animal while injecting the contrast material. The use of deep sedation and general or spinal anaesthesia has been recommended by others in man and

nimals (Steinbach *et al* 1957, Finsterbusch *et al* 1970, Puranen & Kaski 974 and Boobbyer 1978).

In the normal metatarsus, the injected contrast material was rained from the site of injection to opacify the medullary venous hannels. There were more than one medullary vein in the metatarsal one. The venular network of distal metaphyseal region converges and adiate proximaly as fine venous channels adjacent to endosteal surfaces. n addition to this, a separate venous channel that had an undulating ourse with most part in the centre of medullary cavity was also isualised. These findings are contrary to those reported by Singh & Nigam (1980). They described a single medullary vein in the normal metatarsal bone as central medullary vein. More than one venous channels has also been reported by Thomas *et al* (1982) in rabbits. The medullary vein during its course in the diaphysis receives unopacified blood from its tributaries so that the contrast material becomes diluted as it approaches the nutrient foramen. For this reason it becomes less visible as it traverses the shaft and nutrient vein may not be seen (Steinbach *et al* 1957). The four sets of extraosseus veins demonstrated in the osteomedullograms of the normal metatarsus are in agreement with previous anatomical and osteomedullographic reports (Sisson & Grossman 1961, Singh & Nigam 1980).

Osteomedullograms of infected metatarsi showed that medullary vessels were dilated and had sacculations. This indicates disturbance in the venous circulation and the observation is also supported by delayed clearance of the contrast material from the medullary cavity of the infected metatarsal bone. Similar observations had been made previously for infected tibia in calves (Sangwan 1994, Singh 1995). In bone with osteomyelitis , there is build up of inflammatory exudate due to lack of a true lymphatic system in the bone. Therefore, there is inadequate drainage of the affected area which leads to venous engorgement. Thus osteomedullograms of osteomyelitic bone show dilated venous channels which have sacculations or a tortuous course with increased number of tributaries.

The histopathological changes observed in the animals of control group were almost similar to those already reported (Bjorksten 1980, Hall

et al 1983, Nade 1983, Singh 1992, Singh 1995). The pathological changes included infiltration of macrophages in the marrow, osteolytic changes, granulation tissue proliferation in the marrow and both endosteal and periosteal reactive new bone formation. The structural signs of new bone formation were consistent with the radiological observations of presence of osteosclerosis around the inflammatory foci radiolucencies. Such histopathological changes are mainly related to inflammation and the reactive host defence mechanism.

Prevention of bone infection through prophylactic antibiotic therapy is a perpetual challenge to orthopaedic surgeons. An effective antibiotic given before, during and after surgery significantly reduces the postoperative infection rate (Boyd *et al* 1973, Pavel *et al* 1974, Gustilo & Anderson 1976). Bowers *et al* (1973) found that prophylactic regimen beginning within 24 hours after surgery did eliminate the observed tissue changes associated with infection but failed if regimen began after 24 hours. The bacteria contaminating an operative site must be susceptible to the antibiotic. Initially the organisms are few in number and concentrated in location and because of high metabolic activity necessary for replication, they are vulnerable to the effective antibiotic. At later stage, dispersal of bacteria into leukocytes and damaged tissues make them relatively inaccessible and may be accompanied by diminished metabolic activity that makes them less susceptible to the antibiotic.

The minimum inhibitory concentration (MIC) values are essential to calculate dosage regimen of antibiotics based on pharmacokinetic studies. In the present study, the MIC of oxytetracycline against *Staphylococcus aureus*, the organism used to induce bone infection, was found to be 2 μg/ml. In earlier clinical study of osteomyelitis in dogs, the MIC of oxytetracycline against *Staphylococcus aureus* has been reported to be in the range of 0.25 to 16 μg/ml (Hirsh & Smith 1978). In another experimental study, the *in vitro* MIC of oxytetracycline against *Staphylococcus aureus* has been reported to be 0.7 μg/ml (Washington & Sutter 1980). The variations in the MIC values appear to be because of different strains of microorganisms used in different studies and their individual susceptibility to the antibiotic used. The *in vitro* efficacy of an antibiotic *i.e.* MIC may not often correlate well with the clinical response

of an infected animal because of variations in the internal host environment and the laboratory conditions. Thus, the final determination of antibiotic efficacy in specific clinical situations requires well controlled trials rather than theoretic modelling based on *in vitro* activity. Only then the clinical significance of apparent *in vitro* activity of an antibiotic can be appreciated (Fry *et al* 1985).

In the present study, mean peak serum concentration of oxytetracycline was observed at six hours after its intramuscular administration in uninfected calves. Afterwards, the concentration started declining and at 24 hours it was much higher than the MIC value. The peak serum concentration of oxytetracycline has been reported at four hours after its intramuscular administration in cattle (Punch *et al* 1985) and in buffalo (Mohashabde *et al* 1992, Sadekar 1993). Whereas, Davy *et al* (1985) obtained peak blood level at eight hours post administration in castrated Fresian cattle. These variations could possibly be attributed to the differences in the bio-availability and pharmacodynamics of different commercial products used in different studies.

Oxytetracycline was detected in bone marrow at 8 hours after its administration and the peak bone marrow levels were attained at 18 hours. Bone marrow levels of oxytetracycline remained in a narrow range from 12 hours onwards *i.e.* up to 24 hours. This can be attributed to unique ability of oxytetracycline molecules to form a complex with bone calcium, that consequently prolongs the elimination processes. The present study was undertaken on the premise that the measurement of antibiotic in bone marrow following parenteral administration of a single dose could be used as an index of the potential antibiotic efficacy. The concentration of antibiotic in the bone marrow has been used to assess the suitability of parenteral administration of antibiotics for the prevention and treatment of experimental bone infection in calves (Singh 1992).

Based on the observed pharmacokinetic profile of oxytetracycline in calves and its MIC against *Staphylococcus aureus*, the dosage regimen for its intramuscular administration was computed. Our such dosage regimen for these calves was in close accordance with Verma & Paul (1983), who calculated it for buffalo calves, but not with Pilloud (1973).

The MIC of chloramphenicol *in vitro* against *Staphylococcus aureus* was found to be 4.0 μg/ml. Hirsh & Smith (1978) in their studies on clinical osteomyelitis in dogs found MIC of chloramphenicol ranging between 0,5 and 4.0 μg/ml. However, in an experimental study, the *in vitro* MIC of chloramphenicol against *Staphylococcus aureus* was as high as 8 μg/ml (Washington & Sutter 1980). The variations in the MIC values appeared to be due to different strains of the test microorganism and their individual susceptibility to the antibiotics used in different studies.

After intramuscular administration of chloramphenicol at the rate of 20 mg/kg in calves, its serum concentration reached the peak at 6 hours. This is couple of hours later than the report of Sisodia *et al* (1973). This variation can be attributed to differences in the bioavailability of the drug from the injection site, vehicle used, stabilisers added, solvent *etc* in the pharmaceutical preparations.

In the present study the mean serum concentration of chloramphenicol equal or above the MIC (4 μg/ml) remained between 4 and 12 hours. It implies that to maintain the MIC, its injection is to be repeated at least not later than 12 hours. Moreover, chloramphenicol could not be detected in any bone marrow sample collected over a period of 24 hours from uninfected calves.

Poor uptake of most of the antibiotics by non-inflamed bone may at times result in their too low bone marrow concentration to be detected by microbial assay procedure (Norden 1975, Smilack 1976, Summersgill *et al* 1982). This also explains the relative lower bone marrow content of oxytetracycline than the corresponding serum level. There could be many other reasons for this low bone marrow content. The antibiotic may bind to bone tissue and is not available in free form in bone marrow that could be detected in plate diffusion assay. Viscous nature of the bone marrow may also impair diffusion of the antibiotic in the agar medium. Poor diffusion consequently precludes the formation of zone of inhibition in tune with the actual bone marrow concentration. Bone vascular architecture and histoanatomy, physical properties, protein binding, metabolism and excretion of the antibiotic are some other factors that can influence their uptake by the bone tissue (Smilack *et al* 1976).

Summersgill and his associates were able to measure bone concentration of chloramphenicol only at 1 hour (1.8 μg/g) and at 2 hours (0.8 μg/g) after its intravenous administration at the rate of 20 mg/kg in rats. Thereafter, chloramphenicol could not be detected (Summersgill *et al* 1982).

The plate diffusion assay used in the present investigation was not sensitive enough to detect chloramphenicol concentrations below 3 μg/ml. Even if the chloramphenicol level below 3 μg/ml are detectable, it is still lower than the MIC of 4 μg/ml. Therefore, the use of chloramphenicol as a prophylactic antibiotic for osteomyelitis or bone infection due to *Staphylococcus aureus* is not justified.

Calves receiving prophylactic oxytetracycline did not show clinical signs of osteomyelitis, however, during first few days after inoculation local oedema and mild lameness were observed. This clinical picture can be related to postsurgical pain and early intraosseous venous congestion (Phillips *et al* 1967).

The typical radiological signs of osteomyelitis were not observed during the course of study in the calves receiving prophylactic oxytetracycline. However, in one calf progressive sclerotic and reparative changes were noticed during fourth and sixth postinoculation week. This reveals the effectiveness of prophylactic oxytetracycline regimen in most of the calves. An intramuscular loading dose of 4.56 mg/kg followed by a daily maintenance dose of 2.97 mg/kg of oxytetracycline is recommended to prevent bone infection in the bovine.

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SUMMARY & CONCLUSIONS

Metatarsus of calves was inoculated with *Staphylococcus aureus* and sterilised saw dust to study the efficacy of intramuscular administration of oxytetracycline and chloramphenicol (20 mg/kg) in preventing the development of bone infection.

Results were compared with untreated control animals. Osteomyelitis was induced by infusing 4 ml inoculum of *Staphylococcus aureus* (7×10^9 organisms/ml) along with sterile saw dust as foreign nidus, directly into the medullary cavity of test bone. The minimum inhibitory concentrations of the test antibiotics *in vitro* against *Staphylococcus aureus* were calculated.

For disposition studies, serial blood and bone marrow samples were collected at predesignated intervals over a period of 24 hours. Harvested serum and bone marrow, thus obtained, were subjected to assay studies by 'large plate microbial assay' method. *Bacillus cereus* var. *mycoides* (ATCC 117788) and *Sarcina lutea* (ATCC 9341) were used as test organisms for oxytetracycline and chloramphenicol, respectively.

The peak serum concentration of oxytetracycline (16.125 ± 0.279 mg/ml) and chloramphenicol (6.925 ± 0.271 μg/ml) were observed at 6 hours after their intramuscular administration. The peak bone marrow level of oxytetracycline (3.975 ± 0.165 μg/ml) was recorded at 18 post administration hour, while chloramphenicol was not detected in the bone marrow samples at any interval.

On basis of pharmacokinetic studies, initial loading and subsequent maintenance doses of oxytetracycline were computed as 4.56 mg/kg and 2.97 mg/kg/day, respectively, to be administered intramuscularly. The thus computed dose regimen was used in prophylactic trails.

The prophylactic schedule started on the day of inoculation (inducing infection) and continued for subsequent 4 consecutive days. As chloramphenicol was not detected in the bone marrow samples, the prophylactic trials with this antibiotic were not undertaken.

Prophylactic efficacy of oxytetracycline was evaluated on the efficacy of clinical signs, plain radiography, osteomedullography, cultural examination and histopathological studies. Experimental comparison was sought with infected untreated control calves.

In the control animals, clinical signs of reduced general activity, decreased appetite, lameness, local swelling, pain and drainage were observed at different intervals. However, these clinical signs were absent in the animals of prophylactic group except for local swelling and lameness during first 3 to 4 days after inoculation of infection.

Radiological examination of the test bone of control animals revealed increases medullary density, radiolucent foci, osteolytic changes, purulent tracts, endosteal and periosteal bony proliferations and sequestrum at different intervals. In two animals lysis of distal epiphyseal plate was also noticed. The radiographic changes were absent in the animals of prophylactic group except in one animal where progressive sclerotic and reparative changes were observed.

Osteomedullograms of normal metatarsal bone were helpful in demonstration of both intra and extraosseous venous channels. In control animals osteomedullograms showed dilated, tortuous or sacculated extraosseus and medullary vessels. Delayed clearance of the contrast material was also evident. However, these osteomedullographic changes were not seen in the animals of prophylactic group, except one animal where mild stasis of the contrast material was noticed in distal metaphyseal region.

Cultural examination revealed presence of *Staphylococcus aureus* in he bone marrow samples of control animals, but the metatarsus of the animals of prophylactic group were found sterile on bacteriological examination.

The histopathological studies revealed infiltration of macrophages, necrotic foci, granulation and fibrous tissue proliferation in the marrow replacing the fatty marrow, osteolysis and reactive periosteal and endosteal new bone formation in the animals of control group. These histopathological changes were not seen in the animals where fibrous and bony proliferative changes were noticed.

The following conclusions could be drawn from the results of the present study:-

- A loading dose of 4.559 mg/kg of oxytetracycline followed by maintenance dose of 2.974 mg/kg intramuscularly after every 24 hours for 4 days effectively prevents development of *Staphylococcus aureus* bone infection.

- The use of chloramphenicol in preventing bone infection should not be recommended if the minimum inhibitory concentration of this antibiotic against the causative organism is more than 3 μg/ml.

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Prophylactic Efficacy of Chloramphenicol and Oxytetracycline in Bone Infection in Cattle; Thesis submitted by Dr Ramesh Kumar for MVSc in Surgery & Radiology; Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana.
Word Processing: Macintosh, System 7.1, Microsoft Word v. 5.1; *Printed on:* Apple Style Writer-II. *Photocopy:* Micky Enterprises, Model Town, Hisar. .

FIGURES

Photographs as figures from 3 to 26

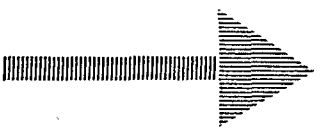


Figure 3: A calf from control group, 6 weeks after inducing infection. Note diffused swelling of the right metatarsal region *(arrow)* in comparison to contralateral limb.

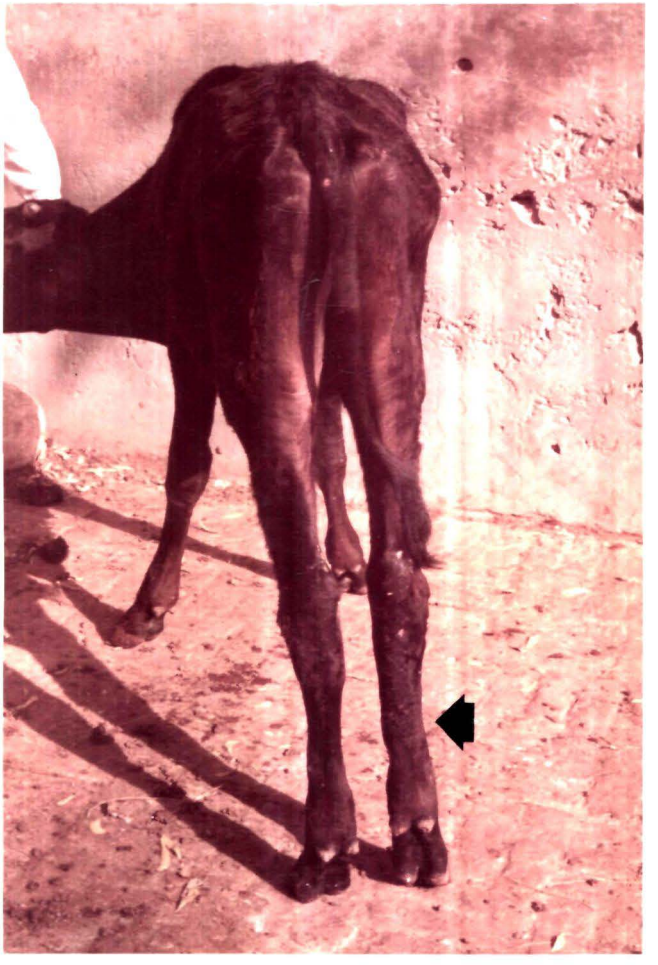
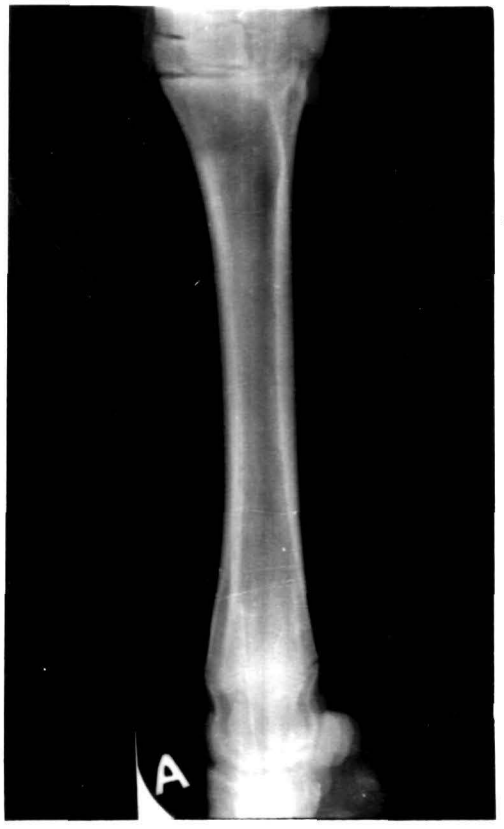


Figure 4-A: Medio-lateral radiograph of the normal metatarsus of a calf.

Figure 4-B: Medio-lateral radiograph of the infected metatarsus of a calf at 2 week interval *(Control Group)* showing soft tissue swelling of the metatarsal region. Arrow indicates site of inoculation.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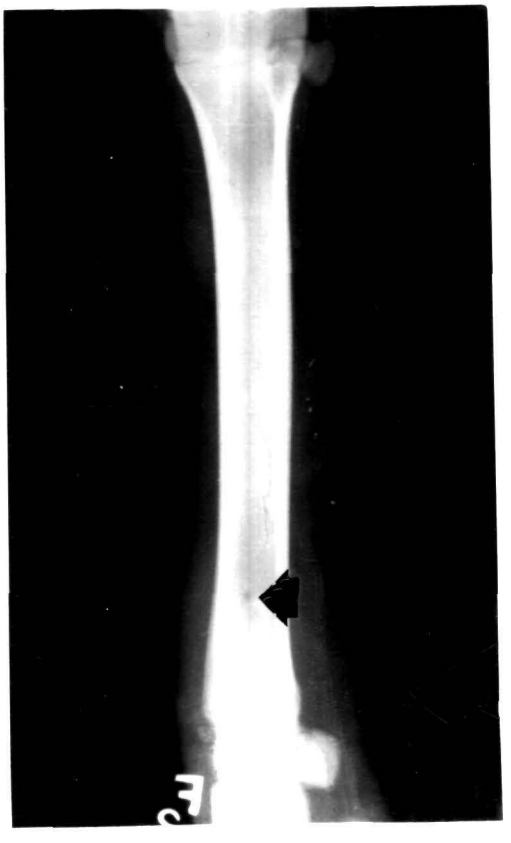
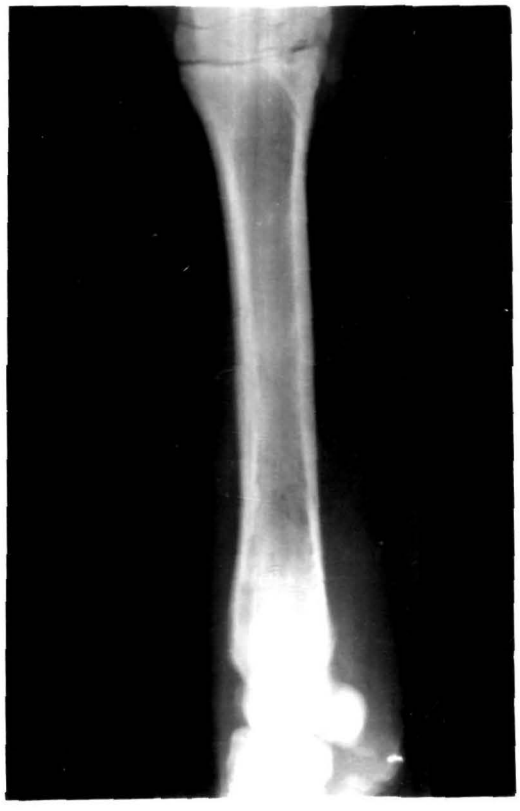


Figure 5-A: Medio-lateral radiograph of the infected metatarsus of a calf at 2 week *(Control Group)*. Note mild periosteal reaction, roughening of endosteal surface and radiolucent foci adjacent to the site of inoculation.

Figure 5-B: A close-up view of figure 5-A.

m = Periosteal reaction.
n = Roughening of endosteal surface.
o = Radiolucent foci.



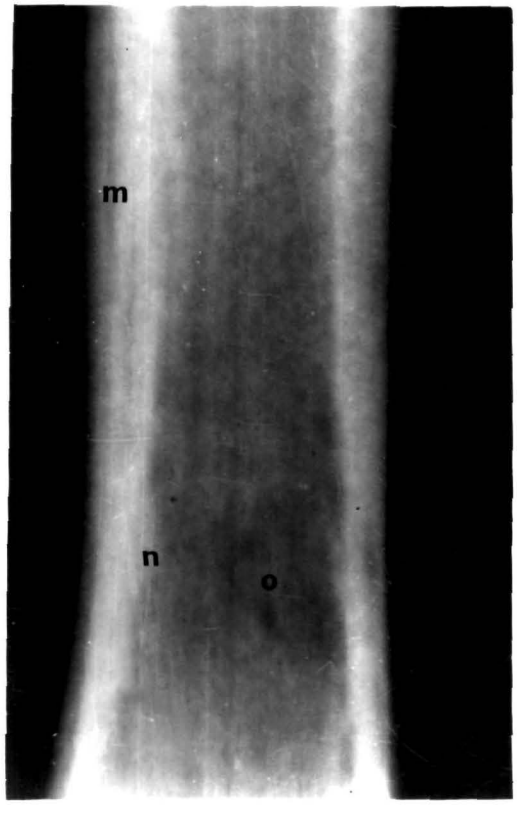

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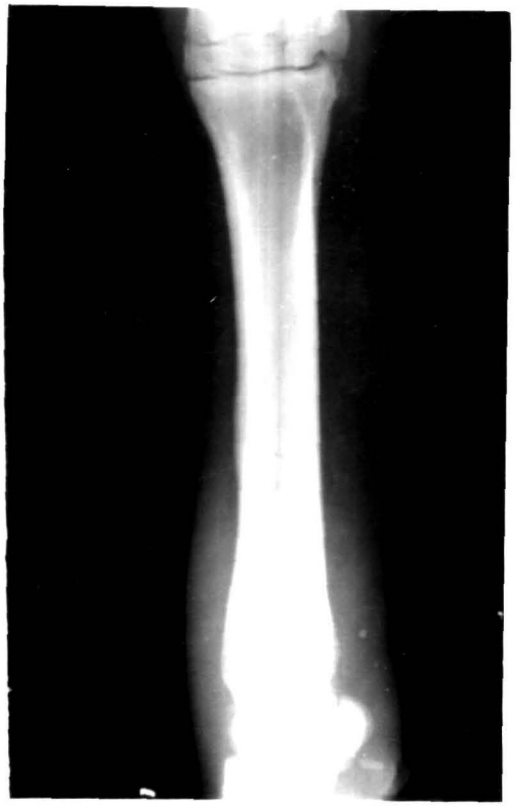
Figure 6-A: Medio-lateral radiograph of the infected metatarsus of a calf at 4 week *(Control Group)* showing periosteal reaction, radiolucent foci and roughening of endosteal surface.

Figure 6-B: A close-up view of figure 6-A. Note lamellar periosteal reaction.



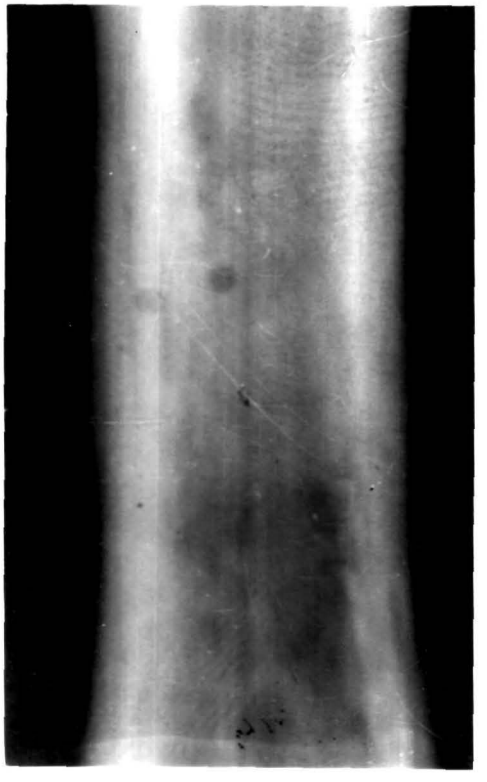
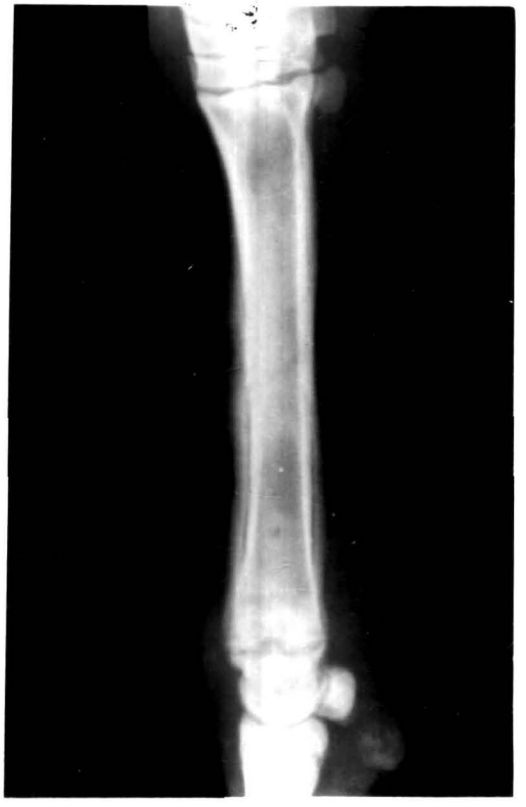


Figure 7-A: Medio-lateral radiograph of the infected metatarsus of a calf *(Control Group)* at 4 week. Note lamellar periosteal reaction and lysis of distal epiphyseal plate.

Figure 7-B: A close-up view of figure 7-A.



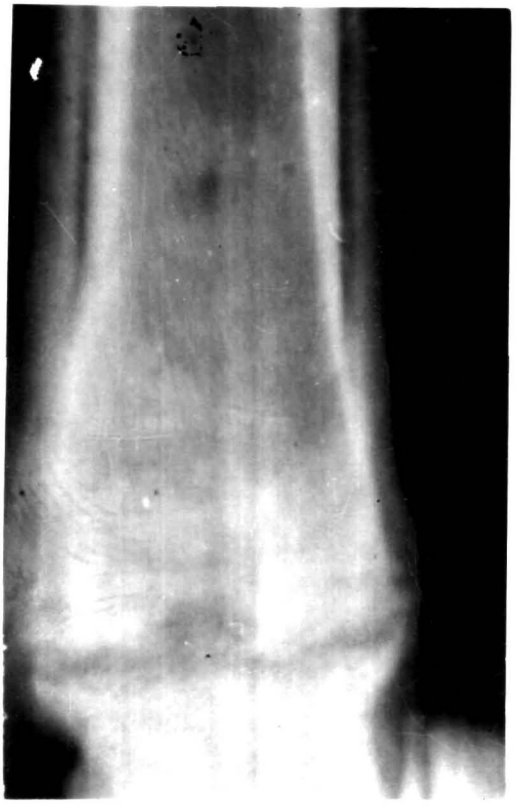


Figure 8: Medio-lateral radiograph of the infected metatarsus of a calf *(Control Group)* at 6 week. Note sub-periosteal and cortical purulent tract, extensive osteolytic changes and periosteal reaction.

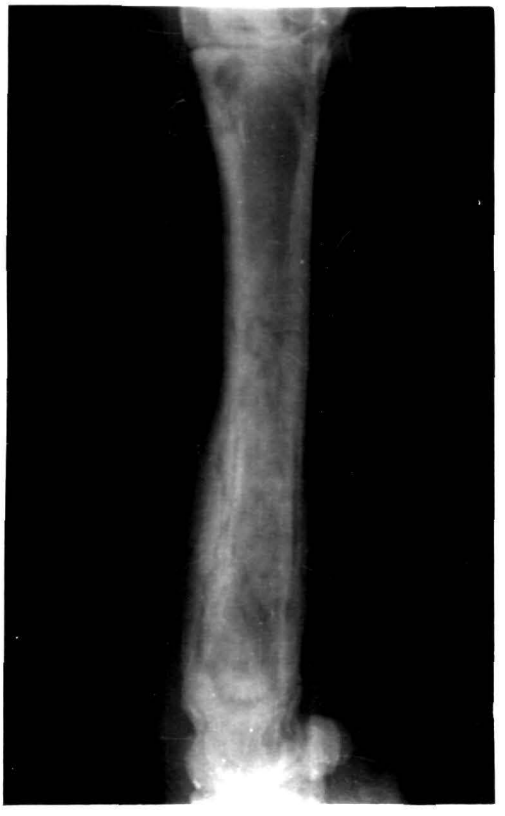
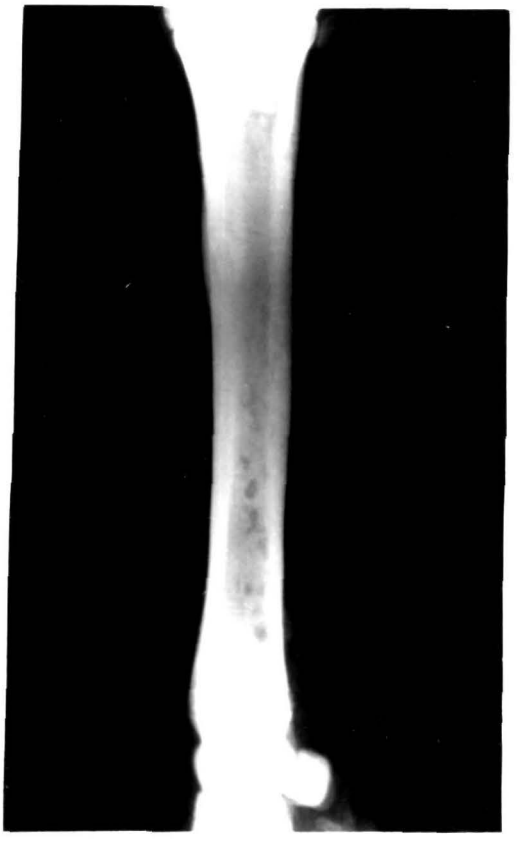


Figure 9-A: Medio-lateral radiograph of the infected metatarsus of a calf *(Control Group)* at 6 week. Note multiple radiolucent foci in the marrow and periosteal reaction.

Figure 9-B: A close-up view of figure 9-B.



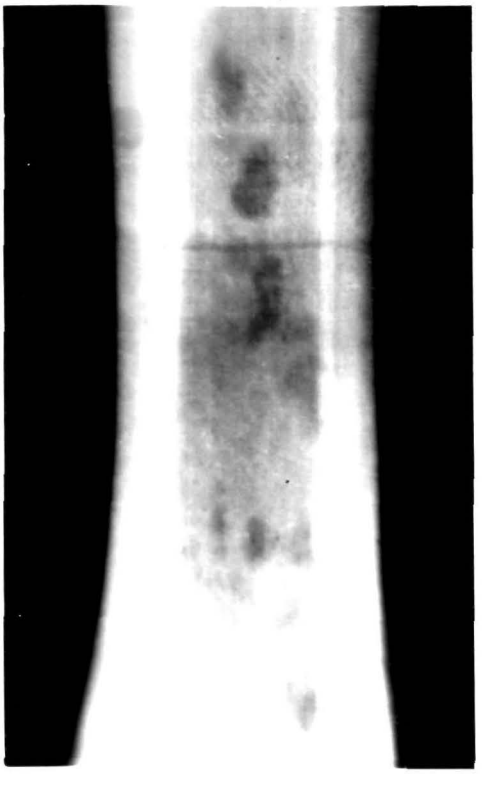
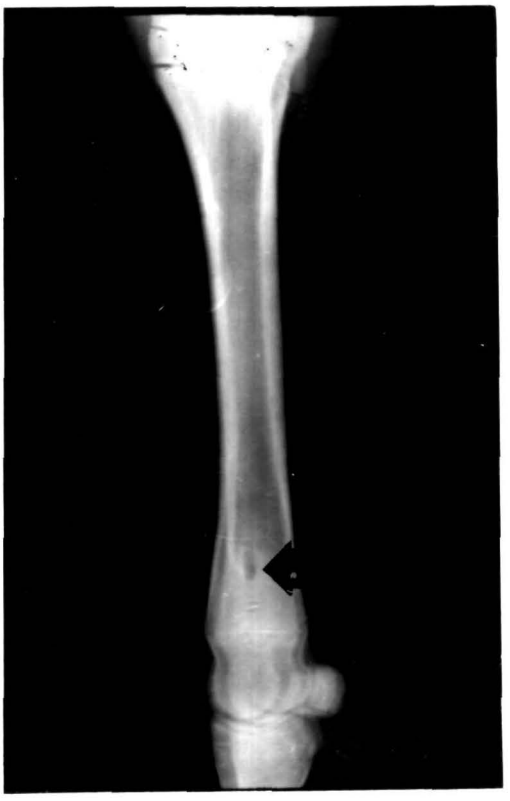


Figure 10-A: Medio-lateral radiograph of the infected metatarsus of a calf *(Prophylactic Group)* at 2 week. Note normal appearance of the test bone and surrounding soft tissue. Arrow indicates site of inoculation.

Figure 10-B: Medio-lateral radiograph of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week, showing no radiographic changes.



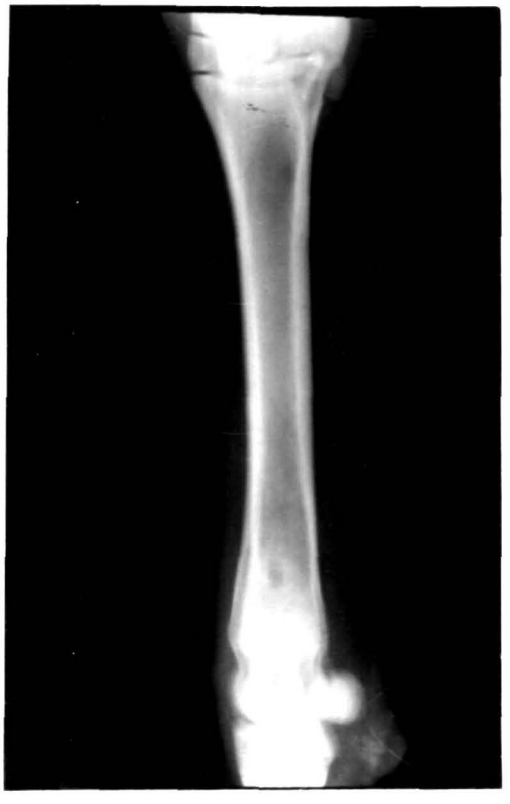


Figure 11: Medio-lateral radiograph of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week. Note slight lytic changes around the site of inoculation.

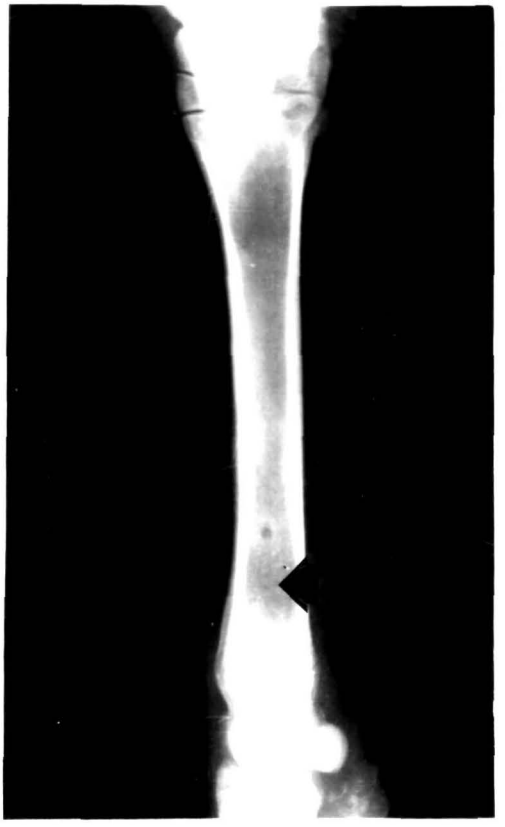
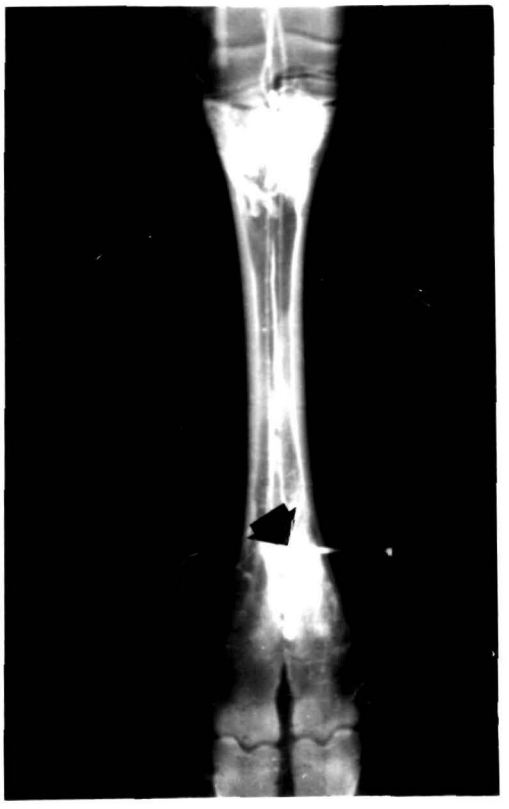


Figure 12-A: Planto-dorsal osteomedullogram of the normal metatarsus of a calf. Note medullary veins and venular network. Arrow indicates hypodermic needle in the marrow cavity.

Figure 12-B: A close-up view of the figure 12-A.

m = Medullary vein.
n = Venular network.



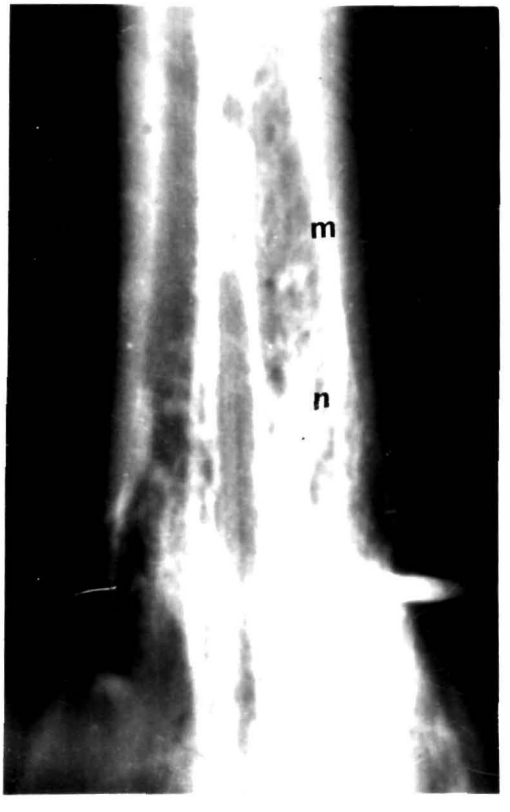

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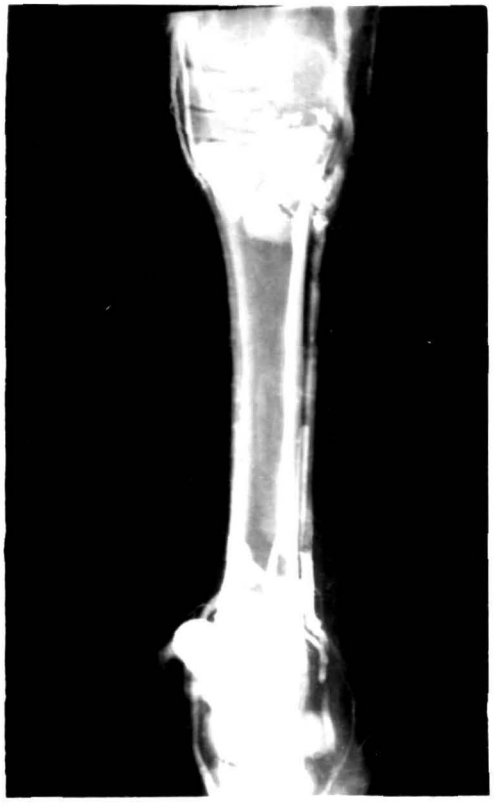
Figure 13: Medio-lateral osteomedullogram of the normal metatarsus of a calf, showing extraosseus as well as intramedullary venous channels.

Figure 14: Planto-dorsal osteomedullogram of the normal metatarsus of a calf.

m = Medial-planter metatarsal vein.
n = Lateral-planter metatarsal vein.
o = Perforating tarsal vein.
p = Proximal-planter arch.
q = Distal-planter arch.



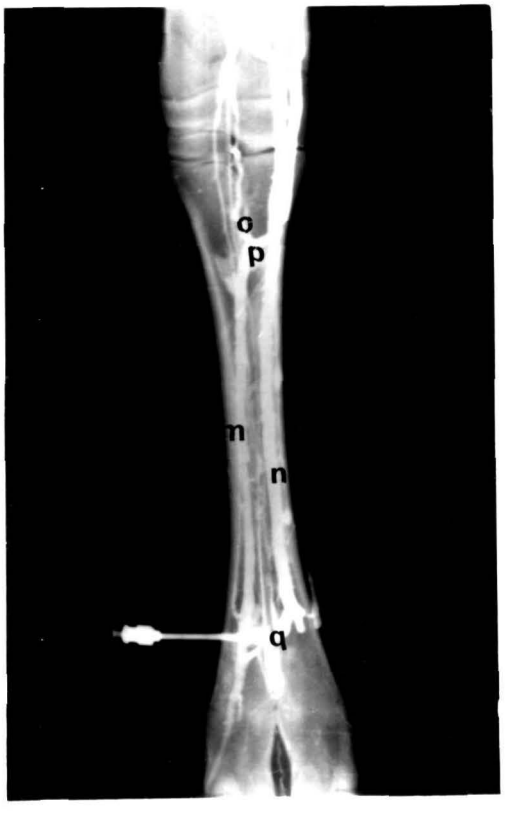
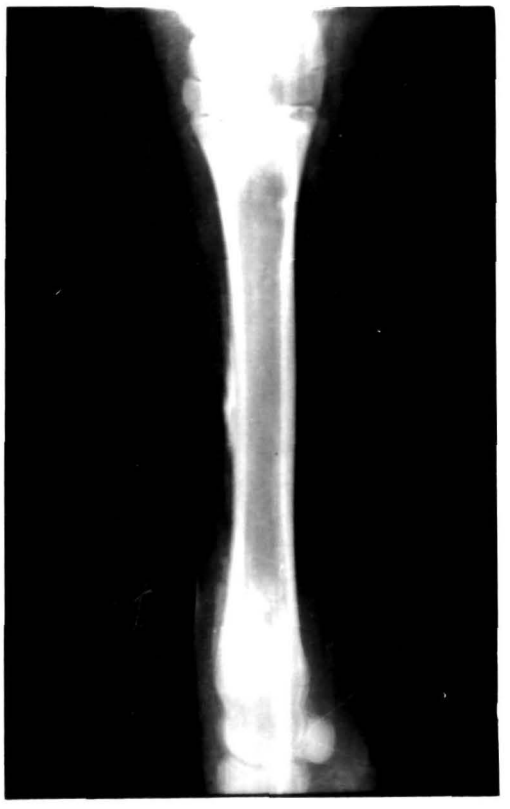

o
p
m
n
q

Figure 15: Medio-lateral osteomedullogram of the normal metatarsus, 10 minutes after injection of contrast material. Note the clearance of contrast material from the medullary cavity.

Figure 16: Medio-lateral osteomedullogram of the infected metatarsus of a calf *(Control Group).* Note the dilated and sacculated medullary venous channel.



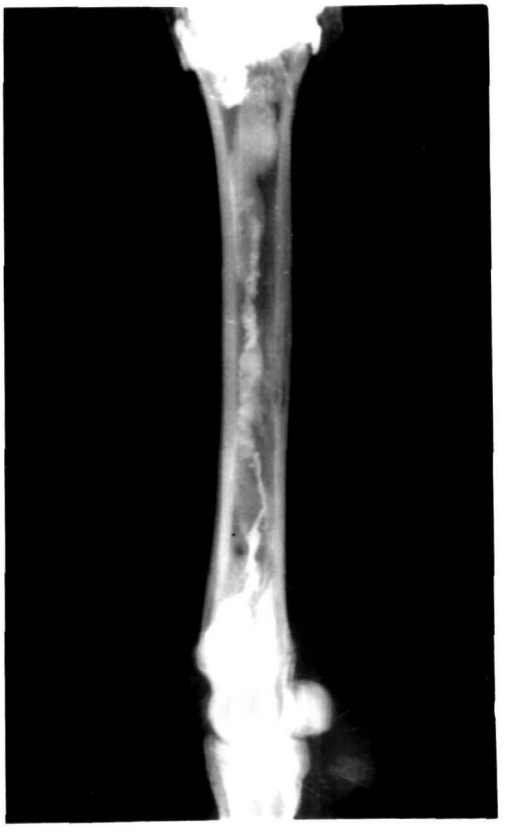
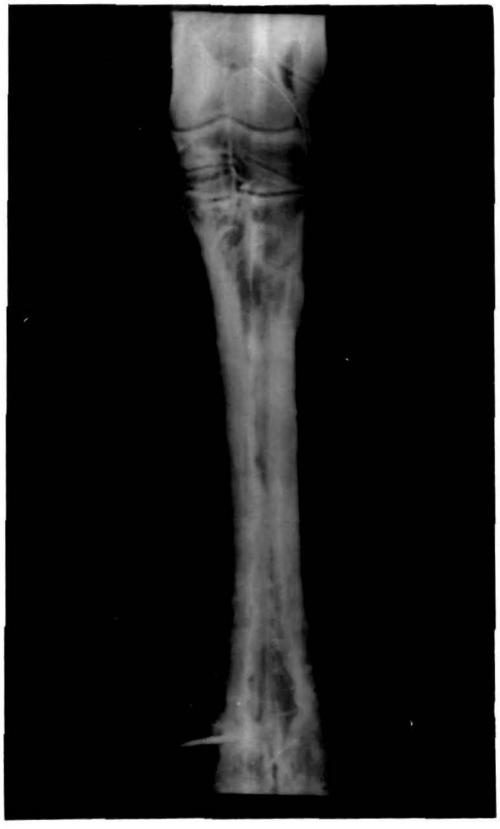


Figure 17: Planto-dorsal osteomedullogram of the infected metatarsus of a calf *(Control Group)* at 6 week, showing increased vascularity of the metatarsal region.

Figure 18: Planto-dorsal osteomedullogram *(20 minutes)* of the infected metatarsus *(Control Group)* at 6 week. Note stasis of contrast material and increased intramedullary density.



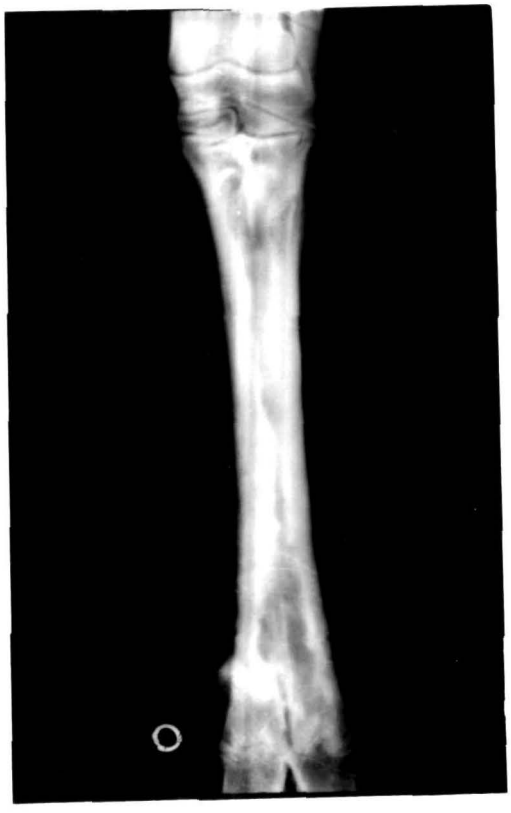


Figure 19: Medio-lateral osteomedullogram of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week, showing normal vascular pattern.

Figure 20: Medio-lateral osteomedullogram of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week. Note stasis of contrast material in the distal metaphysis and increased intramedullary density.

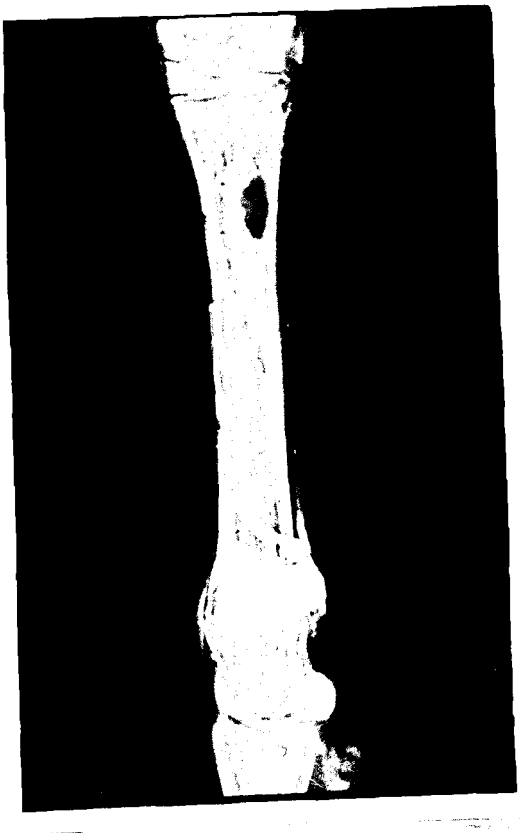


Figure 21: Medio-lateral osteomedullogram (10 minutes) of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week, showing almost complete clearance of contrast material from the medullary cavity.

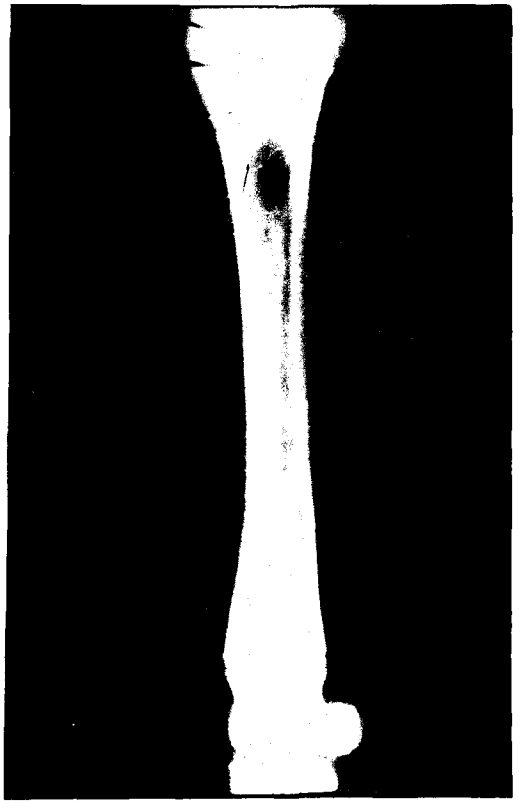
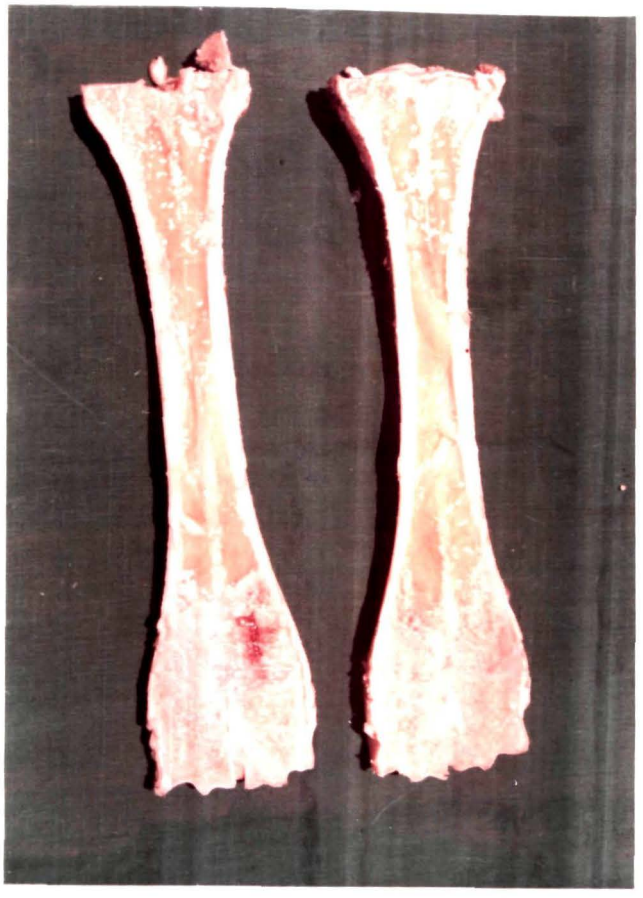


Figure 22-A: Longitudinal section of the normal metatarsus of a calf.

Figure 22-B: Longitudinal section of the infected metatarsus of a calf *(Control Group)* at 6 week. Note haemorrhagic marrow having purulent tracts and reactive new bone formation.



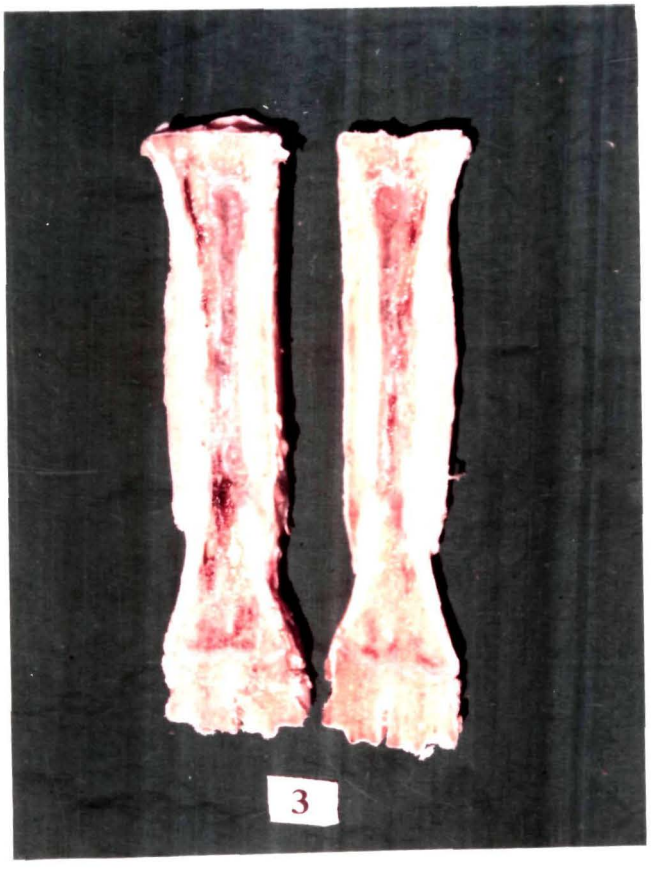

3

Figure 23: Longitudinal section of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week. Note near normal texture of medullary content and the cortices.

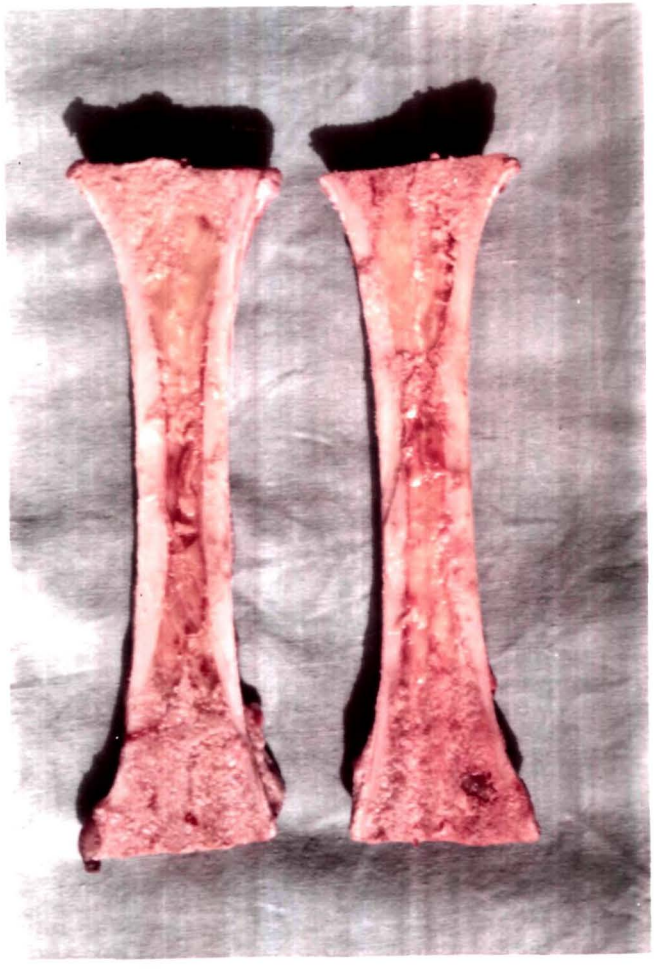
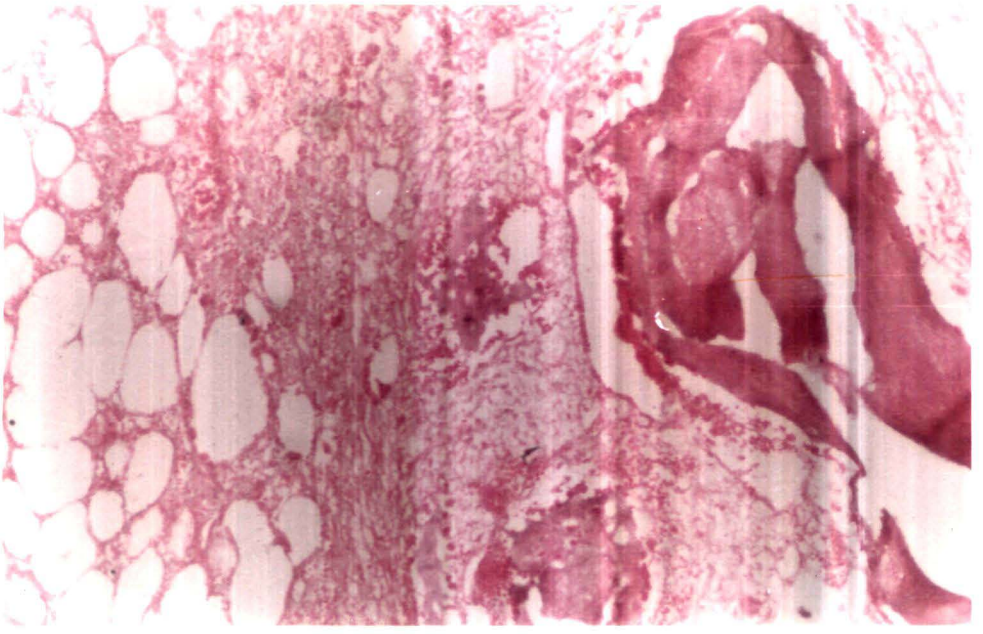


Figure 24: Microphotograph of the infected metatarsus of a calf *(Control Group)* at 6 week. H & E 50X
q = Necrotic foci.
r = Degenerative changes.
s = Fibrous tissue proliferation in the marrow.

Figure 25: Microphotograph of the infected metatarsus of a calf *(Control Group)* at 6 week, showing endosteal new bone formation.
H & E 50X



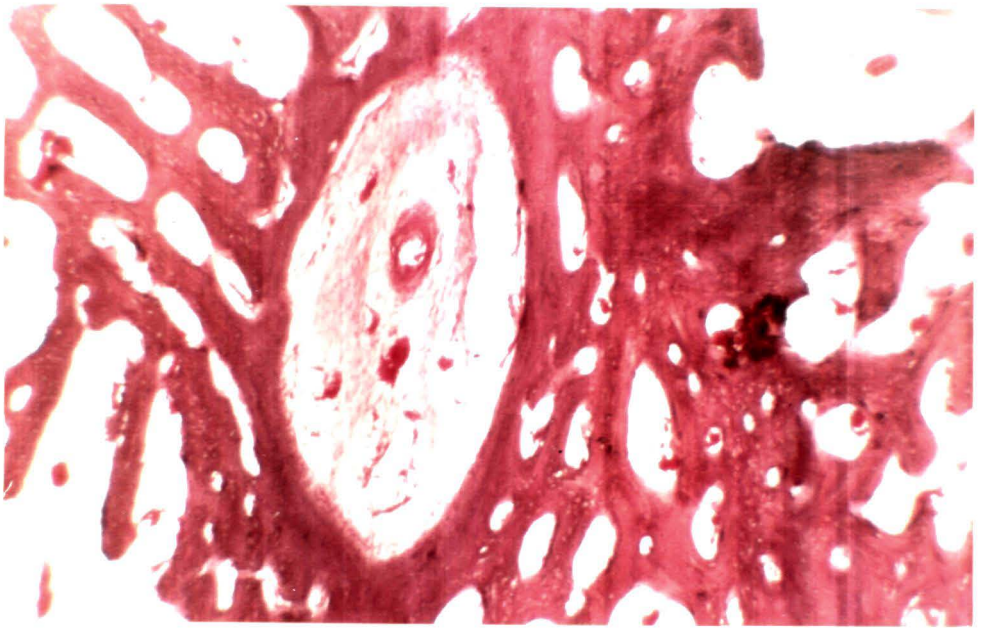


Figure 26-A: Microphotograph of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week, showing normal compact bone.
H & E 50X

Figure 26-B: Microphotograph of the infected metatarsus of a calf *(Prophylactic Group)* at 6 week, showing normal bone marrow.
H & E 50X