MOLECULAR CLONING AND CHARACTERISATION OF LAMBDA TOXIN ENCODING GENE OF *Clostridium Perfringens*

Thesis

SUBMITTED IN PARTIAL FULFILMENT OF THE
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OF

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IN

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BY

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Certificate

Certified that the research work embodied in this thesis entitled **Molecular Cloning and Characterisation of Lamda Toxin Encoding Gene of *Clostridium perfringens*** *submitted* by ***Dr.Sandeep J. Akare****, Roll No. 467 for the award of the Degree of* ***Doctor of Philosophy*** *of Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.*

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Certified that the thesis entitled **Molecular Cloning and Characterisation of Lambda Toxin Encoding Gene of** *Clostridium perfringens submitted by* ***Dr.Sandeep J. Akare****, Roll No.467, in partial fulfilment of the requirements for the degree of* ***Doctor of Philosophy*** *of Indian Veterinary Research Institute, embodies the original work done by candidate. The candidate has carried out this work sincerely and methodically.*

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of the degree of Doctor of Philosophy of Indian Veterinary Research Institute.

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Signature of the External Examiner

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To.....

Mom and Dad

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[SANDEEP AKARE]

ABBREVIATIONS

A	:	Absorbance
APS	:	Ammonium persulfate
ATP	:	Adenosine triphosphate
bp	:	Base pairs
DNA	:	Deoxyribonucleic acid
dNTP	:	2-deoxy nucleoside-5 triphosphate
D.W.	:	Distilled water
EDTA	:	Ethylene diamine tetracetic acid
Fig.	:	Figure
g	:	Grams
h	:	Hour(s)
IPTG	:	Isopropyl-beta-D-thiogalactopyranoside
Kb	:	Kilobase pair
kDa	:	Kilodalton
LB	:	Luria Bertani
M	:	Molarity/Molar
mM	:	Millimolar
min	:	Minutes
mg	:	Milligram
Mol. wt.	:	Molecular weight
µg	:	Microgram
µl	:	Microlitre
ml	:	Millilitre

ng	:	nanogram
OD	:	Optical density
PAGE	:	Polyacrylamide Gel electrophoresis
PCR	:	Polymerase chain reaction
RCM	:	Robertson's cooked meat medium.
RE	:	Restriction endonuclease
RNase	:	Ribonuclease
RT	:	Room temperature
rpm	:	Revolutions per minute
SDS	:	Sodium dodecyl sulphate
Sec.	:	Second
TBE	:	Tris borate EDTA
TE	:	Tris EDTA
TEMED	:	NNN'N`-Tetramethylene diamine
Tris	:	Tris (hydroxy methyl) amino methane
µCi	:	Microcurie
µM	:	Micromolar
v/v	:	volume by volume
viz.	:	namely
vol	:	Volume
w/v	:	Weight by volume
X-gal	:	5 Bromo-4-chloro-3-indolyl-ß-D-galoacto pyranoside
°C	:	Degree Celcius

Contents

INTRODUCTION

The genus Clostridium includes spore-forming bacilli which typically stain Gram positive and grow in an anaerobic atmosphere. Clostridial organisms are distinguished by their ability to produce heat resistant endospores and a variety of exotoxins. Clostridia have wide distribution in soil, fresh water, marine sediments etc. and they are frequently excreted in the faeces following ingestion from pastures, vegetables and fruits. While most clostridial species are saprophytic, some species live as commensals in the intestinal tracts of animals and man. The organisms are involved as primary pathogens or secondary invaders in many human and animal disease conditions such as botulism, tetanus, gas gangrene and enterotoxaemia. Almost all pathogenic clostridial species are, motile rods and produce substances toxic to host animals and man. The pathogenicity of different species varies according to the toxins they produce.

Clostridium perfringens is one of the ubiquitous organism among clostridial species. It is common inhabitant of the gastrointestinal tracts of human and animals and also occurs in the soil. It consists of various types of organisms varying in their

metabolism, toxin production and pathogenic potential (Niilo, 1980). *Clostridium perfingens* is responsible for numerous toxic maladies in human beings and occasionally causes enterotoxaemic diseases of economic significance in domestic animals such as necrotic enteritis, enteretoxaemia, lamb dysentry, equine colitis, neonatal toxaemia, etc. (Songer, 1996).

C. perfringens produces up to 17 potential extracellular toxins and enzymes including α–toxin, β-toxin, ε–toxin, θ–toxin, μ-toxin, λ–toxin, ν-toxin, ι-toxin, k-toxin, enterotoxin, non α–δ–θ hemolysin, neuraminidase, sialidase and factors responsible for vigorous metabolic activity of the organism. These toxins and enzymes act as virulence factors and the pathogenesis is mediated by interaction with different toxins and enzymes. Many of these virulence factors are simple hydrolytic enzymes secreted by the organism as part of its saprophytic life style in the soil, where they are involved in the putrefaction processes. Though some of these toxins and enzymes have been characterized biochemically and genetically, their role in pathogenesis yet remains to be completely defined. With recent advances in techniques to carryout biochemical and molecular anlaysis of *C. perfringers* type strains, the role of certain virulence factors in pathogenesis of ailments caused has been demonstrated (Rood, 1998). Lambda (λ) toxin is one such virulence factor produced by most type B and E strains and certain type D strains of *C. perfringens* (Rood, 1998).

A caseinase gene, *lam*, which encodes a thermolysin zinc-metalloprotease, designated as lambda toxin, has been recently cloned from *C. perfringens* type B strain (Jin *et al.*, 1996). The lambda toxin is secreted as a proenzyme which is processed to mature enzyme, a monomeric protein of 36 kDa. The mature protein has the HE XXH zinc binding motiff and consensus sequences of thermolysin family. The thermolysin family consists of virulence factor activating metalloproteases of

pathogenic bacteria like *Vibrio cholerae* and *Listeria monocytogenes* (Booth *et al.*, 1984; Poyart *et al.*, 1993).

The purified lambda toxin has been shown to cleave a variety of biologically important substances such as immunoglobulins, complement C3 component, fibrinogen, various collagens, fibronectin, etc. Intradermal injection of purified lambda toxin increases vascular permeability and results in haemorrhagic oedematous lesions. These properties suggest that it is a potent virulence factor involved in invasion and tissue destruction. However, a more important role of lambda toxin in activating ε-prototoxin has been suggested. Epsilon toxin, the most potent of *C.perfringens* toxins is secreted extracellularly as inactive prototoxin and is activated by proteolytic cleavage in gastrointestinal tract causing fatal enterotoxaemia in ungulates (Worthington *et al.*, 1973). Secreted inactive epsilon prototoxin is proteolytically cleaved and activated to >1000 fold active epsilon toxin in gastrointestinal tract. The typsin and chymotrypsin have been shown to cleave and activate ε-prototoxin in *in-vitro* studies (Hunter *et al.*, 1992). Trypsin also inactivates beta toxin and hence the *in-vivo* activation by trypsin and chymotrypsin alone seems doubtful considering the diseases like lamb dysentry involving role of multiple toxins like epsilon and beta. The suggested role is supported by *in- vitro* N and C- terminal cleavage and activation of epsilon prototoxin on incubation with purified lambda toxin. The studies on lethality of epsilon toxin in mice suggested that lambda toxin activates epsilon prototoxin to a greater extent than trypsin (Minami *et al.*, 1997).

In India little work has been carried out on molecular pathogenesis of epsilon toxin (Goswami *et al.* 1996) and there is little awareness about lambda toxin and its role in pathogenesis of enterotoxaemia. Considering the reported economic losses

due to ε -toxin mediated enterotoxaemias in India, research in this area assumes importance. The ability to obtain high level expression of λ -toxin gene will help in elucidation of role of lambda toxin in ε-toxin mediated diseases. It may pave the way for developing recombinant λ-toxin prophylactic possibly as an adjunct to ε - recombinant toxoid. It would also help in seeking the long term goal of generating recombinant multivalent fusion protein consisting of protective antigenic epitopes of various clostridial virulence factors. Hence the present study is proposed to be carried out with the following objectives :

1. ***To clone and characterise the lambda-toxin encoding gene fragment of Clostridium perfringens.***

2. ***To attempt expression of lambda-toxin gene in E. coli.***

REVIEW OF LITERATURE

2.1 THE ORGANISM - *CLOSTRIDIUM PERFRINGENS*

C. perfringens was originally isolated by Welch and Nuttal in 1892 from a decomposing human cadaver and was initially named as *Clostridium perfringens welchii*.

C.perfringens enterotoxaemias are acute, highly fatal intoxications that affect lambs, calves, piglets and occasionally foals. The diseases are caused by the major exotoxins of *Clostridium perfringens* types B, C and D and occasionally types A and E. The *C. perfringens* type A occurs in the intestinal tract of humans and animals and in most soils. The *C. perfringens* types B to E are more adopted to survival in the intestines but in outbreaks of diseases they survive long enough in soils to infect other animals (Nillo, 1980; Quinn *et al.*, 1994).

2.1. Identification

Identification of *C. perfringens* is mainly carried out on the basis of morphological, cultural and biochemical characteristic and pathogenesis in laboratory animal test (Sterne and Batty, 1975).

2.1.2 Morphology

C. perfringens is a short fat rod shaped (2-6 µm x 0.8-1.5 µm) Gram-positive organism occurring singly or in pairs. *C. perfringens* is the only species among Clostridia that produces a polysaccharide capsule in animal tissues and is non-motile. The spores are oval, central or subterminal and are rarely produced except in intestinal tract of humans in food poisoning cases.

2.1.3. Cultural characteristics

C. perfringens is relatively aerotolerant and grows in temperature range of 37-47°C. Growth is enhanced in the presence of glucose or blood. *C. perfringens* colonies produce a narrow complete hemolytic band and a wider partial hemolytic zone around them. *C. perfringens* produces a marked opalescence caused by alpha-toxin (lecithinase) referred to as Nagler reaction. The growth in Robertson's cooked meat medium gives pink appearance to the meat particles.

2.1.4 Classification

The *C. perfringens* species is a very heterogeneous group of organisms with respect to their metabolic byproducts, toxins and pathogenic potential. The species is divided into five types from A to E based on the ability to produce any of the four major lethal toxins (Table 2.1). As these toxins are antigenic, typing is achieved by neutralisation of the lethal toxins with type-specific antisera using mice or guinea pigs as test animals. In addition, the various types produce additional virulence factors some of which possess pathogenic potential (Niilo, 1980).

Table 2.1 : Toxins and hydrolytic enzymes produced by *C. perfringens*

C. perfringens	Toxins produced											
Type	α	β	ε	ι	δ	θ	κ	λ	μ	ν	Nm	En
A	+++	–	–	–	–	+	+	–	+	+	+	+
B	+	++	+	–	+	+	+	+	+	+	+	+
C	+	++	–	–	+	+	+	–	+	+	+	+
D	+	–	++	–	–	+	+	+	+	+	+	+
E	+	–	–	+	–	+	+	+	–	+	+	+

Nm : Neuraminidase; En : Enterotoxin

2.2. MOLECULAR GENETICS OF C. *PERFRINGENS*

2.2.1 Organisation of the *C. perfringens* chromosome

C. perfringens was the first Gram-positive bacterium for which a genetic map was elucidated (Canard and Cole, 1989). Strain CPN50 was found to have 3.6 Mb chromosome by pulsed-field-gel electrophoresis. A total of 24 genes or gene regions and 10 ribosomal RNA operons were mapped. The RNA operons are located on either side at the origin of chromosomal replication, *ori C* and are confined to only one-third of the chromosome (Canard and Cole, 1989; Cole and Canard, 1997). All 10 rRNA operons have been cloned and four of them have been partially sequenced (Gariner *et al.*, 1991). The CPN50 map revealed 100 mapped markers (Katayama *et al.*, 1996). Also clustering of several genes encoding extracellular toxin and enzymes within a 250 kb region near *ori C* was found. These genes included *plc*, *pfoA*, *colA* (encoding kappa toxin) and *nag H* (the putative μ toxin gene). The *pfo A* and *col A* genes are located on a 10 Kb apart while *nagH* is located on the same 30 Kb *Apa* I- *Sma* I restriction fragment as *col A* (Katayama *et*

al., 1996; Ohtani *et al.*, 1997). The *nan H* and *nan I* genes encoding sialidases are located within 200 kb of each other. Another 200 kb from *nan I* is *psp A* gene, a homologue of which encodes a putative surface protein that is a virulence factor in *Streptococcus pneumonae*.

Other potential virulence genes identified by genome scanning include *pfo S* gene, which has similarity of *pto R* encoding *pfo A* activator and *cop R* similar to a putative transcriptional activator from *Pseudomonas syringae*. There is no evidence that *pfo S*, *cop R* or *psp A* are involved in virulence of *C. perfringens*. The *vir RS* operon, which is important in regulation of extracellular toxin production and virulence, is located near the putative replication terminus, on the opposite side of the chromosome to the virulence gene region (Katayama *et al.*, 1996).

The comparison of genome organisation of the virulence gene regions of 10 diverse *C. perfringens* isolates representing all 5 toxin types identified 3 hypervariable regions. One of these region spans the plc gene locus, this gene is present at the same site in all strains, but the size of the region between tbe *plc* gene and *rrn* gene varies. Similary variation was observed in *pfo S* to *nag H* regions, although their map positions remained unaltered. It is not known as to why the *plc*, *pfo A*, *col A* and *nag H* genes are located in more variable chromosomal regions. The final hypervariable region had *cpe* gene coding enterotoxin and was present only in one tested strain. All five toxin types of *C. perfringens* had similar genome organisation and ability to produce different toxin was due to acquisition or loss of specific toxin gene (Canard and Cole, 1989).

Phylogenetic position of *C. perfringens* based on 16 S rRNA gene sequences of *C. septicum* and *C. chauvoei*, has been determined (Kulmert *et al.*, 1996) similarly Forsblom *et al.* (1995) used *rrn B* operon to ribotype *C. perfringens* strains and classified them into 18 ribotypes. Daube *et al.* (1996) characterised 123 field strains by DNA hybridisation of epidemiological marker, *Hind III* RFLP with 5

poobes specific for 16 S-rRNA gene, sialidase gene, alpha, theta and mu toxin gene and confirmed genetic variation and mobilization of virulence genes.

2.2.2 Location of toxin genes on extrachromosomal elements

Many of the extra cellular toxin genes, including *etx* epsilon toxin gene and ß-toxin structural gene *cpb*, are plasmid determined (Canard *et al.*, 1992; Duncan *et al.*, 1978; Katayama, 1996). The analysis of *I-Ceu I* digests of 16 *C. perfringens* isolates confirmed chromosomal location of *plc*, *pfo A*, *col A* and *nag H* gene. In contrast *etx*, *cpb*, *iab*/ibp (i-toxin), *lam* (λ-toxin) and urease genes were located on large extra chromosomal elements that did not contain *I-Ceu* I sites (Dupuy *et al.*, 1997; Katayama, 1996).

The differentiation of *C. perfringens* typeA isolates from isolates of types B to E is dependent on the ability of latter isolates to produce various combination of ß-toxin, ε-toxin and i-toxin, which depends on acquisition or loss of extra chromosomal elements that contain the structural genes encoding those toxins (Rood *et al.*, 1997).

2.2.3 Genetic manipulation of *C. perfringens*

2.2.3A Transformation of *C. perfringens*

Transformation based on electroporation have been standardised for vegetative *C. perfringens* cells (Allen and Blascheck, 1988). Cloning of gene from *C. perfringens* has been helped by availability of improved methods of transfer with enhanced transformation efficacy (Mahony *et al.*, 1988). Use of lysostaphin increases number of transformants at optimal concentration of 2-20 µg/ml, Strain 13 is currently the only *C. perfringens* strain that yielded enough transformants to enable direct cloning of genes in *C. perfringens* (Rood and Cole, 1991).

2.2.3B *C. perfringens- E. coli* shuttle plasmids

Shuttle vector capable of independent replication and selection in *C. perfringens* as well as in *E. coli* was developed for the first time by Roberts *et al.* (1988). The shuttle plasmid was designated pHR 106. Subsequently Bannam and Rood (1993) reported the construction of two *C. perfringens- E. coli* shuttle vectors. These carried single antibiotic resistance gene which could express in both the systems. PJIR750 and PJIR751 plasmids had chloramphenicol and erythromycin resistance genes respectively. Both possessed pUC18 derived MCS and LacZ gene which enabled direct selection of recombinant *E. coli.*

2.3 DISEASES CAUSED BY *C. PERFRINGENS*

Clostridial diseases are mostly categorised as

i) Enterotoxaemias - including all the conditions affecting the intestinal tract and parenchymatous organs

ii) Gas gangrene - including the conditions in which myonecrosis and toxaemia predominate.

iii) Neurotropic disorders - including conditions in which the nervous system is primarily affected.

C. perfringens may be the most widely occuring pathogenic bacterium and certainly is the most important cause of clostridial enteric diseases in domestic animals. *C. perfringens* is present in the soil and in the intestines of healthy animals and man disease occurs when the intestinal organisms begin to multiply unusually rapidly and produce toxins. But little is known about the conditions which provide the suitable microenvironment for this to take place. The diseases are caused by the major exotoxins (enterotoxins) of *C. perfringens* type B, C and D and occasionally type A and E organisms. These are summarised follows :

C. perfringens type A

C. perfringens type A is the causative agent for wound contamination, anaerobic cellulitis, gas gangrene and enteric diseases(Hatheway, 1990).

Enterotoxaemia in lambs, known as yellow lamb disease, occurs when the population of nursing lambs is high (Flemings, 1985). A similar condition has been reported in goats and calves (Russel, 1970) . Type A is causative agent of acute enterotoxaemia in sheep and calves referred to as sudden death.Type A causes necrotic enteritis in poultry (Al-Sheiky and Truscott, 1977), intestinal clostridiosis in adult horses (Wierup and Dipierto, 1981) and enteric diseases in suckling and feeder pigs (Olubunmi and Taylar, 1985).

In India type A has been reported in enterotoxaemia of goats and necrotic enteritis of chicken (Phukan *et al.*, 1997; Das *et al.*, 1997).

C. perfringens Type B

C. perfringens type B is the causative agent of lamb dysentry which occurs mainly in lambs and sometimes in foals and calves (Sterne, 1981), Lamb dysentery usually develops during the first few days of life, although older lambs may be involved as the outbreak progresses (Dalling, 1926). Infection is acquired from environment or dam and the number of organisms in gut increases rapidly especially with heavy lactation in dams. The result is enterotoxaemia accompanied by enteritis and extensive haemorrhage and ulceration of small intestine (Frank, 1956). Pathogenesis of lamb dysentry is not clear and it is not known whether the individual effects of α, β, ε -toxin predominate or there is additive/synergistic effect (Songer, 1996).

Lamb dysentry was the first of the enterotoxaemias caused by clostridia to be distinguished from perinatal *E. coli* enteritis. incidence is as high as 30% with

case fatality approaching 100%. Chronic disease, called pine, in older lambs is manifested by prolonged abdominal pain without diarrhoea. *C. perfringens* type B has also been found associated with haemorrhagic enteritis in goats, calves and foals (Stubbings, 1990).

In India neonatal lamb dysentery has been reported due to *C. perfringens* type B infections (Harbola *et al.*, 1988).

C. perfringens Type C

Infections with *C. perfringens* type C have been reported in pigs, cattle, sheep, horses, chicken, dogs and humans (Mackinnon, 1989). Piglets are more commonly affected than other young animals (Johnson *et al.*, 1992). Morbidity and mortality rates up to 30-50% and 50-100% respectively have been reported (Ohnuna *et al.*, 1992).

Type C enterotoxaemia in adult sheep commonly named as "struck", is reported to cause rapid death (Songer, 1996). The organism causes diarrhoea and colic in foals (Drolet *et al.*, 1990) and enteritis in dogs (Turk *et al.*, 1992). In India necrotising enteritis has been reported in piglets by Harbola *et al.* (1983).

C. perfringens Type D

C. perfringens type D causes enterotoxaemia in sheep also known as pulpy kidney disease or overeating disease, which occurs in sheep of all ages except new borns (Timoney *et al.*, 1988). It is most prevalent in lambs aged 3-8 weeks, in fattenning lambs and in adult animals grazed on luxurious pastures (Popoff and Boquet, 1984).

C. perfringens type D is normal inhabitant of the soil and also of the intestines of healthy sheep where it produces small amounts of toxin without affecting

the well being of the hosts. Enterotoxaemia may be precipitated by sudden change to a more concentrated diet and overeating, resulting in unusually high rate of multiplication of organisms and production of high concentrations of toxin causing total toxaemia (Popoff, 1984). The sudden multiplication of organisms is suggested to be related to drop in acidity, while production of ε -toxin is favoured by presence of excess dietary starch in small intestines. High concentration of toxin increases permeability and facilitates its absorption (Niilo, 1993). Primary target of ε -toxin is the central nervous system, where it produces foci of liquefactive necrosis, perivascular oedema and haemorrhages, especially in meninges (Buxton, 1978).

C. *perfringens* type D occasionally cause enterotoxaemia in calves, young cattle, camels and horses (Songer, 1996). In India type D enterotoxaemias have been reported in goats by various workers (Kulshreshtha *et al.*, 1972; Chakrobarty *et al.*, 1980; Harbola and Uppal, 1981; Parhi *et al.*, 1993).

C. perfringens type E

C. perfringens type E is an apparently uncommon cause of enterotoxaemia in lambs, calves and rabbits (Songer, 1996) and recently isolates have been obtained from calves with hemorrhagic enteritis (Meer and Songer, 1997). However, type E remains of uncertain overall importance in animal enterotoxaemic diseases (Songer, 1998).

Enterotoxigenic *C. perfringens*

A role for enterotoxigenic strains, particularly of *C. perfringens* type A in etiology of diarrhoeal conditions in several animal species, had been reported (Istrada - correa and Taylor, 1989; Nillo, 1993). In one study, *C. perfringens* enterotoxin (CPE) production in up to 12% of isolates from cattle, sheep and chickens with enteritis had been reported (Nillo, 1978) and in another study, genotyping revealed

that about 5% of isolates were enterotoxigenic, with most of those being type A (Songer and Meer, 1996; Meer and Songer, 1997). Enterotoxin gene was detected by DNA hybridisation in 14% of samples from horses, 22% from cattle and 10% from poultry (Tschirdewah *et al.*, 1992).

2.4 TOXINS OF *C. PERFRINGENS*

C. perfringens organisms produce variety of toxic and enzymatic substances which have been studied in details because of their importance in relation to identification of strains and the pathogenesis of disease. These toxins, except enterotoxin, are synthesized and released by intact growing cells and are true exotoxins (Pollock, 1962). *C. perfringens* toxins are proteins and are susceptible to heat and proteolytic enzymes. The major toxins α, β, ε, θ, i and enterotoxin have been well characterised already due to their direct involvement in disease production. Other toxins and enzymes, though less well studied, also seem to act as important virulence factors. The various *C. perfringens* toxins and antigenic components are as follow (Niilo, 1980; Hatheway, 1990; Rood, 1998).

Toxin or Antigen	*C. perfringens* types	Characteritics
α	A-E	Lecithinase, calcium dependent phospholipase-C, hemolytic, lethal, necrotizing.
β	B,C	Necrotizing, lethal, inflammatory, trypsin labile
ε	B, D	Protease activated prototoxin, increases capillary permeability, potent neurotoxin, cerebral oedema, lethal
ι	E	Ia ADP-ribosylates actin; Ib mediates binding, dermonecrotic, lethal
δ	C	Hemolytic

θ	A-E	Thiol activated cytolysin, hemolytic, oxygen labile, cause tissue necrosis
Enterotoxin	A-E	Sporulation dependent
κ	A-E	Collagenase, necrotizing
λ	B,D,E	Nonspecific zinc metalloprotease, tissue destruction and invasion, activation of other toxins viz θ and ε.
μ	A-E	Hyaluronidase
υ	A-E	Deoxyribonuclease
Urease	A	Enzyme occasionally found
Neuraminidase	A	Enzyme occasionally found
Fibrinogenase	A	Enzyme occasionally found

2.4.1 Alpha toxin

Alpha toxin is a multifunctional phospholipase produced in varying amounts by all types. It is principal lethal toxin of *C. perfringens* causing hydrolysis of membrane phospholipids in erythrocytes, platelets, leukocytes and endothelial and muscle cells resulting in lysis or other form of cytotoxicity (Elder and Miles, 1957; Smith, 1979). It is hemolytic, necrotising and potently lethal (Rood and Cole, 1991). It plays a key role in pathogenesis of *C. perfringens* mediated gas gangrene infection by promoting local cell membrane disruption and in other diseases, like necrotic enteritis of fowl, where it causes extensive damage to intestinal villi (Smith, 1979).

α–toxin is a zinc metallo-enzyme of 30 amino acid residues with molecular weight of 43 kDa and is reversibly inactivated by calcium chelating agents (Kreg and Kent, 1984).

The alpha-toxin gene, *cpa*, which is located on chromosome, has been cloned and expressed by Titball *et al.* (1993). In one study genetically truncated N-terminal (1-249 aa) and C- terminal (247-370 aa) fragments were assayed as prophylactics. The antibodies against N-terminal fragment neutralised phospholipase-C activity but not hemolytic activity and were non-protective in mice on challenge with the toxin or the organism. On the other hand, antibodies against C-terminal fragment of α-toxin neutralised both phospholipase-C and hemolytic activities and were protective, (Williamson and Titball, 1993).

2.4.2 Beta toxin

The β-toxin is a highly trypsin-sensitive protein (Sakurai and Duncan, 1978) which is responsible for mucosal necrosis and possibly for central nervous system signs in *C. perfringens* induced disease in domestic animals (McDonel, 1986; Reynaurd *et al.*, 1986). It is a 41 kDa monomeric protein, labile to heat and oxidation (Sakurai and Fujii, 1987). Experimentally, purified β-toxin caused rise in blood pressure and electrocardiographic disturbances in rats (Sakurai, 1984). It induced haemorrhagic intestinal necrosis and dermonecrosis and was found lethal to mice and guinea pigs (Lawrence and Walker, 1976; Sakurai and Fujii, 1987). It was reported cytotoxic to hamster ovarian cells (Reynaurd *et al.*, 1986).

Like ε-toxin it is encoded on a large uncharacterised plasmid (Katayama, 1996). The structural gene, *cpb*, has been cloned and it encodes a single polypeptide with a 27 aa signal sequence, which upon secretion is cleaved to produce a 309 aa residue long trypsin-sensitive extracellular toxin (Hunter *et al.*, 1992; Steinporsdottir *et al.*, 1995).

Recently an antigenically distinct β-toxin designated as β2-toxin has been cloned from some type-C strains isolated from enterocolitis cases in horses and piglets (Gilbert *et al.*, 1997). β2-toxin was found lethal for mice and cytotoxic for

cell lines and seems to be causing digestive disturbances in horses and piglets. β2-toxin gene (*cbp 2*) encodes 265 amino acid residue product while the mature β2-toxin is 235 amino acid residue protein with molecular weight of 28 kDa. β2-toxin does not exhibit sequence homology with β-toxin and is only weakly immunologically realated with β-toxin (Herthozl *et al.*, 1999).

Epsilon toxin

Epsilon toxin is most potent of the various *C. perfringens* toxin and is produced by types B and D. It is of great veterinary interest because it causes a rapidly fatal enterotoxaemia amongst ungulates which is commonly referred to as pulpy kidney or overeating disease (McDonell and McClane, 1988). The epsilon toxin is produced as the minimally toxic prototoxin which is converted into > 1000 fold toxic form by proteolytic cleavage in gastrointestinal tract (Payne and Oyston, 1997). Although little is known about the mode of action of epsilon toxin, it is lethal, dermonecrotoxic (Buxton, 1978) and its toxicity is estimated to be 3.2 x 10^6 i/v minimal lethal dose per mg in mice (Sakurai and Fujii, 1987). It primarily appears to increase vascular permeability in the brain, kidneys and intestines and is responsible for pathology observed in both types B and D enterotoxaemias (Payne and Oyston, 1997). The epsilon toxin structural gene (*etx*) is encoded on a plasmid, but little is known about this large plasmid (Blaschek and Solberg, 1981; Katayama *et al.*, 1996). The epsilon toxin gene, *etx*, encodes a single polypeptide that has a 32 aa signal peptide sequence. Proteolysis of additional 13-14 N-terminal aa from the secreted protein by trypsin forms the active 283 aa toxin (Harvard *et al.*, 1992; Hunter *et al.*, 1992). The prototoxin can also be activated by cleavage with *C. perfringens* λ-toxin (Minami *et al.*, 1997). As the immunity can be conferred by vaccination with epsilon toxin preparation, the gene for epsilon toxin *etx* represented a major target for biotechnologists. This gene has been cloned (Hunter *et*

al., 1992) and expressed to produce a second generation candidate vaccine (Rood and Cole, 1991; Goswami *et al.*, 1996).

Iota Toxin

The Iota toxin (ι) is the last of the four major lethal toxins produced by *C. perfringens* and is normally associated with type E strains. Iota toxin increases vascular permeability, provokes necrosis and is lethal. Like ε-toxin, it is reportedly produced from a protoxin by proteolysis (Ross *et al.*, 1949). The toxin consists of two distinct polypetide chains Ia and Ib (Stiles and Wilkins, 1986). The smaller polypeptide possess ADP-ribosylating activity and seems primarily responsible for iota pathogenesis (Simpson *et al.*, 1987).

Iota toxin shares attributes of structure and activity with the toxin of *C. spiroforme* and with C2 toxin of *C. botulinum* types C and D. The genes encoding both fragments of iota toxin have been cloned and characterised (Perelle *et al.*, 1993).

Theta toxin

All five types of C perfringens produce a lethal hemolysin, theta toxin , also known as O-hemolysin, perfringolysin-O or thiol-activated cytolysin. Theta toxin (θ) is member of a family of thiol-activated cytolysins produced by a diverse group of gram-positive bacteria including *Bacillus*, *Streptococcus*, *Clostridium* and *Listeria* Genus. These cytolysins use cholesterol as receptor and form pores in target membranes. The θ-toxin plays role in tissue necrosis associated with *C. perfringens* gas gangrene and is responsible for the depletion of polymorphonuclear leukoyctes in the affected zone altering their morphology, metabolism and migration (Stevens *et al.*, 1988).

The theta toxin structural gene *pfo A* has been cloned and expressed (Tweten 1988a; Canard and Cole, 1989). The θ-toxin is translated as 494 residue preprotein from which a 28-residue signal peptide is removed on secretion to medium. Mature θ -toxin has a molecular weight of 54 kDa (Tweten, 1988a).

Kappa Toxin

Kappa toxin is a 120 kDa collagenase produced by *C. perfringens* and appears to be involved in tissue necrosis, alongwith other toxins such as phospholipase- C and thiol-activated hemolysin (Kameyama and Akama, 1971; Rood and Cole, 1991). The kappa toxin encoding gene *col A* has been cloned and sequenced by Mastsushita *et al.*, 1994).

The μ toxin

The μ-toxin is a hyaluronidase which degrades hyaluronic acid, an important connective tissue constituent to release glucosamine (McDonel, 1986). The *nag H* gene encoding a β-N-acetylglucosaminidase has been cloned and sequenced. The gene is present in all *C. perfringens* isolates examined and its product is a 97,000 Da secreted protein. Primary role of *nag H* protein/μ-toxin in cell wall biosynthesis or autolysis has been suggested, while its action as virulence factor seems secondary (Canard *et al.*, 1994).

Neuraminidase

All *C. perfringens* types produce a neuraminidase or sialidase, which cleaves acylneuraminic acid residues from glycoprotein, glycolipids and oligosaccharides. Neuraminidase may have a role in disease pathogenesis owing to its capacity for destruction of receptor molecules on cell surface or for disrupting connective tissue (McDonel, 1986). A gene *nan H*, encoding neuraminidase, a cytoplasmic protein of 42.7 kDa has been cloned and characterised (Roggentin *et al.*, 1988; Roggentin *et al.*, 1989).

Enterotoxin

The enterotoxin is one of the best studied virulence factors of *C. perfringens*. The enterotoxin is the only known *C. perfringens* toxin that is not secrted from vegetative bacterial cell but is produced during sporulation. Enterotoxin accmulates in large quantities intracellularly and is liberated with lysis of bacteria in the gut. It is processed to a more active form by proteolysis by trypsin (Richardson and Granum, 1983; Granum, 1990). The inactive enterotoxin is a 35.3 kDa monomeric protein which is activated by removal of 24 N-terminal aa residues by trypsin.

Enterotoxin is a cytotoxic protein involved in membrane pore formation, altered permeability, inhibition of macromolecular synthesis, Ca^{2+} mediated cytoskeletal disintegration and cell lysis (McClane and Wnek, 1988; McClane, 1994). The enterotoxin is a single 35.3 kDa heat labile polypeptide that acts on epithelial cells of gastrointestinal tract to cause fluid and electrolyte losses (McClane, 1996).

The enterotoxin structural gene, *cpe* was first cloned in seperate segments (Hanna *et al.*, 1989; Iwanijka *et al.*, 1989; Van Damme-Jongsten *et al.*, 1989) and then in its entirety (Czeczulin *et al.*, 1993). In *C. perfringens* strains isolated from food poisoning outbreaks in humans, the *cpe* gene is chromosomally determined while in enterotoxin-producing *C. perfringens* isolates of animal origin, the *cpe* gene is located on a 100-120 kb plasmid (Collie and McClane, 1998).

Lamda toxin

Lambda toxin is produced by most type B and E strains and some type D strains of *C. perfringens* which cause enteritis and enterotoxaemia in domestic animals (Bidwell, 1950; Hatheway, 1990). The culture supernatant of lambda toxin producing *C. perfringens* type B strain revealed high levels of caseinolytic activity which was shown to be inhibited by metal-chelating agents. The casein-hydrolyzing

activity of lambda toxin producing strain is highest during late log phase. The casein-hydrolyzing activity of the lambda toxin is maintained in 5 mM $CaCl_2$ but lost if dialysed against 50 mM Tris-Cl, pH 7.5 (Jin *et al.*, 1996). Bidwell (1950) described that dialysis of the ammonium sulphate fraction against water resulted in increased lambda-toxin activity due to removal of inhibitor(s).

Jin *et al.* (1996) purified lambda-toxin by subjecting culture supernatant to 60% ammonium sulphate precipitation followed by sequential application to sephacryl S-100, DEAE sephadex A-25, sephadex G-100 and ether-Toyopearl colouns. They reported need to carry out all precipitation and purification steps in 5mM $CaCl_2$.

Biophysical properties of lambda-toxin

Lambda toxin is a single polypeptide with an estimated moleclar mass of 36 kDa in SDS-PAGE under reducing conditions. Lambda toxin gave molecular mass range of 32-36 kDa by gel filtration of sephacryl S-100. Enzyme activity was detected in a wide pH range of 5 to 8. The optimum activity was noted at pH 7.5.

The enzyme activity was unstable above 42°C. The stability of azocaseinase activity of lambda toxin increases in presence of Ca^{2+}, while metal chelators EDTA and EGTA and zinc-specific chelator 1, 10-phenanthroline inhibits azocaseinase activity. The inhibition is reversible on dialysis against buffer containing Ca^{2+} and Zn^{2+} , suggesting that enzyme requires Zn^{2+} possibly as prosthetic metal ion and Ca^{2+} probably as stabilizer (Jin *et al.*, 1996). Azocaseinase activity was also inhibited by phosphoramidon, an inhibitor of thermolysin and thermolysin related metalloproteases (Weaver *et al.*, 1977).

Biological properties of lambda toxin

Lambda toxin is a monomeric zinc metalloprotease invovled in tissue invasion and destruction (Jin *et al.*, 1996). Lambda toxin mature protein contains the

HEXXH zinc-binding motif and consensus sequence of thermolysin family. Thermolysin family consists of virulence factor activating metalloproteases of pathogenic bacteria like *Vibrio cholerae* and *Listeria monocytogens* (Booth *et al.*, 1984; Poyart *et al.*, 1993).

The purified lambda toxin has been shown to cleave a variety of biologically important substances such as immunoglobulins, complement C3 component, fibrinogen, various collagens, fibronectin, etc (Jin *et al.*,1996). Substrate repertoire of lambda toxin is similar to that of *P. aeruginosa* elastase (Hase and Finkelstein, 1993) which also belongs to the thermolysin family and is homologous to lambda toxin. The intradermal injection of purified lambda toxin has shown to increase vascular permeability resulting in haemorrhagic oedematous lesions. These properties suggest that it is a potent virulence factor of *C. perfringens* involved in invasion and tissue destruction.

Activation of epsilon toxin

Apart from role of lambda toxin in tisue invasion and destruction, a more important role of lambda toxin in activating epsilon prototoxin has been suggested. The epsilon toxin, which is the most potent of *C. perfringens* toxin is secreted as inactive prototoxin. The secreted inactive epsilon prototoxin is proteolytically cleaved and activated to >1000 fold active epsilon toxin in gastrointestinal tract. Hunter *et al.*, (1992) has shown trypsin and chymotrypsin activation of epsilon prototoxin in *in-vitro* studies, but *in-vivo* activation is not yet resolved. Trypsin, however, is a strong inactivator of beta-toxin. Hence role of trypsin and chymotrypsin in *in-vivo* activation of toxins needs elucidation indiseases like lamb dysentery involving both epsilon and beta toxins. The lambda toxin has been shown to cleave N and C terminals of epsilon toxin in *in-vitro* studies making a case for lambda toxin mediated epsilon activation *in-vivo*. The epsilon prototoxin activa-

tion is achieved by cleavage of 10 and 29 amino acid residues at N and C terminals respectively, reducing molecular mass of epsilon prototoxin from 32.5 kDa to 30.5 kDa and rendering it biologically active. The studies on lethality of epsilon toxin in mice suggested that lambda toxin activated epsilon prototoxin to a greater extent than trypsin and LD_{50} of lambda toxin cleaved epsilon toxin was reported to be 110 ng/kg body weight in mice (Minami *et al.*, 1997).

Lambda-toxin encoding gene *lam*

The *lam* gene has been reported to be located on a plasmid with a 70 kb nucleotide length in *C. perfringens* type B strain NC 1B 10691, while it was reported to be on a 3.2 kb plasmid in strain ATTC 3626 B (Blaschek and Solberg, 1981). The *lam* gene is probably located on different plasmids in different strains. Jin *et al.* (1996) cloned the partial *Hinc* II digest of plasmid of *C. perfringens* strain NCIB 10691 in pUC18 and selected the clone with caseinase activity. The *lam* gene ORF consists of 553 amino acid residues with molecular mass of 35, 272. The ORF begins at 196 bp which is located at the begining of a typical signal peptide.

The N-terminal amino acid of mature enzyme is seperated by approximately 200 amino acids from signal peptide indicating that the lambda toxin is secreted as a proenzyme and thereafter processed to the mature enzyme.

The lambda toxin amino acid sequence shows a HEXXH motif as well as consensus sequence around the three zinc-binding sites dictated homologous to that of the thermolysin family (Jiang and Bond, 1992; Minami *et al.*, 1997). The thermolysin family inlcudes virulence factors (metalloproteases) of pathogenic bacteria (Hase and Frankelstein, 1990). The predicted amino acid sequence of lamda-toxin shows that it is a zinc-metalloprotease homologous to *Bacillus thermoproteolyticus* thermolysin (Holmes and Matthews, 1982) and *P. aeruginosa* elastase (Thayer *et al.*, 1991).

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All the chemicals utilised in the present study were of Molecular Biology grade.

Agarose (low EEO), Acrylamide, Ammonium persulphate, ATP, Bis-acrylamide, Bromophenol blue, Calcium chloride, Coomassie-brilliant blue, Ethidium bromide, Ethylene diamine tetra acetic acid (EDTA), Glycine, IPTG, Lithium chloride, Lysozyme, Magnesium chloride, Potassium acetate, Proteinase-K, RNase (DNase free), Sodium chloride, Sodium acetate, N, N, N, N-tetramethyl ethylene diamine (TEMED), Trizma base, Urea and X-Gal were purchased from Sigma chemicals, USA. Boric acid, Glucose, Glycerol, Glacial acetic acid, 8-Hydroxy quinilone, Hydrochloric acid, Isopropanol, Methanol, Magnesium sulphate, Polyethylene glycol 6000, Phenol, Sodium hdyroxide and Sodium citrate were from BDH, Liverpool. Bacto tryptone, Bactoyeast extract and Bactoagar were obtained from Hi-Media, India.

Restriction enzymes, T4 DNA ligase, Calf intestinal alkaline phosphatase, Taq DNA polymerase, dNTP, Klenow enzyme, Random primer labelling kit and DNA and protein molecular weight markers were obtained from Bangalore Genei, India.

DIG labelling and detection kit was purchased from Boheringer Manheim, Germany while the T7 sequencing kit was from Pharmacia biotech.

3.1.2 Other materials

Hybond nylon membranes for nucleic acid transfer were from Amersham, U.K., X-ray film from Konica, radioisotopes [^{33}p] α dATP (3000 Ci mmol^{-1}) and [^{35}S] α dATP (3000 ci mmol^{-1}) were purchased from M/s BRIT, Bombay and M/s Jonaki BRIT, Hyderabad, respectively. All the glasswares used in this study were of Borosil make. The plastic wares utilised were of Axygen Sceintific or Tarson make.

3.1.3 Bacterial cultures

The strains of *C. perfringens*, *E. coli* and Pasturella used in this study were already available in Gene Expression Lab, National Biotechnology Centre, IVRI, Izatnagar. The *C. perfringens* strains/isolates used in the present study are type A NCTC 8237, type B NCTC 3110, type C CSL, type D : NCTC 8376, RIWA 759, CWD 78, goat isolates Hyderabad and Mukteshwar, sheep isolate Avinagar and Giraf isolate.

3.1.4 Plasmid vectors and host cells

Plasmid vectors pGEMT (Promega)and pQE 30, 31, 32 (Qiagen) and host strains of *E. coli*, JM109 (Promega)and M15 (Qiagen) were utilised in this study.

3.1.5 Oligonucleotide primers

Oligonucleotide primers derived from published sequence of lambda toxin gene *lam* of C. perfringens type B (Jin, 1996) with linker at 5' end having a restriction site were obtained from M/s Bangalore Genei.

Primer	Primer sequence with R.E. site in bold	R.E. site	Position of annealing
Lam-1 (Forward)	CCCGAGCTCTAATGAAAAAAATATTAATA	*Sac* I	196-213
Lam-2 (Reverse)	CCCAAGCTTTTAATTAATTCCTAC	*Hind* III	1857-1842

3.1.6 Media, buffers and reagents

Different media, buffers and reagents used in various experiments were prepared as described in Sambrook *et al.* (1989) and are detailed in the appendix.

3.2 METHODS

3.2.1 Growth of Clostridial organisms

All clostridial organisms were grown and maintained in Robertson's cooked meat medium (RCM). Prior to inoculation, the medium was heated in boiling water bath for 5 minutes and then cooled to room temperature. Using sterile Pasteur pipette 2-3 drops of seed culture was inoculated into fresh RCM. Simultaneously the seed culture was streaked on blood agar to check aerobic contamination. Both RCM and blood agar tubes were incubated at 37°C for 18 hours in stationary condition.

For isolation of genomic DNA from clostridial cultures, the growth from RCM was inoculated into production medium (appendix), which also was heated and cooled prior to inoculation like RCM. Production medium was incubated at 37°C overnight in stationary condition.

3.2.2 Isolation of genomic DNA of *Clostridium* species

The 40 ml of overnight growth in production medium was centrifuged at 8,000 rpm for 10 min in SS-34 rotor (Sorvall RC-5 B) in polypropylene tubes. Supernatant was discarded and cell pellet was washed with 4 ml GTE buffer (appendix). The cell pellet was resuspended in 3.6 ml GTE buffer by gentle vortexing and 0.4 ml(20ug/ml) lysozyme was added. Cell suspension was incubated for 2h in water bath at 37°C. To this 0.42 ml of 10% SDS and 0.2 ml of 0.5 M EDTA (pH 8.0) were added. The cell suspension was mixed throughly and cell lysis was achieved at room temperature (RT) for 10 min. The cell lysate was treated with RNase (20 µg/ ml) at 37°C for 30 min. Finally proteinase K (50µg/ml) treatment was carried out at 50°C for 3 hours. This was then sequentially extracted once each with equal volume of phenol, phenol : chloroform : isoamyl alcohol (25 : 24: 1) and chloroform : isoamyl alcohol (24:1). Finally the aqueous phase was added with 1/10th volume of 3M sodium acetate pH (5.2) and 0.6 volume of isopropanol. The precipitation was carried out by gentle mixing of two phases and incubation at RT for 15 min. The precipitated DNA was spooled out with micropipette tip in a microfuge tube, was washed once with 70% alcohol and air dried. The DNA pellet was reconstituted in TE buffer and qualitative and quantitative estimations by agarose gel electrophoresis and spectrophotometery (OD 260 nm and OD 280 nm) were carried out.

3.2.3 Amplification of lambda toxin gene (*lam*) by polymerase chain reaction (PCR)

PCR was carried out in thin wall PCR tubes in 50 µl reaction volume. Each reaction had approximately 50-100 ng of template DNA (genomic DNA), 50 mM

KCl, 10mM TAPS, pH 8.8, 1.5 mM $MgCl_2$, 0.1% gelatin, 200 µM each of dNTP, 1µM of each primer and 1.5 units of Taq DNA polymerase enzyme. The reaction were overlayed with mineral oil. Thermocycling was carried out in PTC-100 (MJ Research, USA) as follows : initial denaturation at 94°C for 5 min was followed by 30 cycles each at 94°C for 1 min, 42°C for 1 min and 72°C for 1 min 30 sec. This was followed by 7 min final extension at 72°C. The PCR amplicons were anlaysed by agarose gel electrophoresis. Once the PCR was standardized, the distribution of lam gene in various *C. perfringens* types/strains/isolates was screened by PCR amplficiation. Further the PCR amplicons were dot blotted and hybridized by non-isotopic DIG labelled lam gene probe. The probe preparation and hybridisation was as described in section 3.28.

3.2.4 Elution of specific PCR product from agarose gel

The elution of DNA fragment from agarose gel was carried out using Clean gene kit (Bangalore Genei). The PCR amplicons were electrophoresed in 0.8% agarose gel in 1X TBE and the DNA fragment of interest was excised out from gel with scalpel blade without direct exposure to UV light. Gel piece was sliced and placed in a microfuge tube and 3 vol of sodium iodide solution (from kit) was added. Dissolution of gel was carried out at 50°C for 15 min. Then 20 µl of glass milk suspension (from kit) was added and the tube was gently vortexed and kept at room temperature for 20 min. This was followed by centrifugation at 10,000 rpm for 1 min. in a microcentrifuge. The pellet was twice washed with wash buffer (provided with kit) and air dried. Then the pellet was resuspended in 20 µl of autoclaved distilled water and kept at 50 °C for additional 10 min. The elution was carried out by pelleting the glass suspension at 10,000 rpm for 1 min and the DNA in the aqueous phase was transffered to a fresh tube and DNA recovery was estimated by agarose gel electrophoresis.

3.2.5 Cloning of *lam* gene of *C. perfringens*

The lam gene was cloned in pGEMT vector as described by Sambrook *et al.* (1989). The procedure utilised is briefly as follows :

3.2.5.1 Ligation

About 25 ng of pGEMT vector (Promega) and 50 µg of eluted *lam* gene PCR product were ligated in a 10 µl ligation rection with 1 x ligase buffer, 3 U of T4 ligase enzyme and 1 mM ATP. The ligation was carried out in a circulating water bath at 16°C for 20 hours and the reaction was subsequently stored at -20°C.

3.2.5.2 Preparation of competent cells

The *E. coli* strain JM109 were made competent by TSS method. Single colony of the bacterial strain was inoculated in LB broth with ampicillin (100 µg/ml) and grown overnight at 37°C with shaking. About 50 ml of LB broth was then inoculated with overnight grown JM109 culture at 1:200 ratio and was incubated at 37°C with vigorous shaking till OD_{600} reached between 0.2-0.3. The cells were pelleted at 6,000 rpm for 10 min at 4°C in sterile polypropylene tubes. The supernatant was discarded and tubes were inverted on tissue paper for about 1 min to drain traces of medium.The cell pellet was resuspended in 5 ml of TSS. Cell aliquots of 200 µl were made in sterile microfuge tubes and were stored on ice for 1 hour. The cells were finally stored at –70°C for future use.

3.2.5.3 Transformation

The 5 µl of the ligation reaction was mixed in 195 µl of ice cold TCM (appendix) and was then added to 200 µl of competent cells. The contents were gently mixed and stored on ice for 1 hour. the plasmid coated competent cells were subjected to heat shock at 45°C for 2 min in a circulating water bath and were immediately transferred back on ice for 5 min. To these competent cells 600 µl of

SOC (appendix) was added and the cells were further incubated at 37°C for 45 min in shaker incubator. The cells were then plated on to LB agar plates with X-gal, IPTG and ampicillin.

3.2.5.4 Screening of recombinant clones

The *lam* gene cloned into pGEMT vector designated as pGEM λ was screened by selecting ampicillin resistant white colonies in presence of IPTG and X-gal. Confirmation of recombinant clones was carried out by colony blot hybridization as well as restriction endonuclease digestion of recombinant plasmid.

3.2.5.4.A. Colony blot :

Twenty white and 5 blue colonies were picked up randomly and plated on LB plate supplemented with ampicillin. After incubating the plate for 24 h at 37°C, the plates were kept at 4°C for 1h. A nylon membrane slighlty shorter than the size of the plate was placed on the colonies. After 2 min the membrane was carefully removed and was gently placed with colony side up on a 3 mm Whatman filter paper saturated with denaturation solution (0.5 M NaOH and 1.5 M NaCl) for 5 min and finally on 0.5 M Tris and 1.5 M NaCl for 10 min,drying the filter paper on absorbent sheets in between each step. The membrane was baked at 80°C for 2 h and was hybridized with non-isotopic DIG *lam* gene probe as described in section entitled hybridisation.

3.2.5.4B Confirmation by restriction enzyme digestion:

The colonies giving positive signals were inoculated in 3 ml LB broth (with ampicillin 100 mg/ml) and were incubated at 37°C overnight in shaker incubator. 1.5 ml overnight cultures was centrifuged in microfuge tubes at 8000 rpm for 5 min in a microcentrifuge. The supernatant was dicarded and drained off and cell pellet was resuspended in 200 ul of P1 solution (50 mM Tris, 10 mM EDTA, pH

8.0) by gentle vortexing. To this 200 ul of P2 solution (0.5 M NaOH, 1% SDS) was added and incubated for 5 min at RT. Finally 200 ul of chilled P3 solution was added to the tube and mixed gently and stored on ice for 10 min. The tubes were centrifuged at 15000 rpm for 10 min and the supernatant was transferred to a fresh microfuge tube and extracted once with PCI. The aqueous phase was precipitated by adding 0.6 vol of isopropanol and incubating at RT for 10 min. The plasmid DNA was pelleted at 1500 rpm for 10 min, washed once with 70% alcohol and air dried. The pellet was resuspended in autoclaved distilled water.The plasmids were double digested with *Sac* I and *Hind* III and were analysed by agarose gel electrophoresis.

3.2.6 Large scale isolation of plasmid DNA

Large scale plasmid DNA was isolated by alkaline lysis method (Sambrook *et al.*, 1989). *E. Coli* strains JM 109 and M 15 cells harbouring recombinant plasmid were inoculated in 250 ml LB medium supplemented with 50µg/ml ampicillin. Bacterial cultures were grown overnight at 37°C in an orbital shaker and cells were harvested at 5000 rpm for 20 min in a Sorvall SS34 rotor at 4°C. The cell pellet was resuspended in 9 ml of ice cold solution I and incubated on ice for 10 min. 20 ml of ice cold solution III was added, contents mixed by inverting the tubes and incubated further for 20 min on ice. The tubes were centrifuged at 12,000 rpm for 40 min at 4°C in Sorvall SS34 ortor. The supernatant was transferred to a fresh tube and DNA precipitated at room temperature for 10 min with 0.6 volume of isopropanol. Pellet was washed with 70% ethanol followed by centrifugation at 8000rpm for 30 min. Finally, the DNA was dried and resuspended in 500µg of 1 X TE, pH 8.0.

In order to obtain DNA of high purity for the sequencing reaction, an equal volume of ice cold 5 M lithium chloride solution was added and incubated on ice for 10 min. Suspension was centrifuged at 10,000 rpm for 15 min at 4°C. DNA was

precipitated at room temperature for 10 min by addition of 500µl of isopropanol. The pellet obtained on centrifugation at 10,000 rpm at room temperature for 15 min was dried and resuspended in 500 µl of 1 X TE, pH 8.0. An equal volume of polyethylene glycol solution (13%PCG; 1.5M NaCl) was added and suspension was incubated on ice for 20 min. Solution was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was dried and resuspended in 40µl of 1 X TE, pH 8.0.3 µl of RNase (10mg/ml) was added and solution incubated at 37°C for 30 min. DNA was extracted with PCI and reprecipiteted with 0.1 volume of 3 M sodium acetate and two volumes of absolute alcohol at-70°C for 1 h. Solution was centrifuged at 12,000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 500 of ul of TE.

3.2.7. Restriction mapping of cloned *Iam* gene fragment:

The clones which released the fragment of expected size were further subjected to restriction endonuclease analysis. The recombinant clones were double digested each with *Pst* I, *Pvu* II, *Bgl* II, *EcoR* I in combination with *Hind* III. The restriction digestion was carried out following manufacturer's instructions. The R.E. digests were subjected to agarose gel electrophoresis.

3.2.8 Hybridization of *Iam* gene PCR amplicon to *C. perfringens* genomic DNA

3.2.8.1 Dot blot

Dot blots of DNA samples were performed in minifold dot blot apparatus obtained from Schleigher and Schuell. Nylon membrane and 3 mm Whatman filter paper were placed in minifold apparatus after soaking in 2 X SSC. The assembly was connected with vaccum pump. 3-4µg of each DNA sample was diluted to 50 µl in TE buffer (pH 8.0) and denatured by adding 50 µl of 1M NaOH at room

temperature for 20 minutes. Then 400 µl of neutralization buffer (1.5 M NaCl, 1.0M Tris Cl, pH 7.5) was added and samples were kept at RT for further 10 min. The samples were then loaded into wells of dot blot apparatus. Vaccum was applied till the samples were sucked completely. The nylon membrane was baked at 80°C for 2 hours in vaccum oven and were stored at 4°C untill hybridisation.

3.2.8.2 Southern blotting of *C. perfringens* genomic DNA

About 10 µg of each genomic DNA of *C. perfringens* types were digested overnight with 30 U of Hind III restriction enzyme. The digested DNA was electrophoresed in 0.8% agarose gel at 5 V/cm of gel. After completion of electrophoresis, the gel was placed in 0.2 N HCl for 15 min to carryout depurination. This was followed by denaturation in 1M NaOH and 1.5 MNaCl solution for 30 min. Finally the gel was kept in 1M Tris HCl, pH 7.5 and 1.5 M NaCl for 45 min to acheive neutralisation. The gel was washed briefly in distilled water each time between treatments.

The Southern blot assembly was prepared as described by Sambrook *et al.* (1989). The inverted gel was placed on a 3 mm Whatman paper wick dipped in 10X SSC. Positively charged nylon membrane (Amersham) was placed over the gel avoiding any air bubble in between. Three sheets of 3 mm Whatman filter paper of the size of gel were placed on the nylon membrane. Over this a stack of paper towels was placed. A weight of 0.75 kg was placed over the stack of paper. The capillary transfer was carried out for 24-30 hours. The assembly was dismantled and the nylon membrane was baked at 80°C for 2 hours in vaccum oven. The nylon membrane was covered in saran wrap and was stored at 4°C untill hybridisation..

3.2.8.3 Non-isotopic probe preparation

The *lam* gene PCR fragment eluted from agarose gel was labelled by non-isotopic method using DIG DNA labelling kit from Bochringer Mannheim, Germany.

The reaction mixture was constituted as follows :

Heat denatured lam gene DNA fragment	10 µl (1µg)
Hexanudeotide random primer mixture	2 µl
DIG dNTP mixture	2µl
Water	5µl
Klenow enzyme	1µl

The labelling reaction was carried out at 37°C for 8 hours and then stopped with 2 µl of 0.5 M EDTA.. Labelled DNA was precipitated by adding 2.5 µl of 4 M lithium chloride and 75 µl of prechilled ethanol and left for 30 min at -70°C. DNA was pelleted by centrifugation at 10,000 rpm for 15 min in a refrigerated microfuge and was washed once with 70% ethanol. Labelled DNA was dissolved in 50 µl TE and was stored at -20°C till use.

3.2.8.4 Isotopic probe preparation

The *lam* gene PCR fragment eluted from agarose gel was labelled by isotopic method using Random primerlabelling kit (Bangalore Genei).The reaction mixture was constituted as follows :

Heat denatured lam gene DNA fragment	4µl (50ng)
Hexanudeatide random primer mixture	2 µl
dCTP, dGTP and dTTP mix (5mM each)	2µl
20mM DTT	2ul
Random priming buffer	2ul
[^{33}p] α dATP (3000 Ci mmol^{-1})	4ul
Klenow enzyme	2µl

The labelling reaction was carried out at RT for 8 hours and then stopped with 2 µl of 0.5 M EDTA. The probe was purified by Qiagen quick purification column and was preserved at -70 till use.

3.2.8.5 Hybridization

Nylon membranes of dot and Southern blot were prehybridized in hybridization oven (Techne hybridizer) for 3 hours in prehybridization buffer (composition-appendix I). Following prehybridization about 200 ng of DIG-labelled probe / 50 ng of radiolabelled probe was denatured by boiling for 5 minutes and immediately chilled on ice. The denatured probe was added to prehybridization buffer. Hybridization was carried out for 18 hours. The hybridized membranes were washed twice for 15 min in low stringency wash buffer (2 X SSC, 0.1% SDS) at 37°C. This was followed by two 30 minutes washing in high stringency wash buffer (0.2 X SSC, 0.5% SDS) at 50°C.

3.2.8.6 DIG Detection

Hybridization signals were detected following protocol of non-isotopic DIG DNA labelling and detection kit. Following the stringency washings the membrane were washed twice for 15 min at 37°C in detection buffer 1 (100 mM Tris Cl, 15 mM MNaCl pH 7.5). This was followed by blocking in buffer 2 (1% blocking reagent provided with the kit in buffer 1) at 37°C for 30 min and 2 washings of 15 min each at 37°C with buffer 1. Then the membranes were incubated with anti-DIG conjugate at 37°C for 1 hour (4 µl of supplied conjugate diluted to 10 ml in buffer 1).

Again two fifteen min washing with buffer 1 at 37°C were carried out. Following this the membranes were incubated in buffer 3 (100 mM Tris Cl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min at 37°C. Then the colour development reaction was carried out in 10 ml buffer 3 added with 45 µl NBT and 35 µl X-phosphate supplied with the kit. The colour development was allowed for 15-45

minutes and then was stopped by addition of buffer 4 (10 mM Tris, 10mM EDTA, pH 8.0). The membranes were photographed and preserved.

3.2.8.7 Autoradiography

The membranes hybridised with radiolabelled probe were placed in saran wrap and were exposed to X-ray films in cassette with intensifying screen at -70 for appropriate time.

3.2.9. Nucleic acid sequencing

Sequencing was carried out by T7 polymerase sequencing kit (Amersham). Both forward and reverse M13 primers were used to read the sequence from either end of the cloned lam gene fragment. Sequencing involved various steps described below. After confirming 190 bp 5' sequence and 240 bp 3' sequence of cloned lam gene by T7 sequencing further data was generated by automated seqencer at Delhi University.

3.2.9.1 Denaturation of ds DNA

The plasmid isolated from one miniprep was dissolved in 32 ul to which 8 ul of 2M NaOH was added and DNA was left at RT for 10 min. To this 7 ul of sodium acetate (pH 4.8), 4 ul distilled water and 120 ul of chilled ethanol was added and the tube was kept on ice for 10 min. The DNA precipitate was pelleted at 13,00 rpm for 10 min and washed once with 70 % alcohol, dried and dissolved in 7 ul nuclease free water.

3.2.9.2. Sequencing reaction

To the denatured DNA 2µl of annealing buffer and 1µl of forward/reverse primer was added. The tubes were heated at 65°C for 2 min and were allowed to attain RT over a period of 30 min by placing in a water bath. 2µl of labelling mix, 0.5µl of ^{35}S-dATP(5µCi), 1µl of 0.1 M DTT and 2µl of T7 polymerase(diluted 1:8 in

enzyme dilution buffer) were added to annealed DNA and the reaction was incubated at 37°C for 5 min. In meantime 2.5µl of termination mix (A,C,G and T) were taken in four seperate 0.5 ml tubes and kept at 37°C for 1-2 min.in a water bath. From the tube containing labelled mixture, 3.5 µl mix was added to each prewarmed tube containing termination mix (A,C,G and T), The four tubes were incubated at 37°C for 5min. in a water bath. The reaction is stoped by adding 5 µl stop solution to each tube. The sequencing products were stored at-20°C till further use.

3.2.9.2 Preparation of sequencing gel

Sequencing gel plates were cleaned thoroughly by detergent and washed with triple distilled water. The cleaned glass plates were dried and wiped with tissue paper dipped in methanol. The inner side of shorter glass plate was siliconized by treating with repelcote to prevent the gel from sticking to the plate. Both the plates were sealed together with tape after placing the spacer. The gel mixture (60ml) was prepared by adding 27.35g urea, 6 ml 10x TBE, 9 ml 40% acrylamide- bisacrylamide solution, 200µl of 20% APS, 20 µl TEMED and distilled water to make 60 ml.

The reagents were mixed and the gel mixture was poured inside the glass cassette carefully using 25 ml pipette avoiding air bubbles. The combs were inserted with flat edge towards the gel and the gel was allowed to polymerize for 30-45 min.

After polymerization of the gel, combs were removed and the top surface of the gel was washed with 1 X TBE. After removing the tape from the bottom of the plate, sequencing apparatus was assembled by fixing the cassette properly. The combs were inserted with shark teeth edge downwards, with the teeth about 0.1-0.2 mm inside the gel. The upper and lower chambers of the electrophoresis assembly were filled with 1 X TBE buffer. Pre-run was given for 30-45 min at 60 m amp (75 Watts).

3.2.9.3 Running of sequencing gel

The sample were heated at 95°C for 5 min before loading the sequencing gel. The 3.5 to 4 µl of the sequencing reaction mix was loaded with order A, C, G and T. Electrophoresis was carried out at 75 Watt. After 45 min of run, 250 ml of 3 M sodium acetate buffer was added to the lower chamber. The duration of electrophoresis was kept as per the requirement for short and long runs.

After electrophoresis the plates were dismantled from the apparatus. Shorter plate was lifted up carefully, so that the gel remained on the longer plate. The gel was lifted from the longer glass plate with the help of Whatman filter paper No. 3. The gel was then covered with saranwrap and dried on vaccum gel dryer for 30 min. The dried gel was antoradiographed in the X-ray-cassette at 70°C for 3-4 days.

After 3-4 days X-ray films were developed in developer solution for 2-3 min and then rinsed in water before fixing. The films were left in fixer solution for 10-15 min and were rinsed with water to remove fixer. The developed and fixed films were air dried and stored in envelope.

3.2.10 Expression of lambda toxin gene:

Cloning of lambda toxin encoding gene fragment in expression vector was carried out. The PCR amplified lambda toxin encoding gene fragment was digested by *Sac* I and *Hind* III restriction enzyme digestion and cloned in pQE (Qiagen) expression vector. The PQE vector were double digested with *Sac* I and *Hind* III and dephosphorylated by calf intestinal alkaline phosphatase.

3.2.10.1 Preparation of vector:

Since the pQE series vectors lack any visual marker differentiating recombinant clones, the vectors were dephosphorylated by calf intestinal alkaline

phosphatase following *Sac* I and *Hind* III double digestion. About 2 µg linearised pQE vectors of all three reading frames (30, 31, 32) were digested overnight with *Sac* I/*Hind* III in suitable compatible buffer. The completion of digestion was confirmed by agarose gel electophoresis . The linearised vector were then extracted once with phenol:chloroform: isoamyl alcohol, precipitated with 2 volumes of ethanol and pelleted by centrifugation. The resultant pellet was dissolved in 100 µl of Tris 100 mM (pH 8.3). 10 µl aliquot of this was kept separately as non-dephosphorylated vector control during ligation. The 90 µl of vector was further treated with 10 U of calf intestinal alkaline phosphatese at 37°C for 30 min. This was followed by proteinase-K treatment at 100 µg/ml concentration in presence of 0.5% SDS and 5mMEDTA (pH 8.0). The proteinase-K treatement was carried out at 56°C for 1 hour and was followed by phenol:chloroform:isoamyl alcohol extraction and precipitation with 10 vol of 3M sodium acetate pH 8.0 and 2 volume of ethanol. The precipitated vector DNA was washed once with 70% alcohol and resuspended in autoclaved distilled water before ligation.

3.2.10.2 Preparation of insert:

The *lam* gene PCR amplicon was double digested by *Sac* I and *Hind* III double digestion and was eluted out after agarose gel electrophoresis as described earlier. This eluted *lam* gene fragment was used in ligation with PQE vectors.

3.2.10.3 Ligation

Ligation was carried out as described earlier with 1:3 vector insert ratio. The transformation was done in M15 cells and transformed cell were plated on to LB agar plates with ampicillin (100µg/ml) and kanamycin (50 µg/ml).

3.2.10.4 Selection of positive clones:

Screening of recombinant clones of pQE 30, pQE 31 & pQE 32 vectors was done randomly because there was no specific marker for selecting recombinant

clones. Ampicillin and kanamycin resistant clones were screened by PCR amplification of specific 1.68 kb product and were further confirmed by restriction digestion of recombinant plasmid with *Sac* I and *Hind* II for release of the insert DNA.

3.2.10.5 Induction of expression

E. coli cells harbouring the recombinant plasmid pQE were grown overnight in LB broth with ampicillin (100µg/ml) and kanamycin (25µg/ml) at 37°C. About 20 µl of freshly grown culture was inoculated in 5 ml LB medium and incubated at 37°C with vigorous shaking until 0.6 OD_{600} was recorded. The cells were induced by adding 1 mM IPTG and incubated further for 4-5 h. Non-induced cells were maintained as control. Bacterial cells were then harvested by centrifugation at 6000 rpm for 5 min. The cell were analysed by SDS-PAGE to determine the expressed protein.

RESULTS

4.1 Genomic DNA extraction

C. perfringens genomic DNA was extracted from 40 ml overnight culture in the production medium. The DNA yield was satisfactory and varied between 6-8 µg/ml of bacterial culture. Molar absorbance coefficients at wave length 260 nm (A_{260}) and 280 nm (A_{280}) were taken for each sample and the ratio of A_{260} to A_{280} varied between 1.65 to 1.8. The agarose gel electrophoresis of isolated DNA revealed that DNA was relatively intact without smearing and RNA.

4.2 PCR amplification of lambda toxin gene (*lam*)

PCR amplification of *lam* gene encoding lambda toxin ORF was carried out using specific primers (lam 1&2). Thermostable *Taq* polymerase was employed to carry out PCR amplification from *C. perfringens* type D strain NCTC. The primers lam 1&2 were used at annealing temperature 42°C in 30 cycles PCR reaction in presence of 5% glycerol yielding a specific PCR product of 1.68 kb (Fig 1).

4.3 Distribution of *lam* gene in *C. perfringens* type/strains

The PCR amplification of *lam* gene was carried out using genomic DNA of various *C. perfringens* types/strains. The PCR amplification suggested presence of *lam* gene in all *C. perfringens* type D strains viz. NCTC, RJWA, CWD, Avikanagar, Giraf, Hyderabad, Mukteswar. *Lam* gene was also amplified in *C. perfringens* type B NCTC strain, while *C. perfringens* type A and C and *Pasturella multocida* P52 and *E. coli* strain M15 were found negative for presence of *lam* gene (Fig. 2). The dot blot hybridisation of PCR amplicons with DIG-labelled lam gene probe yielded lam gene distribution pattern similar to that obtained by PCR (Fig. 3).

4.4 Elution of specific PCR product from agarose gel

The gel with PCR amplified *lam* gene band was sliced out without direct exposure to UV light, the elution of the DNA was carried out using sodium iodide based Clean Gene kit. The eluted DNA was checked by agarose gel electrophoresis and it was found to be a single band without smearing. The yield of eluted DNA was approximately 60% of that loaded on to the gel.

4.5 Cloning of PCR amplified *lam* gene

The eluted 1.68 Kb *lam* gene PCR product of *C. perfringens* type D strain NCTC was ligated in pGEMT linearised vector by using T4 DNA ligase by incubating at 16°C overnight. The ligation mixture was used for the transformation of competent *E. coli* JM 109 strain prepared by TSS method. Transformed JM109 cells were plated over LB agar plates containing ampicillin, IPTG and X-gal which yielded blue and white clonies. The colonies transformed with recombinant plasmids were white in color due to inactivation of lac Z gene as the foreign DNA was cloned in MCS. The clonies transformed with recircularised pGEMT were

blue in color as intact lac Z gene led to the synthesis of ß-galactosidase, which chemically broke down x-gal to chromogenic substance. The control ligation and transformation results were as expected. In background control all the colonies were blue. In negative control no colony was seen on LB plate containing ampicillin.

4.5.1 Screening of recombinant colonies

4.5.1.1 Colony hybridisation

Twenty white colonies and 10 blue colonies were picked up randomly and plated on to a fresh LB plate supplemented with ampicillin. These were lifted on to a positively charged nylon membrane, were lysed, denatured and baked at 80°C for 2 hours. The membrane was hybridised with 100 ng of DIG-labelled *lam* gene probe. Most of the white colonies gave positive hybridisation signal while none of the blue colony gave hybridisation signal (Fig. 4).

4.5.1.2 Plasmid isolation and confirmation of released insert

Plasmid DNA was extracted from 5 colonies giving positive hybridisation signal in colony blot and from one blue colony. The miniprep plasmid isolation was carried out from 1.5 ml overnight culture and it yielded approximately 1-2 µg of DNA. The plasmids gave 3 bands on agarose electrophoresis representing different farms of plasmid. Recombinant plasmids were heavier and migrated slower than non-recombinant plasmids. The DNA from each of miniprep was dissolved in 20 µl TE and 5 µl was subjected to *Sac* I and *Hind* III double digestion and *Hind* III single digestion.

The size of linearised recombinant clones was 4.68 kb as compared to 3.0 kb of non recombinant pGEMT vector. The double digestion with *Sac* I and *Hind* III released a fragment of 1.68 bp in recombinant clones (Fig 5). The double

digested recombinant plasmids and linearised non-recombinant plasmids were Southern blotted on to the nylon membrane and were hybridised with non-isotopic DIG-labelled *lam* gene DNA probe. The released product gave positive hybridisation signal further confirming cloned *lam* gene fragment. The linearised non-recombinant vector did not hybridise with the probe (Fig. 7 & 8).

4.6 Characterisation of *lam* toxin gene restriction enzyme analysis of clone *lam* gene

The cloned *lam* gene fragment was subjected to double digestion with *Hind* III and *Pst* I/*EcoR*I/*Pvu*II/*Bgl*II. The *Hind* III/*Pst*I double digestion released a fragment of expected 584 bp size. The *Hind* III/*EcoR*I double digestion released three fragments of 1400, 504 and 217 bp size while *Hind*III/*Pvu*II double digestion released fragments of 1057, 561 and 561 bp. The profile generated by these enzymes (Fig. 6) was conformating with restriction profile of published sequence of *lam* gene of *C. perfringens* type B strain NCIB 10691.

4.7 Hybridization

4.7.1 Dot blot

3-4 µg each of denatured genomic DNA from various clostridial strains/ isolates and enteric pathogens were blotted on positively charged nylon membranes and were hybridized with the non-isotopic *lam* gene DNA probe (200 ng) under stringent conditions. Signals of hybridization were detected in *C. perfringens* type B NCTC strain and in all tested strains/isolates of *C. perfringens* type D (viz. NCTC, RIWA, CWD, Avikanagar, Giraf, Hyderabad, Mukheshwar). However, the signals were not detected with *C. perfringens* types A and C, *Pasteurella multocida* strain P52 and *E. coli* strain M15 genomic DNA (Fig. 9).

4.7.2 Southern blot

Approximately 10 µg each of genomic DNA from different type/strains of *C. perfringens*, *Pasteurella multocida* strain P52 and *E. coli* strain M15 were digested with restriction enzyme *Hind* III overnight and were electrophoresed on 0.8% agarose gel. The digestion was complete in all the DNA samples and all lanes in agarose gel revealed banding and smearing up to the bromophenol blue dye front. The electrophoresed DNA was depurinated, denatured and neutralised before transferring on to a positively charged nylon membrane. The transfer was complete in 24-30 hours as found by UV illumination of agarose gel after transfer. The blot was hybridized with 50 ng of isotopic *lam* gene probe for 16 hours under stringent conditions. Hybridization signals were detectable at 4.0 kb region. The *C. perfringens* type B NCTC strain and type D NCTC strain gave signals, while *C. perfringens* types A, C and *E. coli* strain M15 did not give any hybridisation signal (Fig. 10a & 10b).

4.8 Nucleotide sequencing

Sequencing of *lam* gene of *C. perfringens* type D strain NCTC cloned in pGEMT was carried out by Sanger's dideoxy chain termination method. The forward and reverse M13 universal sequencing primers were used for sequencing both ends of the gene. The 4.0 µl of sequencing reaction mix of each A, C, G and T was found to be optimal to obtain uniform well resolved banding pattern and intensity in each lane. Electrophoresis was carried out in 6% denaturating gel for two and half hours at 75 watts. About 190-240 bases could be clearly read (Fig. 11). Similarly the automated sequencing generated 507 bp 5' sequence and 461 bp 3' sequence.

The 507 bp from 5' end of *lam* gene had the incorporated *Sac*I restrictoin enzyme site and initiation codon ATG. There was a single base substitution at

position 284 bp where G was replaced by A as compared to sequence published for *lam* gene of *C. perfringens* type B strain NCIB 10691.

About 461 bp sequence data generated from 3' end of cloned *lam* gene revealed identical nucleotide sequence as compared to the type B strain NCIB *lam* gene sequence except a single bp substitution at 1675 bp from T to C.

4.9 Expression in PQE 30, 31, 32 vectors

The *C. perfringens* type D strain NCTC *lam* gene PCR product was double digested with *Sac*I and *Hind*III. The *lam* gene fragment was eluted from agarose and was ligated in CIAP dephospharylated PQE 32 vector. The recombinant clones were double digested with *Sac*I and *Hind*III enzyme which released 1.68 kb *lam* gene fragment (Fig. 12). The Southern hybridization by DIG labelled probe revealed signal at 1.68 kb (Fig. 13). Further these positive clones were grown to 0.6 OD_{600} and were induced for expresion with 1mM IPTG. The induction was carried out at 37°C for 4-6 hours following which the bacterial cells were lysed and resolved on 12.5% SDS-PAGE to detect hetrologous expression. The SDS-PAGE did not indicate expression of desired protein. Further, the *lam* gene was also cloned in pQE 30 and pQE 31 vectors so that all three possible reading frames for any gene were available for *lam* gene expression. But the expression could not be detected in pQE 30 and pQE31 vectors as in case of pQE32 vector.

Fig. 1 : PCR amplification of *lam* gene

Lane M : pBR 328 DNA-*Bgl* I and *Hinf* I digest marker

Lane 1 : PCR amplified 1.68 kb *lam* gene fragment from *C. perfringens* type D strain NCTC 8376

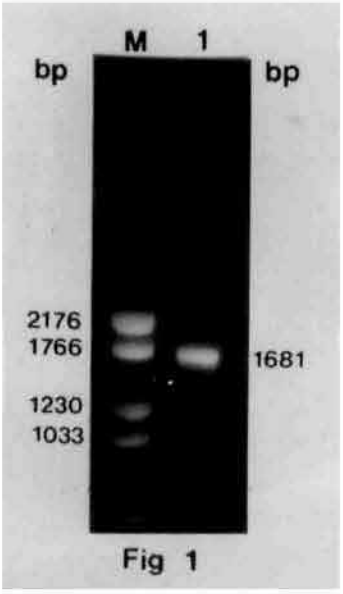


Fig 1

Fig. 2 : PCR amplification of *lam* gene in different *C. perfringen* types/strains

Lane M : pBR 328 DNA-*Bgl* I and *Hinf* I digest marker

Lane 1,2&3 : *C. perfringens* type A NCTC, type B NCTC and type C CSL, respectively

Lane 4,5,6, 7,8,9 & 10 : *C. perfringens* type D strains/isolates NCTC, CWD, RIWA, Avikanagar, Giraf, Hyderabad and Mukteshwar, respectively

Lane 11,12, & 13 : *Pasteurella multocida* P52, *E. coli* M15 and negative control respectively

Fig. 3 : Dot-blot of PCR amplicons with type D NCTC lam gene probe

Lane 1A,1B & 1C : *C. perfringens* type A NCTC, type B NCTC and type C CSL, respectively

Lane 1D,1E, 1F,1G,2A, 2B & 2C : *C. perfringens* type D strains/isolates NCTC, CWD, RIWA, Avikanagar, Giraf, Hyderabad and Mukteshwar, respectively

Lanes 2D,2E & 2F : *Pasteurella multocida* P52, *E. coli* M15 and negative control respectively

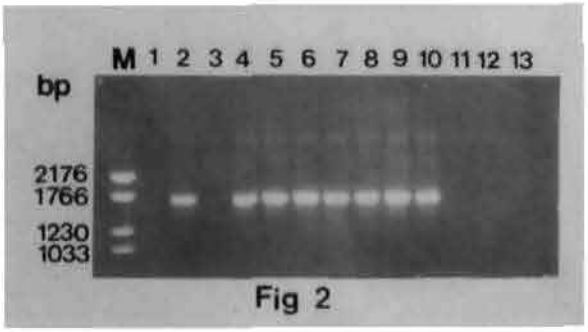


Fig 2

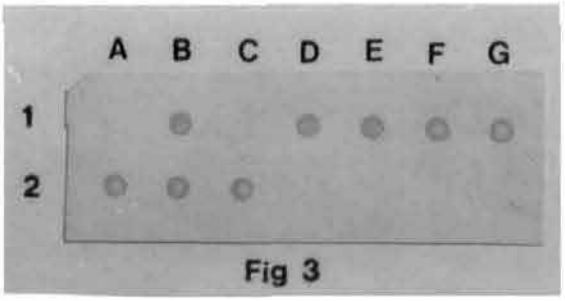
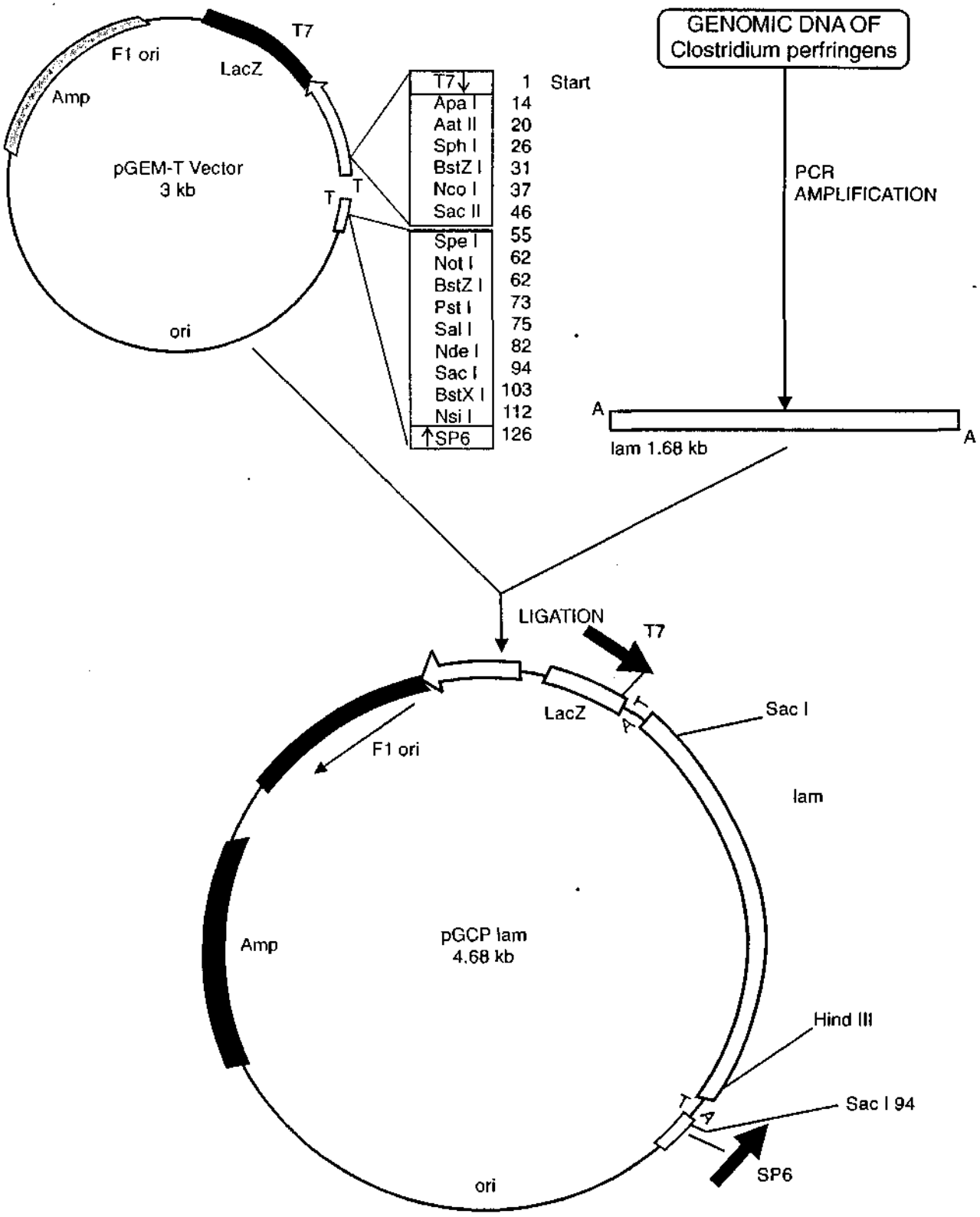


Fig 3



Cloning strategy of PCR amplified lam gene. The PCR amplified 1.68 kb fragment was cloned into the pGEM-T vector to obtain the final plasmid pGCP lam

(Plate 1)

Fig. 4 : Colony blot hybridisation with DIG labelled *lam* gene probe.

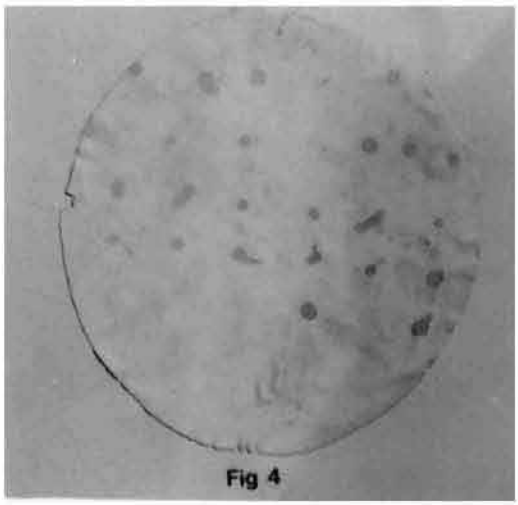


Fig 4

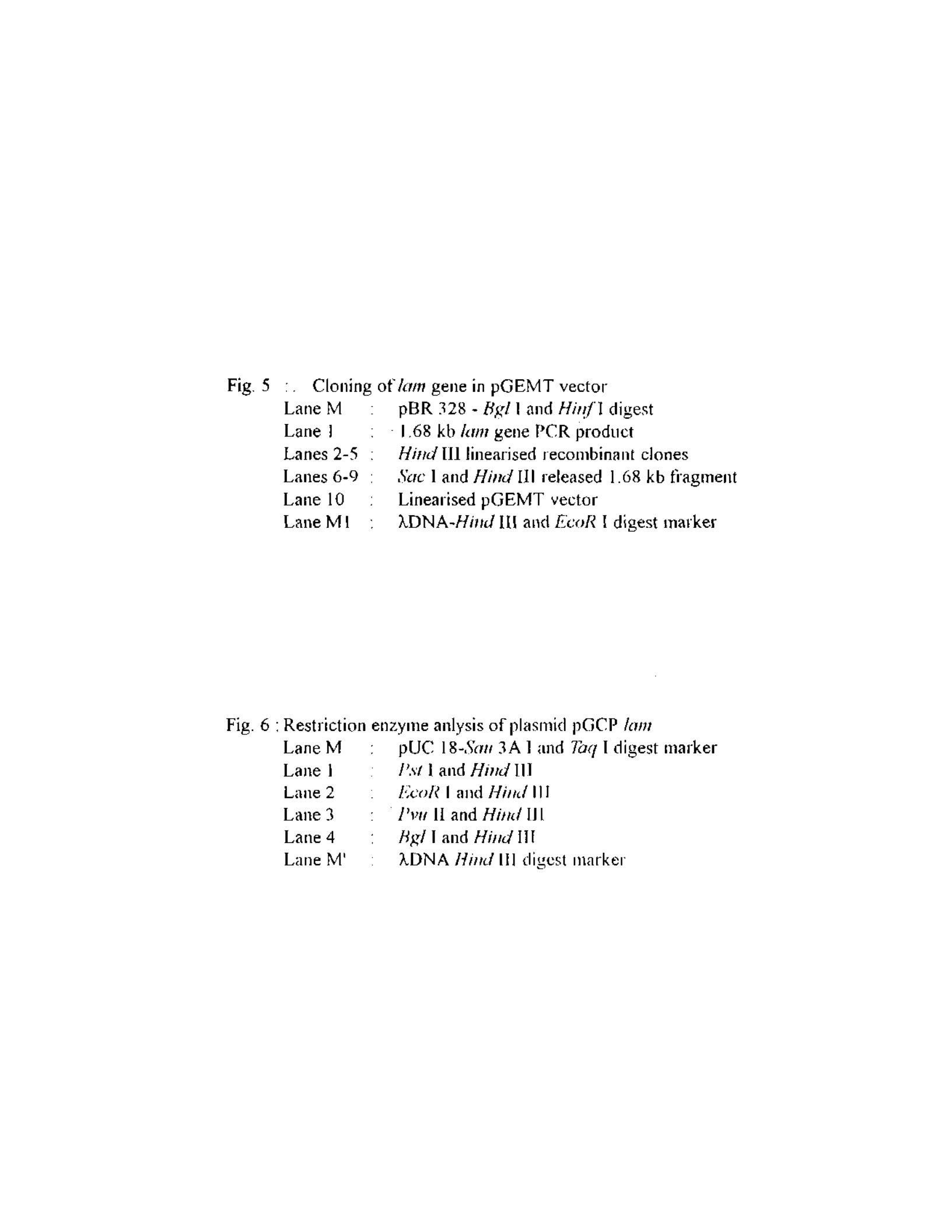
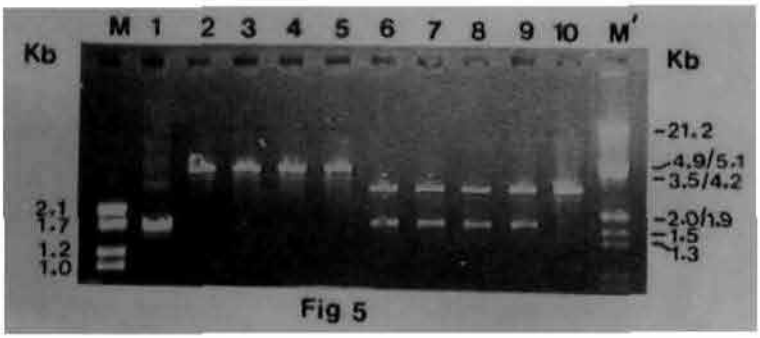


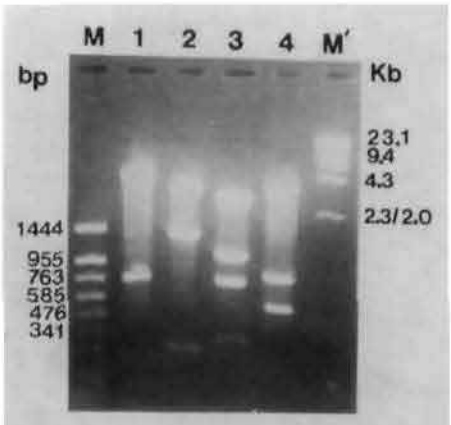
Fig. 5 : Cloning of *lam* gene in pGEMT vector

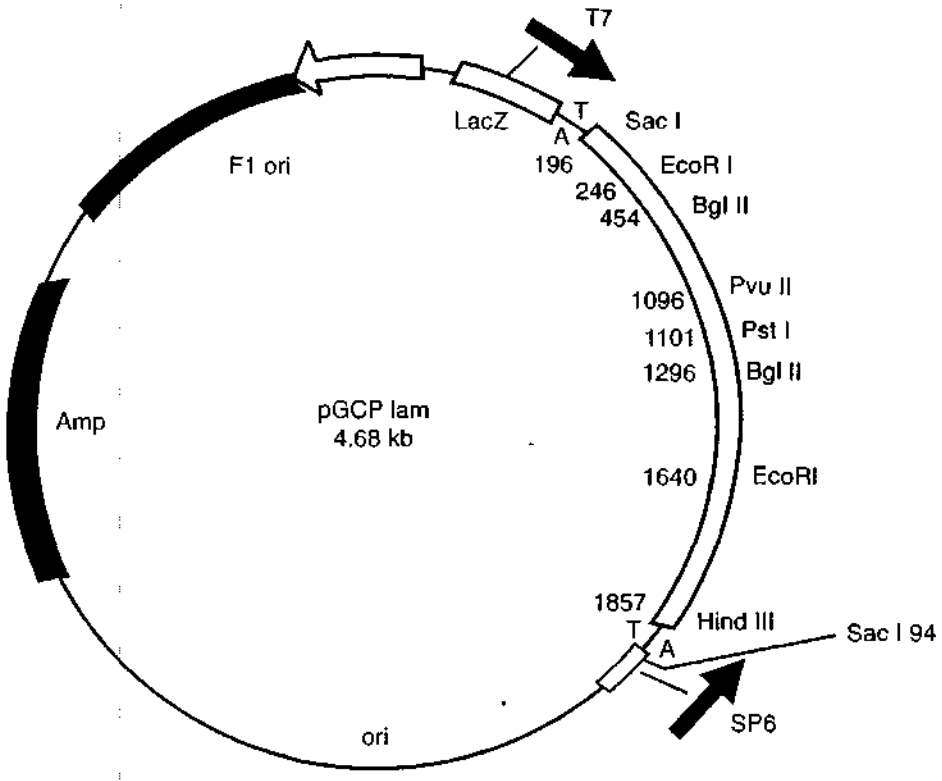
Lane M : pBR 328 - *Bgl* I and *Hinf* I digest
Lane 1 : 1.68 kb *lam* gene PCR product
Lanes 2-5 : *Hind* III linearised recombinant clones
Lanes 6-9 : *Sac* I and *Hind* III released 1.68 kb fragment
Lane 10 : Linearised pGEMT vector
Lane M1 : λDNA-*Hind* III and *EcoR* I digest marker

Fig. 6 : Restriction enzyme anlysis of plasmid pGCP *lam*

Lane M : pUC 18-*Sau* 3A I and *Taq* I digest marker
Lane 1 : *Pst* I and *Hind* III
Lane 2 : *EcoR* I and *Hind* III
Lane 3 : *Pvu* II and *Hind* III
Lane 4 : *Bgl* I and *Hind* III
Lane M' : λDNA *Hind* III digest marker

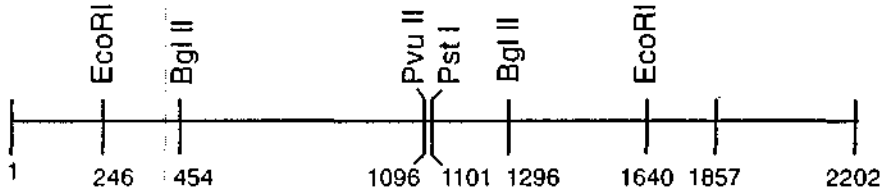

M 1 2 3 4 5 6 7 8 9 10 M'
Kb
Kb
~21.2
~4.9/5.1
~3.5/4.2
~2.0/1.9
~1.5
~1.3
2.1
1.7
1.2
1.0
Fig 5


M 1 2 3 4 M'
bp
Kb
23.1
9.4
4.3
2.3/2.0
1444
955
763
585
476
341
Fig 6

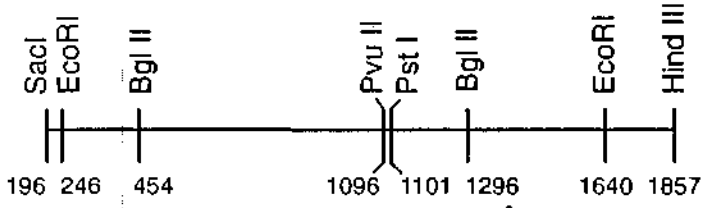


Circular map of PGCP lam

Lam gene of *C. perfringens*
Type NCIB



Lam gene of *C. perfringens*
Type DNCTC



Linear Restriction Map of lam gene

(Plate 2)

Fig. 7&8 : Confirmation of recombinant clones by Southern blot hybridization with DIG labelled lam gene PCR product

Lane M : λDNA *Hind* III digest marker
Lane 1 : 1.68 kb *lam* gene PCR product
Lane 2&3 : Sac I and *Hind* III digested pGCP *lam* clones
Lane 4 : Linearised pGEMT vector

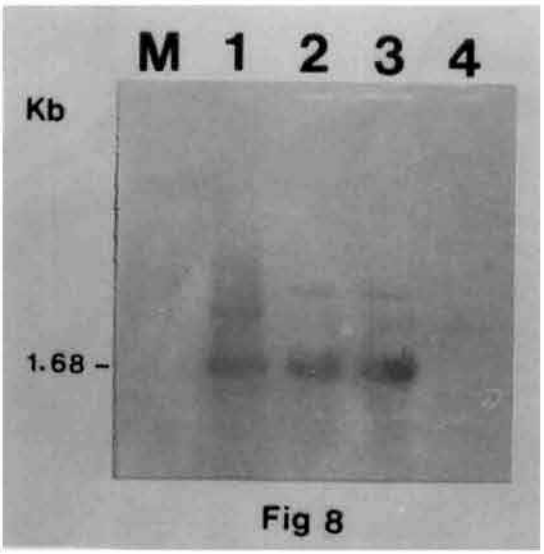


Fig 8

Fig. 9 : Dot blot hybridization of genomic DNA from different *C. perfringens* types/ strains/isolates

1A Lam PCR product	1B A NCTC	1C B NCTC	1D C CSL	1E D NCTC
2A CWD	2B RIWA	2C Avikanagar	2D Giraf	2E Hyderabad
3A Mukteshwar	3B *E. coli*	3C *P. multocida*	3D pGEMT	3E pGCP lam
4A Negative control	4B –	4C –	4D –	4E –

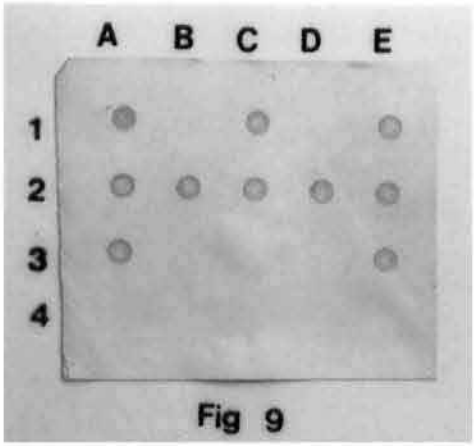


Fig 9

Fig. 10 : Southern blot hybridisation of genomic DNA of different *C. perfringens* types with *lam* gene probe.

a Agarose gel electrophoresis of genomic DNA digested with *Hind* III

b Southern blot hybridized with ^{33}p labelled probe

Lane M : λDNA-*Hind* III marker
Lane 1 : 1.68 kb *lam* gene PCR product
Lane 2 : Sac I and *Hind* III digested pGCP lam
Lane 3 : Type D NCTC
Lane 4 : Type B NCTC
Lane 5 : Type A NCTC
Lane 6 : Type C CSL
Lane 7 : *E. coli* M15

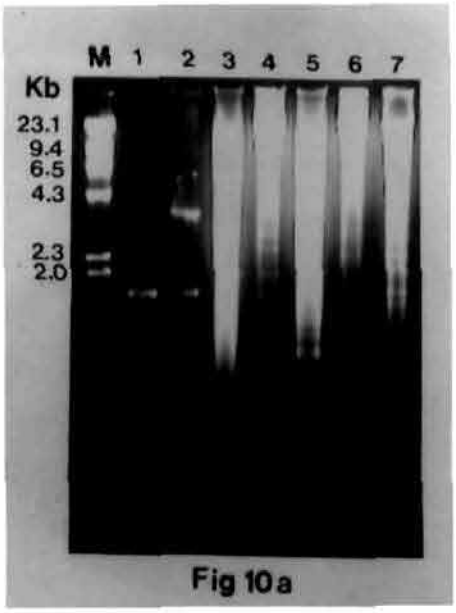


Fig 10a

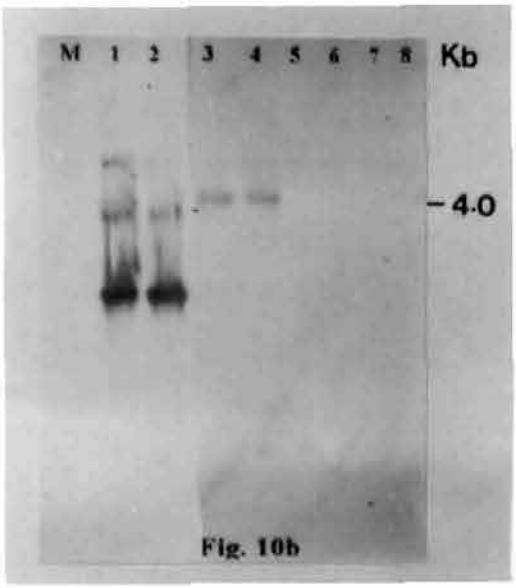


Fig. 10b

Fig. 11 : Nucleotide sequencing of pGEMT cloned *kan* gene

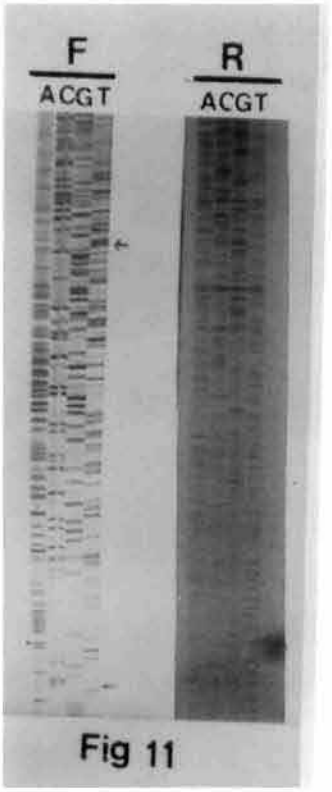


Fig 11

FORWARD SEQUENCE

```
      5'   196
B NCIB    ATGAAAAAAATATTAATATCGCTTCCTACAGTAGCAGCTATTGTAA
D NCTC    ATGAAAAAAATATTAATATCGCTTCCTACAGTAGCAGCTATTGTAA

B NCIB    CAATGAATTCTTCACATATAGTTTCAGCTATTGAAGAAGGACgACA
D NCTC    CAATGAATTCTTCACATATAGTTTCAGCTATTGAAGAAGGACaACA

B NCIB    AATGAAAAGTAAAACAGAAATAATTCAACAGATTTCTGAAGATAC
D NCTC    AATGAAAAGTAAAACAGAAATAATTCAACAGATTTCTGAAGATAC

B NCIB    TAATGGAAAAGAAGAAATATTCATGGATGAAAGTGATGGAGTACA
D NCTC    TAATGGAAAAGAAGAAATATTCATGGATGAAAGTGATGGAGTACA

B NCIB    GATATTTATTAAAGGTAATTTTGATTTAAATACTGGAGTATCAAAA
D NCTC    GATATTTATTAAAGGTAATTTTGATTTAAATACTGGAGTATCAAAA

B NCIB    GATACAGTACTTAGCTATTTTGAAAACAATAGATCTTTTTTTAATT
D NCTC    GATACAGTACTTAGCTATTTTGAAAACAATAGATCTTTTTTTAATT

B NCIB    TTAAAAATAATGACCTTAACTTTAGAATTGATAAATATGAAACTGA
D NCTC    TTAAAAATAATGACCTTAACTTTAGAATTGATAAATATGAAACTGA

B NCIB    TGACCTTGGTTTTACTCATGTAAAATTAAAAGAAACTTATAAAGG
D NCTC    TGACCTTGGTTTTACTCATGTAAAATTAAAAGAAACTTATAAAGG

B NCIB    TAAAGATGTATATGGAAGAGAAATGGACTGTTCACTTTGATAAGA
D NCTC    TAAAGATGTATATGGAAGAGAAATGGACTGTTCACTTTGATAAGA

B NCIB    GTGGAGAAATAAATAGTATTACAGGAACTTTAGAAGATAGAATCC
D NCTC    GTGGAGAAATAAATAGTATTACAGGAACTTTAGAAGATAGAATCC

B NCIB    AATCAATTACAAAAAAAAATACACAGGCAATATCAAGTTCTTCTG
D NCTC    AATCAATTACAAAAAAAAATACACAGGCAATATCAAGTTCTTCTG

B NCIB    CCATAGAGATAGCC
D NCTC    CCATAGAGATAGCC
```

703

Plate 3a : Comparison of nucleotide sequence of lam gene of *C. perfringens* type D NCTC strain 8376 with lam gene of *C. perfringens* type B NCIB 10691. The small bold alphabet indicates change at nucleotide level.

REVERSE SEQUENCE

3' 1396

B NCIB TCTGATGTTTTTGGTGTATTAATTCAAACTTATGAAAAGTATGATG
D NCTC TCTGATGTTTTTGGTGTATTAATTCAAACTTATGAAAAGTATGATG

B NCIB TTAAGAATGGGGGAGATTGGATATTTAATCCTTATGATTGGGTTAT
D NCTC TTAAGAATGGGGGAGATTGGATATTTAATCCTTATGATTGGGTTAT

B NCIB TGGAGATGAAATATATACTCCAGGAATAAAAGGAGATGCTTTAAG
D NCTC TGGAGATGAAATATATACTCCAGGAATAAAAGGAGATGCTTTAAG

B NCIB AAGTCTCGCTAATCCTAAGCTATATGATCAACCAGATCATATGAAA
D NCTC AAGTCTCGCTAATCCTAAGCTATATGATCAACCAGATCATATGAAA

B NCIB AATTACTATAATCTTCCTAATACTGAAAATGGAGATTATTGTGGTG
D NCTC AATTACTATAATCTTCCTAATACTGAAAATGGAGATTATTGTGGTG

B NCIB TTCATATTAATTCAGGAATTCCAAATAAAGCTGCATATAATTTAGC
D NCTC TTCATATTAATTCAGGAATTCCAAATAAAGCTGCATATAATTTAGC

B NCIB ATCTACACTTGGATGTGAAAAAACTGCAAGAATATATTATAGAGCA
D NCTC ATCcACACTTGGATGTGAAAAAACTGCAAGAATATATTATAGAGCA

B NCIB ACTACACAGTATTTTAATAGTACAACTTCATTTGTAGAAGCAAGA
D NCTC ACTACACAGTATTTTAATAGTACAACTTCATTTGTAGAAGCAAGA

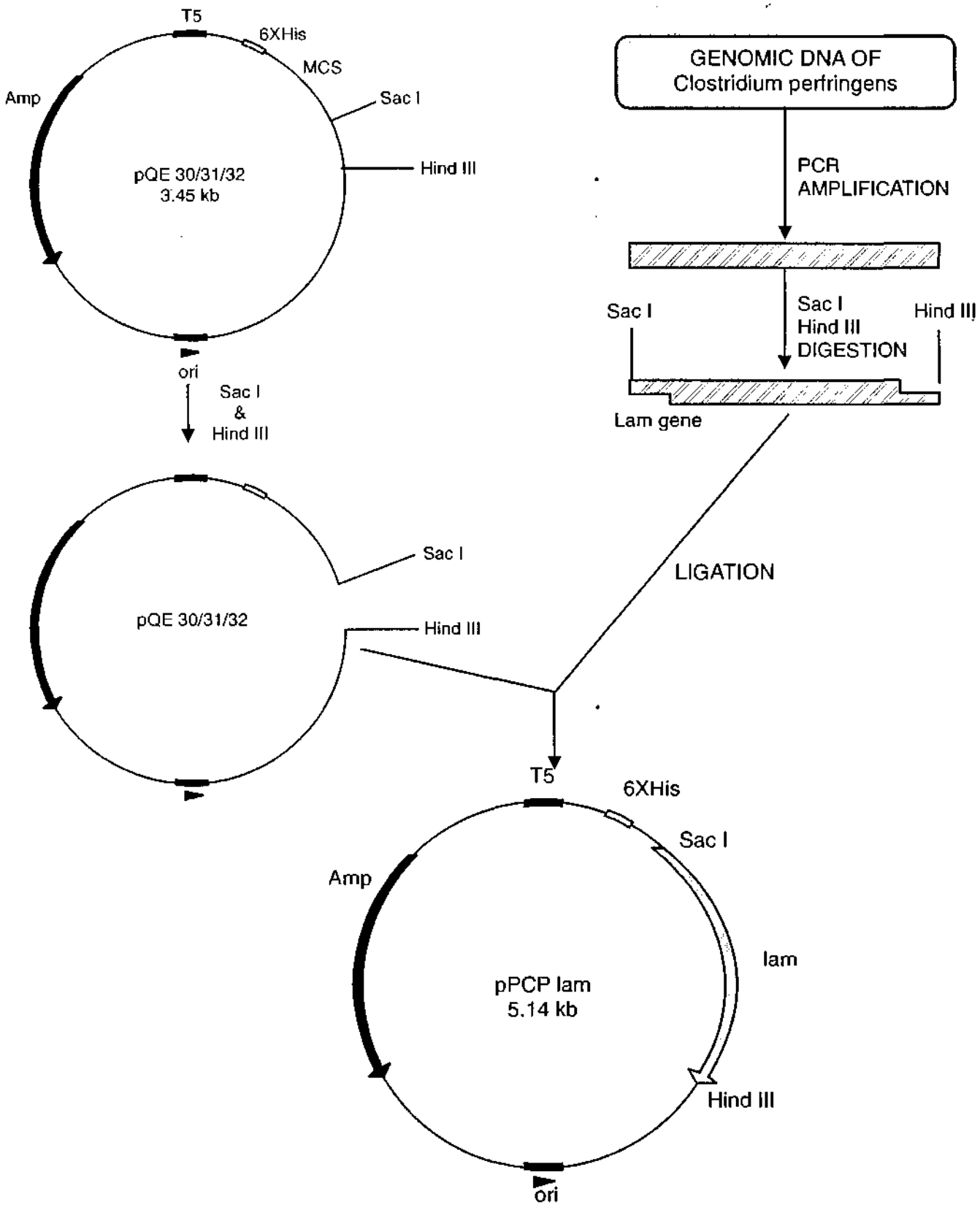
B NCIB CTTGGCTTAGTACAAGCTGCTAAAGATTTATATGTAAATAATTCTT
D NCTC CTTGGCTTAGTACAAGCTGCTAAAGATTTATATGTAAATAATTCTT

B NCIB TAGAAGCTGAAGCTGTAGGTAATGCTTTTTCAAATGTAGGAATTA
D NCTC TAGAAGCTGAAGCTGTAGGTAATGCTTTTTCAAATGTAGGAATTA

B NCIB ATTAA
D NCTC ATTAA

1857

Plate 3b : Comparison of nucleotide sequence of lam gene of C. *perfringens* type D NCTC strain 8376 with lam gene of C. *perfringens* type B NCIB 10691. The small bold alphabet indicates change at nucleotide level.



Cloning strategy of PCR amplified lam gene. The PCR amplified 1.68 kb fragment was digested with Sac I and Hind III and cloned into the Sac I and Hind III digested pQE series vectors to obtain the final plasmid pPCP lam (30/31/32) with the His tag at the N-terminal end under the control of tac promoter.

(Plate 4)

Fig. 12 : Cloning of 1.68 kb lam gene PCR product in pQE 30, 31 and 32 expression vectors

Lane M : λDNA *Hind* III digest marker
Lane 1 : 1.68 kb *lam* gene PCR product
Lane 2 & 3: *Sac* I and *Hind* III digested *lam* recombinant pQE 30 plasmid pPCP *lam*30
Lane 4&5 : *Sac* I and *Hind* III digested *lam* recombinant pQE 31 plasmid pPCP *lam*31
Lane 6&7 : *Sac* I and *Hind* III digested *lam* recombinant pQE 32 plasmid pPCP *lam* 32
Lane 8 : *Hind* III linearised pQE 32 vector

Fig. 13: Confirmation of recombinant pQE, 30, 31, 32 clones by Southern hybridization with DIG labelled lam gene PCR product.

Lane M : λDNA *Hind* III digest marker
Lane 1 : 1.68 kb *lam* gene PCR product
Lane 2 & 3: *Sac* I and *Hind* III digested *lam* recombinant pQE 30 plasmid pPCP *lam*30
Lane 4&5 : *Sac* I and *Hind* III digested *lam* recombinant pQE 31 plasmid pPCP *lam*31
Lane 6&7 : *Sac* I and *Hind* III digested *lam* recombinant pQE 32 plasmid pPCP *lam*32
Lane 8 : *Hind* III linearised pQE 32 vector

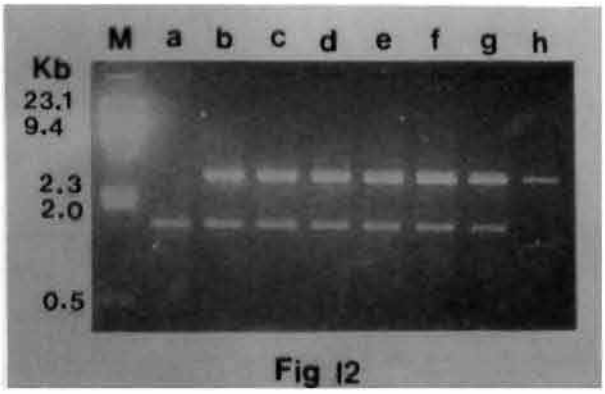


Fig 12

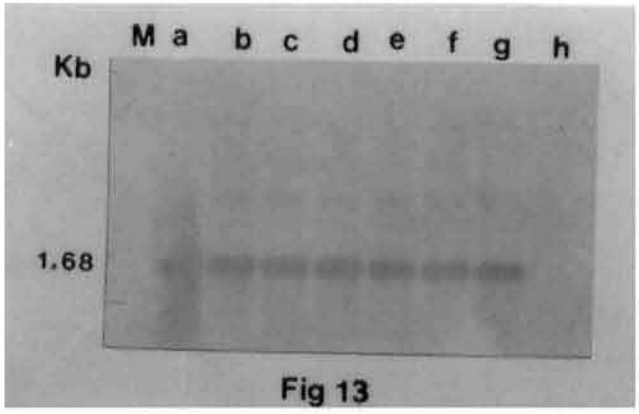


Fig 13

DISCUSSION

Clostridium perfringens is an organism of considerable veterinary importance responsible for several fatal enterotoxaemic conditions in domestic animals due to its various exotoxins and hydrolytic enzymes (Songer, 1996). Many of the virulence factors have been studied to elucidate their pathogenesis and have been characterised genetically and biochemically. Conventionally isolation and purification of these virulence factors has been cumbersome and generated limited amounts of desired protein, hampering the studies to identify their role in molecular pathogenesis. The advent of genetic engineering however, has opened avenues for the production of recombinant proteins on a large scale. The heterologous expression of recombinant proteins in bacterial host, especially in *E. coli*, has time and again proven to be efficient for mass production of proteins. Further the purification of recombinant proteins has been found to be easy owing to fusion affinity tags (Schon *et al.*, 1994; Mukhija *et al.*, 1995). The recombinant *E. coli* expression for *C. perfringens* epsilon-toxin could generate large amounts of recombinant epsilon toxin (Goswami *et al.*, 1996). Hence the present study was undertaken to fulfill the objective of cloning and characterisation of the

lambda-toxin gene of *Clostridium perfringens* for its expression in *E. coli* host system.

C. perfringens was grown in RCM and production medium. A period of 18-20 hours was sufficient for obtaining optimal growth with production of copious amounts of gases (H_2 and CO_2) further assisting in maintenance of anaerobic environment (Rood and Cole, 1991). The absence of solid meat particles in production medium facilitated cell pelleting for isolation of genomic DNA. The yield of DNA ranged between 6-8 µg/ml of bacterial culture. The agarose gel electrophoresis showed that DNA was relatively intact without much shearing and was free of RNA. The ratio of molar co-efficient of extinction at 260 nm and 280 nm wavelengths varied between 1.65 - 1.8 range suggesting sufficient purity of isolated DNA.

The genomic DNA isolation was carried out after Hochulli *et al.* (1987) with minor modifications. The method yielded high molecular weight genomic DNA with little shearing. The yields of DNA were apparently higher then experienced with the methods of Marmur, (1961) and Pitcher *et al.* (1989). The method was also simple as compared with that of Marmur, (1961) and required simple reagents. It avoided the use of hazardous guanidium isothiocyanate which is used in method adopted by Pitcher *et al.* (1989). The method employed, however, to isolate DNA did not differentiate between chromosomal and plasmid DNA and thus the isolated DNA consisted of both chromosomal as well as plasmid components.

Lambda toxin encoding gene fragment was amplified in a polymerase chain reaction using Taq DNA polymerase enzyme. The two primer pairs designed were such that it was possible to amplify complete open reading frame (ORF) of lambda toxin encoding gene. The primers lam 1 and lam 2 yielded the expected

size PCR product of 1.68 kb. The PCR amplified product had flanking restriction enzyme sites for *Sac* I and *Hind* III incorporated in its 5' and 3' sequences respectively. Complete ORF of 1661 bp, which was from 196 bp to 1857 bp of *lam* gene sequence published by Jin *et al.* (1996) along with flanking restriction enzyme, was obtained to further carryout directional cloning and manipulations.

The lambda-toxin is produced by most type B and E and some type D strains of *C. prefringens* (Bidwell, 1950; Hatheway, 1990). In the present studies many *C. perfringous* strains and certain enteric bacteria were screened for the occurence of *lam* gene by PCR. In PCR *C. perfringens* type B NCTC strain and type D seven strains/isolates (viz. NCTC, RIWA, CWD, Avikangar, Giraf, Hyderabad and Mukteshwar), amplified *lam* gene. *C. perfringens* types A and C and other bacteria viz. *Eschereshia coli* and *Pasteurella multocida* were found negative for *lam* gene. Dot blot hybridization was further used to confirm sequence homology of *lam* gene. The PCR products were blotted and hybridised with DIG-labelled *lam* gene fragment amplified from *C. perfringens* type D NCTC strain. The hybridisation signals were obtained in *C. perfringens* type B and type D strains/isolates alone suggesting sequence homology between the *lam* gene amplicons. The presence of lambda toxin gene in all type D strains/isolates used in this study was significant since four of these isolates had been obtained from cases of enterotoxaemia. Such correlation is suggestive of involvement of lambda-toxin in epsilon toxin activation and possible acquisition of sufficient virulence to result in fatal enterotoxaemias.

Dot blot hybridization was further carried out with genomic DNA of various organisms. The hybridization using non-isotopic probe gave *lam* gene distribution pattern which was found similar to the results obtained by PCR amplification. Southern hybridization was carried out to characterize *lam* gene localization. Approximately 10 µg of the total genomic DNA, which consisted both of

chromosomal and plasmid DNA, was used in each lane. Hybridization was carried out with isotopic probe at 10 ng/ml concentration under stringent conditions. Hybridization signals were detectable on autoradiography at about 4.0 kb regions in *C. perfringens* type B NCTC and type D NCTC strains. The *C. perfringens* type A, C and *E. coli* did not give any hybridization signal even on prolonged exposure confirming the absence of *lam* gene sequence in their genomes.

The PCR amplified *lam* gene fragment from *C. perfringens* type D NCTC strain was ligated into pGEMT plasmid vector with insert vector in ratio of 3:1. Transformation was carried out in *E. coli* JM109 strain prepared by TSS method. Colonies were determined as recombinant and non-recombinant based on color develoment. The colonies transformed with recombinant plasmids were white in color due to inactivation of lac Z gene, as the *lam* gene insert was cloned in to multiple cloning site. The colonies transformed with recircularized pGEMT were blue in color as intact lac Z gene resulted in break down of chromogenic substance X-gal present in LB agar plates.

To further identify the recombinant colonies with *lam* gene insert, colony hybridization were carried out employing non-isotopic DIG-labelled probe. Majority of the white colonies gave hybridization signals indicating the presence of *lam* toxin encoding gene. All blue colonies were negative for hybridization with *lam* gene probe. The positive colonies were grown in LB broth containing ampicillin and the plasmids were isolated by miniprep method. The plasmids were digested with *Sac*I and *Hind*III restriction enzymes to check the presence of *lam* gene insert. The double digestion released the desired 1.68 kb *lam* gene fragment.

The cloned *lam* gene fragment was further characterized by restriction enzyme analysis. Four enzymes *Pst* I, *EcoR* I, *Pvu* II and *Bgl* II were used in

combination with *Hind* III. The restriction digestion pattern of cloned *lam* gene suggested single site for *Pst I* at 1101 bp and two sites for *EcoR*I at 246 and 1640 bp positions. Similarly the *Pvu* II digestion revealed site at 1096 bp, while *Bgl* II enzyme had sites at 454 bp and 1296 bp. The restriction map was similar to the restriction map derived from *C. perfringens* type B strain NCIB 10691 *lam* gene sequence suggesting sequence homology of cloned *lam* gene with earlier published *lam* gene sequence Jin *et al.* (1996).

Further cloned *lam* gene was sequenced by employing Sanger's dideoxy chain termination method as described by Murphy and Kavanagh (1988). The commercially available universal sequencing primers were employed to read partial sequences from both the ends of *lam* gene cloned fragment. Using M13 forward primer, nucleotide sequence of 190 bp starting from *lam* primer was obtained. The *Sac* I site and the initiation ATG of *lam* gene ORF was also traced unaltered. The sequence analysis revealed only a single base change at position 284 bp. The G was substituted by A at nucleotide level. This would result in change of amino acid from arginine to glutamine. The reverse M13 primer was used to sequence 3' region of cloned *lam* gene. The nucleotide sequence information of 236 bp for region 1621 to 1857 was obtained. When compared with reported sequence of *C. perfringens* type B strain NCIB strain only a single bp substitution at 1675 from T to C was observed. The 507 bp 5' sequence and 461 bp 3' sequence obtained by automated sequencing revealed no additional nucleotide change. There was no deletion or insertion in the forward and the reverse sequences and the reading frame of ORF seemed to be maintained at least in the sequenced portion of gene.

The *lam* gene encoding PCR fragment was double digested with *Sac* I and *Hind* III restriction enzymes. The *Sac* I and *Hind* III digested fragment was ligated in CIAP dephospharylated pQE 32 Qiagen expression vector. The recombinant

blue white selection was not available and so the screening was carried out by randomly selecting few colonies. These colonies were boiled and the supernatants were employed for PCR amplification of *lam* gene fragment. The colonies, which gave amplicon of 1.68 kb, were further grown in LB broth with appropriate antibiotics and plasmid minipreperation was carried out. The restriction enzyme digestion with *Sac* I and *Hind* III released the desired *lam* gene fragment from pQE32 vector.

These *lam* gene recombinant colonies were grown to 0.6 OD_{600} and were induced with 1mM IPTG for 4-6 hours. The SDS-PAGE was carried out with total cell lysate to detect expressed protein with non-recombinant and recombinant uninduced *E. coli* cell controls. On SDS-PAGE no additional protein band could be detected in any of the recombinant clones.

Since the partial nucleotide sequence was carried out, the chance of some detection/insertion/frame shift could not be ruled out in the portion that was not sequenced. So in an emperical attempt the *lam* gene PCR product was cloned in pQE30 and pQE31 vectors. Togetner the pQE 30, 31 and 32 vectors had all three possible reading frames for any gene. The presence of *lam* gene fragment in pQE30, 31 and 32 was also confirmed by restriction endonuclease analysis and Southern hybridization. The induction of these clones with IPTG did not reveal any expression on SDS-PAGE. The change in temperature during induction from 32°C to 40°C was also tried but it could not induce heterologous expression as observed by SDS-PAGE protein profile. The unavailability of anti-lambda toxin polyclonal/monoclonal antibodies also ruled out western blotting to detect suboptimal / low level heterologous expression of lambda-toxin, if any.

The recombinant expression is a complex phenomenon governed by several known/unknown microenvironmental factors. The recombinant plasmid could

be unstable because of certain insert sequences it carried. This factor, however, appeared unlikely because pQE30, 31 and 32 plasmid vectors with *lam* gene insert could be propogated and isolated with optimal yield. The transcripts generated during heterologous expression of certain genes are unusually unstable and prone to degradation by host cell machinery and perhaps the same inferences held true in the present case. The secondary structure in transcript can stall translation, but since lam gene has 70% A + T contents, this problem can be ruled out in present experiment (De Lamarter *et al.*, 1985).

Apart from above, several specific factors govern recombinant expression. The organism/species specific codon bias leads to stalling of ribosomal complex and immature termination of translation (Robinson *et al.*, 1984). An improper context codons immediately after initiation codon ATG could severely affect the expression (Stormo *et al.*, 1982). Some heterologous proteins expressed in host cell systems may be identified as alien and would be subject to degradation by host cell proteolytic machinary (Bishia *et al.*, 1987). The intramembranous and cytotoxic proteins (like lambda toxin) could be cytotoxic to host cell during heterologous expression, even under uninduced conditions due to low level of constitutive expression of gene (Tabor, 1995). Any single factor or several of these in synergy could have prevented/stalled/down regulated heterologous expression of *lam* gene.

Nevertheless the lambda-toxin encoding ORF cloned in the present study, offers a ready genetic material for further genetic manipulation to achieve expression, which in turn would help in understanding the molecular pathogenesis of epsilon toxin activation. It would possibly be of some prophylactic value in epsilon toxin mediated enterotoxaemic disorders because all the four *C. perfringens* type D Indian isolates were found to carry *lam* gene in the genomes. Presence of

lam gene might be suggestive of imparting virulence to epsilon toxin producing *C. perfringens*, as all the four Indian isolates were originally obtained from diseased animals, implying correlation of *lam* gene with the malady.

Further experiments need to be carried out to pin point the reason for non-expression of *lam* gene in heterologous host system. Briefly complete nucleotide sequencing for *lam* gene and extraction of RNA for *in-vitro* translation to translate the transcript have to be carried out.

SUMMARY

Clostridium perfringens, responsible for several enterotoxaemic diseases in domestic animals, produce up to 17 extracellular toxins and hydrolytic enzymes which act as virulence factors. Lambda toxin, a zinc metalloprotease is one such virulence factor involved in tissue invasion and destruction. Its role in cleavage and activation of epsilon proto-toxin has been lately highlighted. The genetic characterisation of lambda-toxin to acheive its recombinant expression has been considered to be a tool to obtain sufficient quantities for making possible to undertake studies which might help to understand its role in pathogenesis of enterotoxaemias and subsequently assess its role as an adjunct to recombinant epsilon-toxin based prophylaxis. The present studies were, therefore, planned with an objective to clone and characterise lambda toxin from *C. perfringens* to attempt its heterologous expression in *E. coli*.

Polymerase chain reaction for lambda toxin gene amplificaiton was carried out generating a 1.68 kb *lam* gene fragment. The PCR amplification confirmed the distribution of *lam* gene in *C. perfringens* types B and D.The *C. perfringens* types A, C and *E. coli* did not amplify *lam* gene. The presence of lambda toxin

gene in all type D strains/isolates used in this study was significant since four of these were Indian isolates obtained from cases of enterotoxaemias. This underlines the possible role of lambda toxin in epsilon mediated enterotoxaemia and correlation of *lam* gene with clinical enterotoxaemia. The PCR amplified lam gene from *C. perfringens* type D strain NCTC was cloned in plasmid vector pGEMT. The restriction endonuclease analysis of cloned fragment revealed presence of restriction site for *Pst* I at 1101 bp, *EcoRI* at 246 bp and 1640 bp, *BgI II* at 454 and 1296 bp and *Pvu II* at 1096 bp. The generated partial restriction map was in alignment with published type B *lam* gene sequence map. Dot blot hybridisation of genomic DNA from various *C. perfringens* types/strains, *E. coli* and *Pasteurella multocida* generated *lam* gene distribution pattern identical to that obtained by PCR. Southern hybridisation with *Hind III* digested genomic DNA generated signal at about 4.0 kb position only in *C. perfringens* B NCTC and D NCTC. Partial nucleotide sequencing revealed almost complete homology with type B *lam* gene sequence.

The 507 bp 5' sequence from 5' end between 196 bp to 703 bp revealed G to A substitution at 284 bp. Similarly the 461 bp sequence of 3' end from 1396 to 1857 revealed T to C substitution at 1675 bp. The 1.68 kb *lam* gene ORF was cloned in pQE 30, 31 and 32 expression vectors. The recombinant expression of lambda toxin, however, could not be detected by SDS-PAGE or by caseinolytic activity on milk agar warranting further research efforts on this facet.

MINI ABSTRACT

Lambda toxin gene of *C. perfringens* types B and D was amplified in polymerase chain reaction using specific primers lam 1 and lam 2 generating 1.68 kb *lam* gene fragment. The PCR confirmed the distribution of *lam* gene in *C. perfringens* types B and D.The *C. perfringens* types A, C and *E. coli* did not amplify *lam* gene. The presence of lambda toxin gene in all type D strains/isolates used in this study was significant since four of these were Indian isolates obtained from cases of enterotoxaemias. This underlines the possible role of lambda toxin in epsilon mediated enterotoxaemia and correlation of *lam* gene with clinical enterotoxaemia. The *lam* gene from *C. perfringens* type D strain NCTC was cloned in pGEM-T vector. The restriction endonuclease analysis generation partial restriction map which was in alignment with type B lam gene map. The partial nucleotide sequence revealed almost complete sequence homology with published *lam* gene sequence of *C. perfringens* type B. The dot blot hybridisation gave a similar *lam* gene distribution pattern as obtained with PCR. The Southern blot generated hybridisation signal of *lam* gene at about 4.0 kb position in *Hind III* digested types B and D genomic DNA. The *lam* gene clone generated in the present study would be useful for carrying out further genetic manipulations for heterologous expression of lambda toxin, with a view to ultimately develop recombinant multicomponent prophylactic.

लघु सारांश

क्लोस्ट्रिडिम परफ्रिंजन्स टाइप बी एव डी की लॉमड़ा टॉक्सिन जीन को पॉलिमरेस चेन रियेक्शन से विशेष प्राईमर लॉम–1 एवं लॉम – 2 के प्रयोग द्वारा परिवर्तित किया गया । पी. सी. आर. द्वारा सभी सी. परफ्रिंजन्स टाईप डी स्ट्रेन्स/आईसोलेटस् में लॉम जीन की उपस्थिति से लॉम जीन का क्लीनिकल इनटेरोटॉक्सीमीया के साथ संभावित सम्बन्ध निर्देशित हुआ । लॉम जीन खण्ड की पी.जी.एम.टी. वेक्टर में क्लोन किया गया । रेस्ट्रीक्शन एन्डोन्यूकियेज विश्लेषेण तथा न्युक्लिमोटाइडस अनुक्रमण द्वारा क्लोनड लॉम जीन एवं टाईप बी लॉम जीन के न्यूक्लियोटाइड कम में लगभग सम्पूर्ण समानता निर्देशित हुई । डॉट ब्लॉट हाइब्रीडाइजेशन द्वारा लॉम जीन के विभिन्न सी. प्ररफ्रिजन्स टाईपस में उपस्थिति पी.सी.आर. द्वारा प्राप्त परिणामोंरूप थी । साउदर्न ब्लॉट में लॉम जीन हाइब्रीडाइजेक्शन के सीम्नल करीब 4 के. बी. स्तर पर पाये गए। प्रस्तुत अभ्यास मे क्लोन किया लॉन जीन खण्ड जीन वर्णन में उपयोगी हो सकती है । एवं इनटेरोटॉक्सीमीया के पॅथोजीनेसीस अभ्यास एवं रीकॉम्बीनेन्ट मल्टीकंपोनंट प्रोफायलॅकटीक निर्माण में यह उपयोगी हो सकती है ।

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APPENDIX

MEDIUM

Luria-Bertani (LB) medium

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Distilled water	ad. 1 litre

pH was adjusted between 7.2 and 7.5 by 10 N NaOH. Medium was sterilized by autoclaving. LB agar medium was prepared by adding 1.5% agar to LB medium.

Clostridium Production Medium

Beef Extract	3 gm
Yeast Extract	2 gm
NaCl	0.5 gm
Cysteine HCl	0.05 gm

SOB medium

Tryptone	2 g
Yeast extract	0.5 g
NaCl	0.05 g
KCl	0.0186 g
2M Mg^{++}	1 ml
Distilled water	ad 100 ml

SOC medium

SOB	980 ml

lM Glucose	20 ml

Reagents for Genomic DNA extraction

Tris : Glucose : EDTA

1 M Tris (pH 8.0)	5 ml
0.5 M EDTA (pH 8.0)	2.5 ml
1M Glucose	33 ml
Distilled water	ad. 100 ml

10% sodium dodecyl sulphate (SDS)

SDS	10 g
Distilled water	ad. 100 ml

Lysozyme solution (20 mg/ml)

Lysozyme	100 mg
Tris : Glucose : EDTA	add 5 ml

Reagents for Agarose gel electrophoresis

Tris-borate EDTA (TBE) buffer (5 X stock solution)

Trisbase	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Distilled water	

6 X Loading dye/buffer (1000 µl)

Glycerol	300 µl
10% Bromphenol blue	25 µl
Water	675 µl

Intercalating dye

Ethidium bromide	10 mg

Distilled water	1.0 ml
X-gal (1000 X)	
X-gal	25 mg
Dimethyl formamide	1.0 ml
IPTG (1000X)	
IPTG	25 mg
Distilled water	1.0 ml
Ampicillin (1000 X)	
Ampicillin (sodium salt)	500 mg
Distilled water	10 ml
Kanamycin (50 mg/ml)	
Kanamycin	500 mg
Distilled water	10 ml
Reagents for hybridization 20 XSSC	
3 M NaCl	
300 mM sodium citrate, pH 7.0	
Denaturation solution	
N NaOH, 1-5 M NaCl	
Neutralization solution	
1.0 M Tris-HCl, pH 7.5	
1.5 M NaCl	
Pre-hybridization buffer	10 ml
20 XSSC	2.5 ml
50 X Denhardt's	1.0 ml
10% N-lauroyl sarcosine	100 µl

10% SDS	
10 mg/ml denatured, salmon sperm DNA	100 µl

Denhardt's solution (50 X)

Ficoll	1 g
Polyvinyl pyrolidone	1 g
BSA	1 g
H_2O to	100 ml

Hybridization buffer

Prehybridization buffer with Denatured probe at 10 ng/ml concentration

Reagents for SDS-PAGE

Acrylamide/Bisacrylamide (30%)

30 g acrylamide and 0.8 g bisacrylamide were desolved in distilled water to make 100 ml solution.

Upper Buffer (0.5 M TRis.Cl)

6 g of Tris was dissolved in in 80 ml distilled water. The pH of the solution was adjusted to 6.8 and the volume was made upto 100 ml.

Lower Buffer (1.5 M Tris.Cl)

18.16 g Tris was dissolved in 80 ml distilled water and pH was adjusted to 8.8 and volume was made upto 100 ml.

Reservoir buffer [pH 8.3]

Tris	0.025 M
Glycine	0.192 M
SDS	0.1%

PAGE Stain (1000 ml)

Coomassie brilliant blue	1.25 g
Methanol	500 ml

Acetic acid	100 ml
Distilled water	400 ml
2 X sample loading buffer	
Glycerol	2 ml
2-mercapto ethanol	1 ml
10% SDS	4.5 ml
Stacking buffer	1.7 µl
0.1% bromophenol blue	200 µl
Distilled water	600 µl

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