MOLECULAR EPIDEMIOLOGY OF BACILLUS

CEREUS FOOD POISONING

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BY

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A thesis submitted in part fulfilment of the requirement for Doctor of Philosophy of the University of Nairobi.

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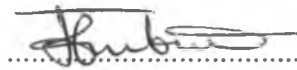
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DECLARATION

This thesis is my original work and has not been presented for a degree in any other

University.

....Date 21/12/98

DR. JACKSON N. OMBUI (BVM, MSc.)

This thesis has been submitted for examination with our approval as university

supervisors.

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....Date 21.12.98

PROF. SAMUEL M. ARIMI (BVM, MSc. PhD)

DEDICATION

....

In memory of my late mother **Rael Nyaboke** who passed away on January 20, 1997

when I was doing this project. Her demise came at a time when we required her most

and especially my young brothers and sisters who are still in school and looked

forward to her love and care. Her departure suddenly reminded me of her invaluable

service and contribution to the family that I had till then taken for granted. I pay

tribute to her. May the Lord rest her soul in eternal peace.

....

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ABSTRACT

In this study, a survey was carried out to determine the prevalence of food poisoning outbreaks in Kenya by use of medical records and annual reports of the Ministry of Health. Thirty seven outbreaks were reported to the Ministry of Health from various parts of the country between 1970 and 1994, and only 13 of these outbreaks, involving a total of 926 people, were confirmed to be due to particular aetiological agents. The confirmed outbreaks were due to *Staphylococcus aureus* (5); *Clostridium perfringens* (1); *Clostridiun botulinum* (2); Plant poisoning (3); and chemical poisoning (2). Vehicle foods that were involved in these outbreaks included milk and milk products (6), meat and meat products (2), cassava (2), white ants(1) and maize flour contaminated with chemicals(2). This study indicated that the occurrence of food poisoning outbreaks in the country is more than was recorded by the Ministry of Health considering the high number (309,191) of food poisoning cases that were treated as outpatients in the period 1988 to 1991 (Anon, 1993).

The study further explored the potential of antibiogram analysis and genetic based techniques in identifying and establishing the toxigenicity of *B. cereus* strains. A total of 153 milk samples consisting of 80 pasteurized and 73 raw were studied. *Bacillus cereus* was isolated from 33(41.2 %) pasteurized and from 27(37 %) raw milk samples. The 60 isolates were tested for production of diarrhoeal enterotoxin and ability to hydrolyse starch. Fifty percent of them produced diarrhoeal enterotoxin and 65 % hydrolysed starch. Although there was a strong correlation between production of

diarrhoeal enterotoxin and hydrolysis of starch, the latter cannot be used in diagnosis of enterotoxigenic *B. cereus*.

Antimicrobial susceptibility tests found *B. cereus* isolates to be resistant to one or more drugs with all strains being resistant to ampicillin and susceptible to streptomycin. The test for carriage of plasmids found 71.7 % of the isolates to harbour between 1 and 5 plasmids with sizes ranging from 0.1 to 60 megadaltons (mDa). No relationship was apparent between carriage of plasmids, antimicrobial susceptibility patterns and enterotoxin production. Bacterial restriction endonuclease DNA analysis (BRENDA) with *EcoR1*, *Hind III*, *BamH1*, Mbo1 and *Dra1* restriction enzymes revealed no common BRENDA type for either enterotoxin positive or enterotoxin negative strains.

A polymerase chain reaction (PCR) test detected the *bceT* gene in 41.1 % of the strains, while a 741 base pair (bp) probe detected *bceT* gene in 44.6 % of the strains. All the *bceT* gene positive strains with the PCR were also positive with the 741 bp DNA probe.

In conclusion, this study has demonstrated that plasmid profile analysis and BRENDA are not useful in differentiating enterotoxin producing strains of *B. cereus* , but can be useful techniques in differentiating individual strains of *B. cereus*. The study further demonstrated that two or more diarrhoeal enterotoxins are involved in *B. cereus* food poisoning, and some strains can produce more than one diarrhoeal enterotoxins. Therefore, during investigation of a suspected *B. cereus* food poisoning, it would be necessary to screen for production of diarrhoeal enterotoxin complex by use of *Bacillus cereus* enterotoxin-reverse passive latex agglutination (BCET-RPLA) test kit, or

western blot, and also detect the presence of *bceT* gene by PCR or a probe derived from the *bceT* gene.

It is suggested that PCR be used to amplify whole *hblA* (Beecher and Macmillan, 1990), *hblC* and *hblD* genes (Ryan *et al.* 1997) that code for the B, L_2 and L_1 proteins of hemolysin BL respectively, to delineate the gene that is specific enough to characterize enterotoxigenic strains of *B. cereus*. The tests should involve direct detection of *hblA*, *hblC* and *hblD* by (1) PCR, and (2) probe prepared from the PCR products on a number of *B. cereus* strains representing both enterotoxin positive and negative strains.

CHAPTER ONE

1   INTRODUCTION

Food poisoning is any illness associated with recent consumption of food, having normally a short incubation period and symptoms with gastrointestinal features (mainly diarrhoea and vomiting), although in some cases there may be neurological and other signs not connected with the alimentary tract (Gracey, 1986). Food poisoning illnesses are caused by a variety of aetiological agents including bacteria, parasites, viruses, chemicals, heavy metals, poisonous plants and animals. Many of these present with diarrhoea and vomiting (Gracey, 1986). Diarrhoea is the fourth cause of mortality in Kenya and is usually due to a variety of causes including food poisoning (Anon, 1991). For example, out of the total number of patients who attended outpatient clinics in Kenya in 1990, 4 % were diarrhoeal cases (Anon, 1991). Food poisoning illnesses occur worldwide, but the prevalence varies from one region to another. The prevalence of foodborne diseases in Kenya is unknown and this was under investigation in this study.

Bacillus cereus food poisoning has been implicated in a number of outbreak episodes in many parts of the world. The first definitive account of *B. cereus* food poisoning was published by Hauge (1955) following the investigation of four outbreaks in Norway involving 600 cases. The food that was implicated in all the four instances was vanilla sauce prepared and stored at room temperature for one day before being served. Samples of the sauce were found to contain 25 to

100×10^6 colony forming units (cfu) of *B. cereus* spores/ml, while corn starch (one of the ingredients) was contaminated with up to 10^4 *B. cereus* spores/gram. The illness was characterized by an incubation period averaging 10 to 12 hours, abdominal pain, profuse watery diarrhoea, tenesmus and nausea that lasted no longer than 12-24 hours (Drobniewski, 1993). Similar outbreaks due to *B. cereus* were subsequently reported from other parts of the world (Goepfert *et al.* 1972; Terranova and Blake, 1978; Gilbert, 1979; Van Netten *et al.* 1990). Mortimer and McCann (1974) reported another type of *B. cereus* food poisoning outbreak that occurred 1 to 5 hours following ingestion of fried rice. The illness was characterised by an acute attack of nausea and vomiting which was followed by diarrhoea in some cases. The illness lasted for less than 24 hours.

Over time *B. cereus* food poisoning was established to be caused by two different toxins, a diarrhoeal enterotoxin (Spira and Goepfert, 1975) and an emetic toxin (Melling *et al.* 1976) that give the two observed syndromes, namely diarrhoea and vomiting respectively. In the diarrhoeal syndrome, patients experience profuse diarrhoea, with abdominal pain and cramps (rarely vomiting or fever), beginning 8 to 16 hours after ingestion of contaminated food. Symptoms resolve within approximately 12 hours. The diarrhoeal enterotoxin may be preformed in the food, or may be produced within the small intestines. Proteinaceous food products such as meat and meat products, milk and milk products are the most commonly implicated foods (Spira and Goepfert, 1972; Kramer *et al.* 1982; Shinagawa *et al.* 1984). The source of contamination is from spores naturally present in food that are able to survive normal cooking procedures (Goepfert *et al.* 1972; Spira and Goepfert, 1972). The spore forming and psychrophilic nature of the organism

enables it to survive pasteurization as well as grow at refrigeration storage temperatures (Coghill and Juffs, 1979).

The vomiting syndrome is characterized by a short incubation period of 1 to 5 hours, nausea, vomiting, abdominal cramps and diarrhoea in about one third of patients. Incubation periods as short as 15 minutes and as long as 12 hours have been reported (Kramer and Gilbert, 1989). The syndrome is associated with ingestion of rice and pasta based foods (Drobniewski, 1993). This is because *B. cereus* is ubiquitous in the soil and contaminates rice plants in the paddy fields. In the restaurants, large amounts of rice that contain *B. cereus* spores are cooked, allowed to cool slowly and used to make fried rice dishes. The rice is usually left at room temperature as refrigeration causes starch to clump the rice grains together. Spores germinate and vegetative cells produce toxin at room temperature. Toxin production is enhanced by addition of protein in the form of egg or meat and subsequent cooking is usually too brief to inactivate the heat stable emetic toxin (Drobniewski, 1993).

The prevalence of *B. cereus* food poisoning varies around the world. In the United Kingdom and United States in the late 1970s and early 1980s, *B. cereus* accounted for 1 to 3 % of reported outbreaks of bacterial food poisoning (about 1 % of actual cases). In the Netherlands, by contrast, 22 % of outbreaks (about 11 % of cases) were caused by *B. cereus* (Kramer and Gilbert, 1989). In Canada, 7 % of outbreaks and more than 2 % of bacteria-related food poisoning cases were reported as being due to *B. cereus* (Kramer and Gilbert, 1989).

There were at least 230 diarrhoeal type *B. cereus* food poisoning outbreaks reported worldwide between 1950 and 1976 (Turnbull, 1981). It was the most common cause of food

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poisoning in Hungary (117 outbreaks). Other countries that reported outbreaks in the same period were Finland (50 outbreaks), the Netherlands (11 outbreaks) and Canada (9 outbreaks). At least 170 outbreaks of emetic type *B. cereus* food poisoning were reported worldwide between 1971 and 1990. More than 110 outbreaks were reported in Great Britain alone between 1971 and 1978 (Shinagawa, 1990). Cooked and fried rice were implicated in 108 of the outbreaks of which 104 were related to boiled rice from Chinese restaurants or " take away " shops (Gilbert, 1979). A total of 73 food poisoning outbreaks due to *B. cereus* (1323 cases) occurred in Japan between 1982 and 1986, with 93 -95 % being of the emetic type. Majority of the outbreaks (73 %) were due to some form of cooked rice, whereas 16 % were due to noodles of various types and 11 % to other foods (Shinagawa, 1990).

The nature of the diarrhoeal enterotoxin is not well understood, whether it is a single factor or is composed of multiple components. Attempts to isolate the factors responsible for the biological activities and to relate them to diarrhoeal activity have resulted in inconclusive findings. For example, Thompson *et al.* (1984) reported the diarrhoeal enterotoxin to consist of three distinct protein components of molecular weights 43, 39.5 and 38 kDa. The three proteins were not active individually, but the combined components were hemolytic, lethal to mice, cytotoxic to vero cells, and positive in ileal loop assay and vascular permeability (VP) test. This toxin complex was only partially purified and its characterization was incomplete. Bitsaev and Ezepchuk (1987) described it as a tripartite enterotoxin factor, which they called DL-toxin. Beecher and Macmillian (1990, 1991) reported the diarrhoeal enterotoxin to be a hemolysin BL consisting of three protein components designated B (35 kDa), L1 (36 kDa) and L2 (45 kDa). None of these components

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had hemolytic or VP activity individually, but maximal activity occurred in the presence of all the three components. This hemolysin BL caused lysis of sheep erythrocytes and elicited vascular permeability in rabbits.

Shinagawa *et al.* (1991a and 1992) on the other hand described the diarrhoeal enterotoxin as a single electrophoretically homogenous protein with a molecular mass of 45 kDa and an isoelectric point of 5.5. These authors purified the single component enterotoxin and demonstrated its VP activity, mouse lethality and fluid accumulation in mouse ligated intestinal loops. They were however unable to show any hemolytic and lecithinase activity and pointed out to some inadequacies in the information available regarding the nature of *B. cereus* enterotoxin. For example, it was not clear whether the enterotoxin produced by all *B. cereus* strains is composed of one or more than one component, and if the enterotoxin produced by different strains is biologically and antigenically the same. This study attempted to explain these inadequacies regarding the nature of the diarrhoeal enterotoxin.

The diagnosis of *B. cereus* food poisoning has relied mainly on bacterial culture and enterotoxin detection. Biological and immunological assay methods have mainly been used to demonstrate the enterotoxigenicity of *B. cereus*. These methods are based on the activity of the toxic protein in culture fluids of suspect strains, or in extract of foods contaminated with *B. cereus* enterotoxin. They are dependent on sufficient amounts of toxin being produced in the absence of interfering products (Roger *et al.* 1990). They cannot detect toxigenic strains that have silent toxic genes, or those strains that do not produce readily detectable levels of toxin, which may be as a

Application of molecular biology techniques in the identification of *B. cereus* food poisoning strains were under investigation in this study.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 PREVALENCE OF FOODBORNE DISEASES IN KENYA

Foodborne diseases as a public health hazard, have received little attention in Kenya (Kasirye-Alemu, 1986). Although there are many newspaper reports of individuals complaining of illness or even deaths resulting from consumption of contaminated food (Anon, 1983; 1989), uninspected meat (Anon, 1970; 1973) and some food crops such as cassava (Anon, 1994a), there is little officially recorded or published data. This indicates the presence of a public health hazard that deserves more attention.

Incidence of foodborne diseases are estimated by analysis of statutory notifications of suspected or diagnosed clinical illnesses, laboratory reports of organisms identified (Galbraith and Young, 1980), and reports from Medical Officers of Health which may or may not have the causative agent identified in the laboratory (McCormick, 1986).

Food poisoning outbreaks are mainly due to bacterial agents. McCormick, (1986) reported 84 % of all food poisoning outbreaks in England and Wales in the period 1980-1984 to be due to

salmonella infection, 11 % due to *Clostridium perfringens*, 3 % to *B. cereus* and 2 % to *Staphylococcus aureus*. In the period 1975-1984, majority of 632 foodborne disease outbreaks reported in Finland were due to *Staphylococcus aureus* (170 outbreaks), *Cl. perfringens* (138), Salmonella infections (55), *B. cereus* (51) and other agents including *Cl. botulinum, E. coli* and *Shigella spp.* (16), and 202 outbreaks of unknown causative agents (Him and Aho, 1986). Pohn and Grossmann (1986) reported foodborne disease outbreaks in the republic of Federal Germany to be mainly due to *Salmonellae* and *Staphylococci, E. coli, B. cereus* and *Campylobacter spp.* Out of 164 outbreaks in the period 1983-1985, Salmonellae were the common causal agents, followed by *Clostridium botulinum, Staphylococcus aureus, escherichia.coli, Campylobacter, Costridium. perfringens, Bacillus cereus, Shigella spp.* and to a lesser extent hepatitis A.

Of the 501 foodborne disease outbreaks reported in Spain in the period 1976-1984, 67.68 % of them were due to salmonellosis, 22.72 % due to *Staphylococcus aureus*, and 9.83 % others (Perez *et al.* 1986). Ezenwa, (1986) reported majority of food poisoning cases diagnosed in Nigeria in the period 1974-1983 to be due to shigellosis, typhoid fever, paratyphoid fever and cholera. Becker, (1982) reported 20.2 % of all food poisoning incidents in the Netherlands to be of bacterial causes, 5.5 % due to chemical poisoning and 74.5 % were of unknown aetiology.

In Kenya, the seriousness of bacterial foodborne illnesses is recognised by the Ministry of Health. To this effect, the National Public Health Laboratory Service (NPHLS) and Nairobi City Council (NCC) Public Health Department screens for *Salmonella spp., Staphylococcus aureus, Clostridium spp.* and *E. coli* in all samples received for routine food quality assurance. *Vibrio cholerae* are bacteria under constant surveillance through water testing and rectal swabs of

suspected cases. In 1984, NPHLS recorded 616 cases positive for cholera, which represented 4.4 % of total samples tested. In 1985, the record was 394 positive, which was 5.7 % of the total specimens received (Anon, 1984; 1985). Cholera still continues to be a public health problem in Kenya going by the occasional media reports of outbreaks of the disease from some parts of the country (Anon, 1997a).

Salmonellosis due to *S. typhimurium* is a public health problem in Kenya (Wamola and Mirza, 1981), and typhoid fever and paratyphoid fevers due to *S. typhi* and *S. paratyphi* are endemic in the country. For example, Jumba *et al.* (1995) found 96 % of Kenyan healthy population to have agglutinin titres of less than 1: 80 for both H and O antigens of *S. typhi*, while 4 % had titres of 1:160 or above, indicating occurrence of the disease in the Kenyan population. Epidemics are often reported from various parts of the country including Marsabit District, (in 1993), Western Province, (in 1992), Kirinyaga and Embu Districts, (in 1992) (Anon, 1992; 1993).

Reports of outbreaks of dysentery due to *Shigella* spp. continue to appear from various parts of the country including an outbreak in Wundanyi, Taita Taveta district (between March and April, 1994) (Anon, 1994b), Samburu District (between Dec. 1993-Feb. 1994) (Anon, 1994b) and Muranga District (Anon, 1997b).

An outbreak of food poisoning due to *Clostridium botulinum* type A among Kenyan nomads was reported by Smith *et al.* (1979a) involving consumption of sour milk. The outbreak involved 16 people, six of whom died. Another outbreak due to *Clostridium botulinum* type A was reported in 1980 in Bungoma district, Western Province, that involved consumption of white ants (*Isoptera*), which are considered a delicacy by some tribes in Western Kenya (Nightingale and

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Ayim, 1980). The outbreak involved six people, five of whom died. Infectious hepatitis A and poliomyelitis viral infections are sometimes transmitted through food and are thought to exist in Kenya (Kasirye-Alemu, 1986). Helminthic and protozoan parasitic infections, which are often acquired through food and water are a heavy burden particularly in children. Infection often arise from direct transfer of polluted soil to the mouth by dirty hands, rather than eating contaminated food (Pamba, 1980).

Cases of fungal food poisoning are important in Kenya, mainly as a result of aflatoxin poisoning involving cereals, pulses, nuts and other foods used for human consumption. A classical case of aflatoxicosis outbreak occurred in 1981 in Machakos district and killed twelve of 20 patients admitted to hospital with hepatitis (Ngindu *et al.* 1982). Eight of the dead belonged to families which had eaten maize that contained as much as 12,000 ppb aflatoxin B_1 and other foods that contained substantially high concentrations of the toxin. The outbreak was attributed to the drought in1980, which caused a food shortage forcing people to consume food that was normally discarded. Muraguri *et al.* (1981) also described aflatoxin B_1, B_2, G_1 and G_2 in some food product especially cereals. These authors found 17.6 % of 91 maize and maize products tested for aflatoxin in Nairobi to contain various levels of aflatoxin B_1, B_2, G_1 and G_2. The highest total aflatoxin level in maize was 290 ppb, while 1050 ppb was found in one sample of raw groundnuts.

Unintentional contamination of food with agricultural chemicals used for crop protection and food preservation is the most common type of chemical food poisoning reported in Kenya (Kasirye-Alemu, 1986). The most common chemicals that have been found to contaminate food

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in Kenya are organophosphates, organochloride pesticides, cyanide and heavy metals (Anon, 1986).

Majority of food poisoning outbreaks occur where mass catering is practised compared to outbreaks in private homes (Pohn and Grossmann, 1986). In urban areas, mass catering and holding of food for long periods at ambient temperatures before consumption is a common practice. Therefore, one may expect a higher number of foodborne disease outbreaks in urban than in rural areas, where food is cooked and eaten immediately (Kasirye-Alemu 1986). This author observed that in the city of Nairobi, customers of food establishments frequently complain about snacks, fish and chips, roasted chicken and meat, sausages, meat and egg sandwiches, meat rolls, "samosas" (spiced ground meat enclosed in a triangle-shaped wheat dough casing), and deep fried pies and buffets for causing diarrhoea and vomiting. This was attributed to long periods of holding food at ambient temperatures, sometimes in warming cabinets maintained between 37-50°C.

2.2   *BACILLUS CEREUS* AND FOOD POISONING

2.2.1 Cellular and colonial morphology

Bacillus cereus is a member of the genus *Bacillus*. The organism is a gram positive, aerobic or facultatively anaerobic rod, which measures 3 to 5 μm long and 1.0 to 1.2 μm wide and tends to occur in chains (Sneath, 1986). The cells contain ellipsoidal spores in central position, which do not swell the sporangium (Gordon *et al.* 1973). Mature endospores may be released

from the sporangium as a result of lysis (Claus and Berkeley, 1986). The organism is normally motile by peritrichous flagella, however non-motile strains are sometimes encountered (Logan and Berkeley, 1984; Logan *et al.* 1984).

Colonies have a dull or frosted glass, appearance that often form an undulate margin from which extensive outgrowths develop and they vary from small and compact to large feathery, spreading type (Sneath, 1986; Claus and Berkeley, 1986). Some strains of the organism may produce a red pigment, pulcherrimin, in a starch medium containing sufficient iron. Other strains produce a yellow green fluorescent pigment in various media. Some strains darken the medium slightly on nutrient agar, while some produce a pinkish brown diffusible pigment (Sneath, 1986; Claus and Berkeley, 1986).

2.2.2 Relationship between *B. cereus* and other *Bacillus spp.*

DNA/DNA hybridization studies of *B. cereus* revealed genetic homogenicity with G + C content of DNA between 31.7-40.1 % (Sneath, 1986). High DNA/DNA homology was reported with *Bacillus cereus, Bacillus thuringiensis, Bacillus anthracis* and *Bacillus cereus var. mycoides* (McDonald *et al.* 1963; Kaneko *et al.* 1978). In a comparative study of 16S rRNA sequences from *B. anthracis var. sterne* and *B. cereus* emetic strain NCTC 11143, 1,446 bases, or 94 % of the total sequences were found to be identical (Ash *et al.* 1991). *Bacillus thuringiensis* and *B. cereus var. mycoides* differed from each other and from *B. anthracis* and *B. cereus* by fewer than nine nucleotides (Ash *et al.* 1991). Sequencing of the 23S rRNA genes derived from polymerase chain

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reaction amplification of chromosomal DNA from *B. anthracis* and an emetic strain of *B. cereus* showed them to be almost identical (Ash and Collins, 1992).

Bacillus thuringiensis can be distinguished from *B. cereus* by its pathogenicity to the larvae of *Lepidoptera* and by the production in the cell of a protein parasporal crystal, of rarely two or three in parallel with spore formation (Sneath, 1986). This structure is formed outside the exosporium and separates readily from liberated spore and in the larval gut, toxin is released from the crystal by enzymatic action (Hofte and Whiteley, 1989). The capacity to form crystal may however be lost in laboratory cultures.

Bacillus anthracis can be distinguished from *B. cereus* by lack of hemolysis on sheep or horse erythrocytes (Kramer and Gilbert, 1989), its susceptibility to ampicillin and capsule formation (Sneath, 1986; Drobniewski, 1993). Virulent strains of *B. anthracis* form capsules of glutamylpolypeptide during *in vivo* multiplication, on agar with bicarbonate under CO_2 which develop mucoid colonies (Drobniewski, 1993). *Bacillus cereus var. mycoides* can be distinguished from other variants of *B. cereus* as it is non-motile and forms distinctive rhizoid colonies on agar (Sneath, 1986). Ability to form rhizoids, however, may be lost. The distinguishing characteristics of *B. cereus* and the related species are listed in table 1.

Table 1: Principal distinguishing characteristics of *B. cereus*, *B. thuringiensis*, *B. anthracis* and *B. cereus var. mycoides.*

Characteristics	Bacillus cereus	Bacillus anthracis	Bacillus thuringiensis	B. cereus var. mycoides
Colony on blood agar	Slight green tinge	Grey white to white	Slight green tinge	Spreading rhizoid colonies
Motility	+	-	+	-
hemolysis[d]	+	-	+	(+)
Lysis by gamma phage	-	+	-	-
Penicillin susceptibility	-	+	-	-
Cystalline parasporal inclusion	-	-	+	-
Tyrosine decomposition	+	-	+	a
Elaboration of anthrax toxin and capsule	-	+	-	-
Possession of plasmids pXO1 and pXO2.	-	+	-	-
Citrate utilization	+	b	+	a
Virulence in mice	-	+	-	-
M'Fadyean reaction in killed mice	-	+	-	-
Growth in 7 % NaCl	+	+	+	a

+, > 84 % of the strains positive; a - 50 to 84 % positive;
b - 15 to 49 % positive; (+) - usually weakly positive
d - on sheep or horse blood agar at 24 h (adapted from Drobniewski, 1993).

2.2.3 Distribution and growth requirements of *B. cereus*

Bacillus cereus is widely distributed in nature and is commonly found in soil (Goepfert *et al.* 1972; Claus and Berkeley, 1986; Ueda *et al.* 1986), milk (Goepfert *et al.* 1972; Coghill and Juffs, 1979; Van Netten *et al.* 1990; Crielly *et al.* 1994), meat and meat products (Shinagawa *et al.* 1984), cereals (Shinagawa *et al.* 1979; 1980) and other dried foodstuffs (Kim and Goepfert, 1971a; Goepfert *et al.* 1972; Claus and Berkeley, 1986). Spores are wide spread and multiplication has chiefly been observed in foods (Johnson, 1984).

Germination and growth of *B. cereus* spores has been reported to occur between 5 and 55°C (Kramer *et al.* 1982; Johnson *et al.* 1983; Johnson, 1984). Goepfert *et al.* (1972) and Kramer *et al.* (1982) reported growth of the *B. cereus* at temperature range of 10-50°C with optimum growth between 28 and 38°C. Johnson *et al.* (1983) found growth to occur from 5-55°C, while, Van Netten *et al.* (1990) reported growth of *B. cereus* strains within a temperature range of 4-37°C with no growth at 43°C.

The range of pH permitting growth of *B. cereus* in laboratory media has been reported to be 4.9-9.3 (Goepfert *et al.* 1972). The minimum pH for growth of *B. cereus* in foods vary from pH 4.5 to pH 5.15 (Pivovarov and Kukhto, 1970; Mikolajcik *et al.* 1973). Raevuori and Genigeorgis (1975) reported initiation of growth of *B. cereus* in meat environment at a pH of 4.35, while growth was observed at a pH of 8.8 with a relatively high inoculum (10^6 cells per 0.01 ml).

The minimum water activity (a_w) value necessary to allow growth of vegetative *B. cereus* cells has been reported to be 0.95 (Scott, 1957; Raevuori and Genigeorgis, 1975). According to Kim and Goepfert (1971a) and Troller (1973), *B. cereus* is able to grow in 7 % NaCl, but not in 10 %. Raevuori and Genigeorgis (1975) investigated the effects of various combinations of pH and NaCl on growth and found it to vary with the strain and the growth medium. Growth rate was found to decrease when *B. cereus* was exposed to media with NaCl concentrations increasing from 0 to 10 %, and when the pH values drifted away from the optimum 6.5-7.0. Smaller concentrations of NaCl were required to inhibit initiation of growth at pH values remote from the optimum. These authors concluded that high NaCl concentrations and extreme pH values prevent growth of the organism. Rappaport and Goepfert (1978) reported a decrease in viability of thermally injured *B. cereus* vegetative cells at pH values less than 6.3 and greater than 7.3, and at concentrations of 2 % NaCl, or greater in plating media.

Bacillus cereus has an absolute requirement for one or several amino acids. Various strains differ in their amino acid requirements, while vitamins are not required (Sneath, 1986). Rappaport and Goepfert (1978) showed that thermally injured cells require amino acids plus purines and pyrimidines (adenine, thymine, guanine and uracil) for their recovery. The recovery requires RNA (but not DNA) synthesis or replication.

2.2.4 Thermal resistance of *B. cereus*

The distribution of thermal resistance among *B. cereus* is phenotypic in character (Vas, 1970). Several workers have determined the thermal resistance of *B. cereus* spores. Ingram *et al.* (1969) reported a D_{100} value of 5 min, with a corresponding z value of 10°C. Similarly, Briggs (1966) observed a D_{100} of 5.5 min (z = 9.7°C) for *B. cereus* spores suspended in distilled water. Gibriel *et al.* (1973) obtained a D_{100} value of 0.8 min and Bradshaw *et al.* (1975) reported a $D_{121.1}$ value of 0.03 min (z = 9.9 °C). Unusual high heat resistance of *B. cereus* strains has been reported. Burgos *et al.* (1972) for example, detected a D_{110} of 11.5 min with spores suspended in one fourth strength of Ringer's solution. Franklin (1970) reported an isolate of *B. cereus* from cream that survived 140°C for 0.2 sec and upon subculturing found a resistant fraction (one in 10^5 to 10^8) that was able to survive 135°C for 4 hours. Bradshaw *et al.* (1975) reported a $D_{121.1}$ value of 2.35 min for a *B. cereus* isolated from abnormal cans of commercially canned soup.

Although *B. cereus* spores are not particularly thermostable, spores of some strains are able to withstand relatively high temperatures, particularly if the food has a high fat content, which seems to have a protective effect (Kramer and Gilbert, 1989). Spores in such food survive cooking, germinate in food during storage and produce enterotoxins. Conversely, some additives such as garlic extract have an inhibitory effect on bacterial growth (Kramer and Gilbert, 1989).

2.2.5 Susceptibility to antibiotics

Susceptibility to antibiotics has previously been used to characterize bacterial strains. For example, Somer et al. (1996) found all aerobic and anaerobic bacteria originating from cases of summer mastitis to be susceptible to penicillin, amoxicillin, amoxicillin-clavulanate, cefoxitin, clindamycin and chloramphenicol. In addition, all anaerobes were susceptible to oxytetracycline and spiramycin. Berkowtz et al. (1990) and Marisol et al. (1998) found Group B Streptococci to be consistently susceptible to penicillin, ampicillin and gentamicin. Barabara et al. (1997) found all Group D Streptococci to be susceptible to ampicillin, vancomycin, and gentamicin with no isolate showing β-lactamase activity. Holmberg et al. (1984) found *Salmonella typhimurium* isolates from 18 of 20 outbreaks to have the same antimicrobial susceptibility patterns. In another subgroup of outbreaks in which isolates unrelated to the outbreak were compared, the outbreak isolates were differentiated from the control isolates four times (44%) by antimicrobial susceptibility testing. Epidemic isolates were multiply susceptible in 14 (70 %) of the 20 outbreak isolates. Aarestrup et al. (1997) found all 431 *Campylobacter* spp. to be susceptible to apramycin, neomycin and erythromycin, while Temblay and Gaudreau (1998) found all 59 *Campylobacter fetus ss fetus* to be susceptible to ampicillin. Similar studies were reported by Bopp et al. (1985) with epidemics associated *Campylobacter jejuni* isolates.

Few studies on the relationship between taxonomy and sensitivity of *Bacillus* species to antibiotics have been done. Kundrat (1963) found Bacillus species except *B. alvei*, *B. laterosphorous* and *B. stearothermophilus* to be resistant to sulfonamides. Resistance to

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erythromycin, streptomycin and chloramphenicol has been observed with strains of *B. lichenformis* (Docherty *et al.* 1981; Yoshimura *et al.* 1983), *B. sphaericus* (Burke and McDonald, 1983; Yoshimura *et al.* 1983) and *B. lichenformis* and *B. sphaericus* (Burke and McDonald, 1983) respectively. According to Bernhard *et al.* (1978), *B. cereus* generally shows high resistance to ampicillin, colistin and polymyxin. The organism produces beta-lactamases that are responsible for resistance to beta-lactam antibiotics. Only a few of the strains studied were resistant to bacitracin, cephaloridin, Kanamycin or tetracycline. The organism is usually susceptible to clindamycin, vancomycin, gentamycin, chloramphenicol and erythromycin.

Bacillus anthracis strains are generally sensitive to penicillin, but one resistant strain has been reported (Sneath, 1986). Some strains of *B. subtilis* were found resistant to streptomycin (Bernhard *et al.* 1978) and resistance to tetracycline has been observed in some strains of *B. cereus, B. lichenformis, B. sphaericus, B. stearothermophilus* and *B. subtilis* (Polak and Novick, 1982). With the exception of tetracyclines, resistance to antibiotics is not generally associated with plasmids in *Bacillus* species (Bernhard *et al.* 1978; Polak and Novick, 1982).

2.2.6 Growth temperatures of *B. cereus* food poisoning strains

Typical environmental strains of *B. cereus* do not normally grow at temperatures below 10°C. However, psychrotrophic strains isolated from dairy products have been reported to grow and produce diarrhoeal enterotoxin at refrigeration temperatures (Christiansen *et al.* 1989; Van Netten *et al.* 1990; Griffiths, 1990). These strains have been implicated in food poisoning, and are

of great concern to the dairy industry (Van Netten *et al.* 1990). Enterotoxigenic strains that can grow at 6°C and above 37°C are regarded as potential food poisoning organisms as they can grow both in the product and in the ileum (Granum *et al.* 1993). Dufrenne *et al.* (1994) found *B. cereus* strains with the capacity to grow at or below 7°C not to differ from those with minimum growth temperatures of ≥ 10°C. Spores of all these strains were able to germinate even at below minimal growth temperature and all produced diarrhoeal enterotoxin.

2.2.7 Differentiation between diarrhoeal and emetic strains of *Bacillus cereus*

Literature concerning the criteria of differentiating the two food poisoning strains of *B. cereus* is scanty. Johnson *et al.* (1982) examined the heat resistance and germination responses of spores of eight strains of *B. cereus* representing diarrhoeal, emetic and atoxigenic origins, and reported no correlation between heat resistance at 85 or 90°C and origin of the strains. These authors reported a more extensive germination of spores in tryptic soy broth at 30°C for diarrhoeal strains than for emetic strains as measured by loss of heat resistance. Investigations to the influence of temperature on germination and growth of spores of *B. cereus* in broth medium and in rice revealed little difference in responses between the diarrhoeal and emetic types except a higher maximum population that was achieved in rice for both strains (Johnson *et al.* 1983). Growth for some strains occurred from 15 to 50°C, while germination was more extensive for diarrhoeal strains in either medium.

It had been proposed that *B. cereus* strains producing emetic toxin are unable to hydrolyse starch (Raevuori *et al.* 1977; Shinagawa *et al.* 1985, Nishikawa *et al.* 1996). Shinagawa (1990) reported a possible relationship between starch hydrolysis by *B. cereus* strains and diarrhoeal enterotoxin production. This author reported that all the nine *B. cereus* strains isolated from diarrhoeal type outbreaks hydrolysed starch, whereas none of the 82 *B. cereus* strains isolated from emetic type outbreaks were able to do so. Shinagawa *et al.* (1979) found isolates from foods to vary in their ability to hydrolyse starch. They found 83 % of the isolates from uncooked rice, 38 % of the isolates from cooked rice, and 71 % (199 of 281) of the isolates from other foods such as meat products, raw meat and meat product additives to be able to hydrolyse starch. Of the 199 starch hydrolysis positive strains isolated from other foods, 108 (54 %) were considered to be enterotoxin positive (Shinagawa *et al.* 1984). These results indicated a possible relationship between starch hydrolysis and diarrhoeal enterotoxin production Emetic toxin production in *B. cereus* strains was reported by Nishikawa *et al.* (1996) to be related to HEp-2 vacuolation factor production. This may be significant in the identification of *B. cereus* strains isolated from different types of food poisoning. *Bacillus cereus* strains isolated from the diarrhoeal type food poisoning and those isolated from uncooked rice that were starch hydrolysis positive were less heat resistant (100°C for 30 min, or 105°C for 5 min) than those isolated from the emetic type of food poisoning and those isolated from cooked rice that were starch hydrolysis negative (Shinagawa *et al.* 1979).

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2.2.8 The diarrhoeal enterotoxin

Turnbull *et al.* (1979) described the diarrhoeal toxin as a single relatively unstable protein of molecular weight approximately 50,000 and isoelectric point of 4.9. Other authors reported the diarrhoeal enterotoxin to be comprised of three protein components, which together, but not individually, produce the known properties of the enterotoxin. For example, Thompson *et al.* (1984) isolated three components with molecular weights of 38, 39.5 and 43 kDa respectively, but from their results, it was unclear whether all of them possessed enterotoxic activity. It was, however, clear that the 39.5 kDa component was enterotoxic. These authors reported immunological cross-reaction between the three components. It was subsequently shown by cross immunoelectrophoresis that these components consisted of distinct distinguishable antigens (Granum and Nissen, 1990), and that the immunological cross reaction reported by Thompson *et al.* (1984) was probably due to contamination by the other two components.

Beecher and Macmillian (1990, 1991) reported the purification of a tripartite hemolysin BL that was immunologically related and had molecular weights and isoelectric points similar to the multicomponent enterotoxin purified by Thompson *et al.*(1984). These hemolysin BL components were designated as B (35 kDa), L1 (36 kDa) and L2 (45 kDa). None of these components had hemolytic or vascular permeability activity individually, but maximal activity occurred in the presence of all the three components.

Granum *et al.* (1993) also demonstrated three protein bands produced by an enterotoxin positive *B. cereus* by western blot technique. The protein bands were of molecular weights 58, 50

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and 40 kDa respectively, and only the 50 kDa protein was shown to be enterotoxic on its own. These authors further showed that almost all enterotoxigenic strains of *B. cereus* co-produce both the toxic and non-toxic components of the "enterotoxin complex". Granum and Nissen (1993) determined the first 14-15 amino acid sequence of the three components of the enterotoxin complex, and reported that the 40 kDa protein was sufficient to cause toxicity to vero cells alone. However, Heinrichs *et al.* (1993) thought that the activity reported by Granum and Nissen (1993) was not solely due to the 40 kDa protein, but rather to a combination of two or more proteins as these were shown to be required in trace amounts to cause hemolysis. The 34 kDa protein N-terminal amino acid sequence was also shown to be the same as that of sphingomyelinase, and that this protein caused hemolysis although traces of at least one of the two other proteins could be detected in the purified proteins.

A complete nucleotide and amino acid sequences of the gene coding for the B component of the hemolysin BL were also determined by Heinrichs *et al.* (1993), and was shown to have the same amino acid sequence as that of the 40 kDa protein reported by Granum and Nissen (1993). This B component was reported to be expressed as a polypeptide of 41 kDa containing a signal peptide that allows for its secretion by *B. cereus* into the culture medium. However, it was not known whether the B component alone was sufficient to cause toxicity without the interference of contaminating protein, or whether disruption of the B gene in *B. cereus* could result in loss of enterotoxin activity.

Shinagawa *et al* (1991a; 1991c and 1992) reported purification of a single enterotoxin component with vascular permeability reaction and mouse lethal activity, but without hemolytic

and lecithinase activity. The molecular weight of this enterotoxin component was reported to be 45 kDa by sodium deodecyl sulphate (SDS) gel electrophoresis, but was thought to be identical to the 39.5 kDa protein of Thompson *et al.* (1984). The nucleotide and amino acid sequence of *B. cereus* enterotoxin *(bceT)* gene that encodes the single component diarrhoeal enterotoxin was determined by Agata *et al.* (1995). The N-terminal amino acid sequence was found to have no homology with the amino acid sequence of the enterotoxin complex. This gene was shown to be present in bacterial strains of *B. cereus.*

2.2.9 Biological characteristics of the diarrhoeal enterotoxin

The diarrhoeal enterotoxin is thought to be a true exotoxin. For a toxin to qualify as an exotoxin, it should meet the following criteria: it should be synthesized and released only by actively growing cells; there should be no appreciable increase in toxin during the stationary phase and intracellular concentration should be negligible at any moment of bacterial growth. *Bacillus cereus* diarrhoeal enterotoxin is synthesized during the growth phase of the organism and meets the above criteria (Thompson *et al.* 1984). This enterotoxin causes fluid accumulation in rabbit ileal loops (RIL) assay (Spira and Goepfert, 1972; Turnbull *et al.* 1979; Kramer *et al.* 1982), increases vascular permeability (VP) in rabbit skin (Glatz *et al.* 1974), cytotoxicity in cultured cells (Bonventre, 1965, Turnbull *et al.* 1979; Kramer *et al.* 1982), and lethality to mice after intravenous injection (Chu, 1949, Thompson *et al.* 1984). Therefore, the toxins described as mouse lethal factor 1 (Chu, 1949; Johnson and Bonventre, 1967), diarrhogenic factor (Spira and

Goepfert, 1972), necrotic factor (Turnbull *et al.* 1979; Thompson *et al.* 1984), vascular permeability factor (Glatz *et al.* 1974; Ezepchuk *et al.* 1979), and edema factor (Spira and Goepfert, 1972) are all part of the same enterotoxin complex.

Spira and Goepfert (1972) reported the VP activity to correlate well with RIL activity. Turnbull *et al.* (1979) were able to isolate a fraction enriched for VP and RIL activities and devoid of phospholipase activity, but this fraction contained a residual hemolysin activity. Ezepchuk *et al.* (1979) also isolated a permeability factor of molecular weight 100 kDa, which was lethal to mice but could not give a RIL response. Garcia-Arribas *et al* (1988) showed that there was no strict relationship between hemolysin and VP activity, while Christianssen *et al.* (1989) found no correlation between hemolysin production by *B. cereus* and cytotoxicity to cultured cells.

Diarrhoeal enterotoxin production is best at a pH range of 6.0 to 8.5, with the optimum production occurring between 7.0 to 7.5. It is most stable in the pH range of 5.0 to 10.0, but loses activity rapidly outside this range (Spira and Goepfert, 1975). It is produced over a temperature range of 18 to 43°C (Johnson, 1984). The production is favoured by low dissolved oxygen, and most is produced during the logarithmic phase (Spira and Goepfert, 1975). Garcia-Arribas and Kramer (1990) found enterotoxin to be synthesized during the late logarithmic growth phase at an optimum temperature of 32°C to 37°C and at pH of 7.5 in brain heart infusion broth supplemented with 1 % (w/v) starch or glucose. Enterotoxin production is delayed by irradiation, which causes reduction in numbers of *B. cereus* in a growth medium (Grant *et al.* 1993). This enterotoxin is heat labile, trypsin and pronase sensitive. It is inactivated at pH below 4.0, and is

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readily degraded by proteolytic enzymes in the stomach and duodenum (Kramer and Gilbert, 1989; Granum and Nissen, 1990).

2.2.10 Mode of action of diarrhoeal enterotoxin

The diarrhoeal enterotoxin causes reversal of fluid and sodium chloride ions absorption, severe necrosis of mucosa and other tissue damage especially in high concentration, glucose and amino acid malabsorption, activates adenyl cyclase through cyclic AMP and alters capillary permeability (Gilbert and Kramer, 1984). Turnbull (1986) reported that purified diarrhoeal enterotoxin complex, cell free culture filtrates and whole cell suspensions produce fluid accumulation in rabbit and mouse ileal loops by activation of adenyl cylase. Singh *et al.* (1992) found that *B. cereus* enterotoxin affected the capillaries of blood vessels locally and also systemically resulting into release of proteinaceous exudates and blood cells. In human beings, this enterotoxin causes diarrhoea by stimulating the adenyl cyclase cAMP system (Smith *et al.* 1981; Turnbull, 1986).

2.2.11 The emetic toxin

The emetic toxin (Cereulide) has a molecular weight of 5 kda (Turnbull *et al.* 1979) and is formed during the late exponential to stationary growth phase and may be associated with sporulation at optimum temperatures of 25 to 30°C. The toxin may be a breakdown product from

foodstuffs supporting the growth of *B. cereus* (Drobniewski, 1993). Agata *et al.* (1996) reported production of cereulide to be associated with a specific class of *B. cereus*. These authors found that the H-1 serovar phenotype was strongly associated with the production of cereulide and that none of the isolates that hydrolysed starch or expressed diarrhoeal enterotoxin activity produced cereulide. The toxin causes emesis in monkey feeding tests and is cytostatic, i.e it inhibits growth of HEp-2 cells and is non-hemolytic (Hughes *et al.* 1988). Szabo *et al.* (1991) reported that the emetic toxin affect the cells by changing the vacuoles and by acid production, cell rounding, cell granule promoting and cytostatic activity. The toxin does not have any hemolytic activity (Mikami *et al.* 1994) and has low antigenicity, a factor that prevents detection by routine immunological means (Melling and Capel, 1978). It is heat and pH stable (surviving 100°C for 90 min), and is resistant to proteolytic enzymes like trypsin and pepsin (Johnson, 1984; Drobniewski, 1993). A comparison of some characteristics of emetic and diarrhoeal enterotoxins is given in table 2.

Table 2: A comparison of some properties of diarrhoeal and emetic enterotoxins.

Properties	Diarrhoeal toxin	Emetic toxin
Nature	protein	Small peptide
Mol. weights	50,000 daltons	< 5,000 daltons
Isoelectric point	4.9 - 5.3	-
Stabilities: heat	destroyed at 55°C for 20 min.	stable at 126°C for 90 min.
pH	unstable < 4 > 11	stable 2-11
Enzymes	pronase and trypsin sensitive.	pepsin and trypsin resistant
4°C for 7 days	reduced activity.	active
Production: in foods	sometimes preformed	preformed
Optimum temp.	32-37°C	25-30°C
Growth phase	exponential	stationary
Antigenicity	Antigenic	Not antigenic

Source: Gilbert and Kramer, 1984.

2.2.12 Diagnosis of *B. cereus* food poisoning outbreaks

Isolation of large numbers (usually $\geq 10^7$ cfu/ml) of *B. cereus* from incriminated foods and clinical specimens is a primary criterion for diagnosing outbreaks of *B. cereus* food poisoning. *B. cereus* has to multiply to a level of at least 10^5 cfu/g or per ml to cause food poisoning (Kramer and Gilbert, 1989). Although enterotoxin may be detected in products with 10^5 cfu/g (or per ml), it is unlikely that the small amounts of preformed enterotoxin will contribute to illness. Thus, more than 10^5 cfu/gram or per ml will be needed to produce enough toxin to cause illness. Since there is a high level of asymptomatic faecal carriage of *B. cereus* in humans (Ghoshi, 1978; Turnbull and Kramer, 1985), proof that a sample of food is responsible for an outbreak requires that the strain of *B. cereus* isolated from the clinical specimens and foodstuff be of the same serotype (and/or biotype or phage type) and be present in significant numbers of $> 10^5$ cfu/g or per ml (Kramer *et al.* 1982).

The outbreak is considered in the context of its epidemiological characteristics including incubation times, clinical symptoms and appropriate toxin production by various strains of *B. cereus*. In episodes of emetic food poisoning, failure to recover *B. cereus* from foodstuffs does not entirely rule it out as the causative organism, since heating after contamination could kill the organism but leave preformed stable emetic toxin intact. Diagnosis is complicated by the natural contamination of many foodstuffs with spores and the detection of *B. cereus* in the absence of clinical symptoms may not always signify a foodborne disease. When symptoms and isolation of organisms from foodstuffs concide, the case for *B. cereus* food poisoning is stronger.

2.2.13 Enumeration and isolation procedures

Various selective and diagnostic media have been developed for-detection and direct isolation of *B. cereus* from foods. They include MYP (mannitol egg yolk phenol red) medium of Mossel *et al.* (1967), KG medium of Kim and Goepfert (1971b) and polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) of Holbrook and Anderson, (1980). All these media rely upon the suppression of gram negative organisms by addition of 5 to 10 µg /ml polymyxin B to the growth medium. In addition, mannitol, egg yolk and an indicator have been incorporated in these media to make differentiation of *B. cereus* easier (Holbrook and Anderson, 1980; Bouwer-Hertzberger and Mossel, 1982). The principal diagnostic characteristic used in all is the egg yolk reaction, the precipitation of hydrolysed lecithin (Holbrook and Anderson, 1980). Surface plating onto these selective media is the common method for isolation and enumeration of *B. cereus* from foods and clinical specimens (Holbrook and Anderson, 1980; Bouwer-Hertzberger and Mossel, 1982). The selective media inoculated with 0.1 ml decimal dilutions of sample homogenates are incubated for 18 to 24 hours at 32-37°C. The suspected *B. cereus* colonies are enumerated to determine the counts per gram or per millitre of the test sample. In cases where few organisms are expected to be present, enrichment procedures are used to isolate the organisms. This may be done with brain heart infusion broth to which polymyxin B and sodium chloride have been added.

The isolates are identified by morphological characteristics based on gram and spore staining and biochemical tests. Identification of *B.cereus* using biochemical properties such as production of acetyl carbinol, liquefaction of gelatin, casein and starch hydrolysis, non production

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of indole, utilization of citrate, production of lecithinase, glucose fermentation and failure to ferment mannitol, arabinose and xylose is carried out using the methods and interpretation of Gordon (1973), Cowan and Steel (1974) and Sneath (1986). Variations in the typical biochemical behaviour of strains from various sources may be observed (Sneath, 1986).

A rapid confirmatory staining method was developed by Holbrook and Anderson (1980), which combine the spore stain and the intracellular lipid staining for confirmation of *B. cereus* strains. The microscopic examination for presence of lipid globules in the vegetative cells of *B. cereus* replaces the need for biochemical testing. The stains show spores to be central or paracentral in position, which do not swell the sporangium. The spores stain pale green to mild green, while lipid globules stain black and the vegetative cytoplasm appears red. Holbrook and Anderson (1980) confirmed that only *B. cereus* of the *Bacillus* species are capable of possessing lipid globules in their vegetative cells when grown on the *B. cereus* selective medium. The microscopic appearance together with the typical colony form in *B. cereus* selective agar medium confirms the identity of *B. cereus*.

2.2.14 *Bacillus cereus* typing

A study of *Bacillus* species antigens by Norris and Wolf (1961) established that the spore antigen possessed the highest species specificity, while the flagella (H) antigen provided the highest degree of strain specificity. A serological typing scheme was subsequently developed, based on *B. cereus* H-antigens (Taylor and Gilbert, 1975; Gilbert and Parry, 1977) for investigation of

foodborne outbreaks. The scheme comprises of 23 agglutination antisera raised against prototype strains isolated from food and clinical specimens (Kramer *et al.* 1982). The scheme proved to be a useful epidemiological tool for the investigation of food poisoning (Ghoshi, 1978), the distribution of serotypes in various foods and food additives (Gilbert and Parry 1977; Blakey and Priest, 1980; Roberts *et al.* 1981), hospital cross infection (Fitzpatrick *et al.* 1979; Gostling *et al.* 1980) and bovine mastitis (Jones and Turnbull, 1981). By application of the serotyping scheme, isolates from the incriminated food can be directly related to those found in clinical specimens (Kramer *et al.* 1982).

Bacillus cereus serovars associated with diarrhoeal syndrome include types 1, 2, 6, 8, 9, 10, 12, and 19; while serovars 1, 3, 4, 5, 8, 12 and 19 have been associated with emetic syndrome (Kramer *et al.* 1982). Serotypes 1, 8, 12 and 19 have been found in association with both emetic and diarrhoeal outbreaks (Gilbert and Parry, 1977; Gilbert, 1979; Kramer *et al.* 1982), indicating that specific serotypes are not exclusively linked to either of the two syndromes. Gilbert and Parry (1977) found serotype 1 to be associated with 70 % of all the emetic syndome incidents reported. The implication that type 1 strains might have greater resistance to heat and subsequently be selected for during the cooking process was subsequently borne out in a study of the heat resistance of spores of *B. cereus* strains from various sources (Parry and Gilbert, 1980).

Biotyping using a combination of biochemical properties including VP reaction, nitrate reduction, citrate utilization, urea decomposition, starch hydrolysis, fermentation of sucrose and salicin has also proved to be a useful epidemiological tool (Kozasa *et al.* 1977; Ueda *et al.* 1986).

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However, the method has not been standardized for routine use. Its application and interpretation is dependent on previously established individual criteria.

Attempts have been made to develop a basic set of phages for use in routine laboratory typing (Lida *et al.* 1985; Ahmed *et al.* 1995). A scheme developed by Lida *et al.* (1985) used six phage types isolated from the soil. This set of phages lysed, specifically, a large number of *B. cereus* at one Routine Test Dilution (RTD). Ahmed *et al.* (1995) described a phage typing scheme for *B. cereus* isolates from outbreaks and sporadic cases of food poisoning using 12 bacteriophages isolated from sewage. These authors reported a good correlation between phage types of strains isolated from suspected foods and those of strains isolated from stools of symptomatic patients. Phage typing along with biotyping has been used successfully in epidemiological investigations (Shinagawa, 1990).

2.3 METHODS FOR DETECTION AND ASSAY OF *BACILLUS CEREUS* ENTEROTOXINS

2.3.1 Biological methods

2.3.1.1 Use of animals

Bioassays of diarrhoeal enterotoxin of *B. cereus* has been done using monkey feeding (Goepfert, 1974; Terranova and Blake, 1978), rabbit ileal loop (Spira and Goepfert, 1972), rabbit vascular permeability reaction test (Kramer *et al.* 1982), rabbit skin test (Glatz *et al.* 1974), Guinea

pig skin (Glatz and Goepfert, 1973) and mouse lethality (Molnar, 1962). Emetic enterotoxin has however been assayed using rhesus monkey emesis test (Melling *et al.* 1976). These methods were found inconvenient and expensive to be used on routine basis, which necessitated the development of alternative methods.

2.3.1.2 Cell culture techniques

Cell cultures have been used to assess various properties of enterotoxins. For example vero cell cultures have been used to study the mode of action (McClane and McDonel, 1980), binding to cells (McDonel, 1980) and biological activity of enterotoxins (McDonel and McClane, 1981). The assay is based on the observation that enterotoxin inhibits plating efficiency of vero cells grown in culture. Foetal intestinal cells have been used in the assay of the biological activity of *B. cereus* enterotoxins (Turnbull *et al.* 1979). Jackson, (1993) described a rapid screening test for enterotoxin producing *B. cereus* using a McCoy cell tissue culture system. HEp-2 cell culture has been used to test for *B. cereus* emetic toxin in culture filtrates and in foods (Hughes *et al.* 1988; Mikami *et al.* 1994).

2.3.1.3 Ligated loop technique

The technique is based on the fact that certain enterotoxins elicit fluid accumulation in the small intestine of susceptible animals. This technique has been employed in studies on the virulence

and pathogenesis of foodborne pathogens (De and Chatterje, 1953). Spira and Goepfert (1972) Turnbull *et al.* (1979) and Shinagawa *et al.* (1991b) described use of ligated loop technique for screening *B. cereus* culture preparations for diarrhoeal enterotoxin activity.

2.3.2 Immunological Methods

2.3.2.1 Aggregate heamagglutination

The method is based on the use of erythrocytes sensitized by aggregated proteins of immune serum (Olovnikov, 1967). Antibodies are introduced into three dimensional protein complexes, so that the active sites of the antibodies are spaced some distance from the surface of the erythrocytes, which become readily accessible to antigenic determinants (Olovnikov, 1967). The test has been used to detect diarrhoeal enterotoxin of *B. cereus* (Gorina *et al.* 1976). Antiserum to *B. cereus* enterotoxins is aggregated by use of gluteraldehyde. The aggregate sensitizes erythrocytes and hemagglutination occurs on addition of enterotoxin. The method was found to be more sensitive detecting upto 0.004 µg/ml of *B. cereus* enterotoxin compared to gel immunodiffusion with a sensitivity of 2-3 µg/ml (Gorina *et al.* 1975). These authors reported the method to give reproducible results and do not require much time for reaction to occur. However, cross-reactions occurred with two closely related microorganisms, *B. anthracis* and *B. megaterium*. The method could be used for detecting enterotoxin in foodstuffs and culture media (Gorina *et al.* 1975; 1976).

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2.3.2.2 *Western immunoblot technique*

Detection of *B. cereus* enterotoxin by use of the western immunotechnique was reported by Agata *et al.* (1995). The method involved separation of *B. cereus* culture supernatant proteins by SDS polyacrylamide gel electrophoresis, which were then transferred electrophoretically to a filter membrane as described by Towbin *et al.* (1979). The blots were probed with rabbit antiserum specific to a *B. cereus* diarrhoeal protein and the binding of rabbit antiserum was detected by goat anti-rabbit immunoglobulin conjugated to the enzyme horse radish peroxidase, which catalizes a chromogenic reaction. The substrate was finally added and a colour reaction formed marking a band containing the desired enterotoxin protein (Agata *et al.* 1995).

2.3.2.3 *Fluorescent immunodot* assay

Fluorescent immunodot assay uses an antiserum prepared against purified enterotoxin. The assay is very specific for *B. cereus* diarrhoeal enterotoxin detecting 50 ng or more of purified enterotoxin (Jackson, 1989). The method is a substrate gel system that utilises nitrocellulose membrane placed on a microfold base and smoothed gently onto a gel surface for observation under a long wave ultraviolet light for fluorescence. Jackson (1989) evaluated the fluorescent immunodot and found it to have adequate sensitivity and specificity.

2.3.2.4 Reverse passive latex agglutination test

Reverse passive latex agglutination is a method where polystyrene latex particles are sensitized with purified antiserum taken from rabbits immunized with purified *B. cereus* diarrhoeal enterotoxin. These latex particles aggregate in the presence of *B. cereus* enterotoxin. The test is performed in v-well microtitre plates, where dilutions of the food extract or culture filtrate are made and a volume of appropriate latex suspension added. If *B. cereus* enterotoxin is present, agglutination occurs due to the formation of a lattice structure, which upon settling forms a diffuse layer on the base of the well. Absence of *B. cereus* enterotoxin, or if enterotoxin is present at a concentration below the assay detection level, no such lattice structure can be formed but instead a tight button is observed. A control reagent for the test consists of latex particles sensitized with non-immune globulins (Kramer and Gilbert, 1989; Granum *et al.* 1993).

In the reverse agglutination assay, the antibody which is attached to the particles react with the soluble antigen. The latex particles do not themselves play a part and are therefore passive. The cross-linking of the latex particles by specific antigen-antibody complex results in the visible latex reaction. *Bacillus cereus* enterotoxin-reverse passive latex agglutination (BCET-RPLA) test kit (Oxoid) is a simple and reliable means of determining diarrhoeal enterotoxin production by strains of *B. cereus* by reverse agglutination assay (Granum *et al.* 1993).

2.3.2.5 Enzyme linked immunosorbent assay (ELISA)

ELISA technique has been used to detect *B. cereus* diarrhoeal enterotoxin. The diarrhoeal enterotoxin visual immunoassay (Tecra-ELISA) detects two non-toxic proteins of the enterotoxin complex (Beecher and Wong, 1994b; Day *et al.* 1994). Tan *et al.* (1997) reported use of a commercially available ELISA kit for detection of Bacillus diarrhoeal enterotoxin (BDE) in a variety of foods and in faeces.

Biological and immunological methods are only useful for detection of strains that produce detectable enterotoxins. The methods cannot be used for studying strains that do not produce readily detectable enterotoxins or those containing silent genes (Roger *et al.* 1990).

2.3.3 Molecular biology techniques

2.3.3.1 Plasmid profile analysis

Plasmids are extrachromosomal genetic elements that represent a dispensible gene pool found in most bacterial species (Birnboim and Doly, 1979). They are double stranded closed circular DNA molecules that range in size from 1.0 kilobase pair to greater than 200 kilobase pairs. They often contain genes coding for enzymes that under certain circumstances are advantageous to the bacterial host (Maniatis *et al.* 1982). Among the phenotypes conferred by different plasmids include: resistance to antibiotics (Helsinki 1973; Bernhard *et al.* 1978; Imanaka *et al.* 1981, Aoki,

1988), production of enterotoxins (Smith *et al.* 1979b; Moseley *et al,* 1983; Iandolo, 1989), production of antibiotics, degradation of complex organic compounds, production of colicins, and production of restriction and modification enzymes (Maniatis *et al.* 1982). Some of the plasmid mediated factors are associated with virulence of bacterial pathogens (Elwell and Shipley, 1980).

Birnboim and Doly (1979) described a method of isolation of plasmid DNA from 0.5 ml of an overnight bacterial culture in 4 hours. The method involves disruption of bacterial outer membrane with EDTA, removal of cell wall by treatment with lysozyme and lysis of internal membranes with alkaline detergent. The chromosome is denatured at acid pH by treatment with 3 M sodium acetate (pH 4.8) and cleared along with cellular debris by microcentrifugation. Plasmid DNA is precipitated by ethanol in the cold and then electrophoresed through an agarose gel, which separates them on the basis of molecular weights during migration towards the anode. Kado and Liu (1981) described a rapid procedure for isolation of small and large plasmids. The method utilizes the molecular characteristics of covalently closed circular DNA that is released from cells under conditions that denature chromosonal DNA by using allkaline sodium deodecyl sulfate (pH 12.6) at elevated temperatures. Proteins and cell debris is removed by extraction with phenol-chloroform resulting in elimination of chromosomal DNA. DNAs of known molecular weights are included as standards on each gel and their relative mobility on the gel is used for calculation of the molecular weights of unknown plasmids (Meyers *et al.* 1976).

Plasmid DNA is important in enteric pathogenesis and in the identification and classification of pathogenic microorganisms (Parisi and Hecht 1980; Schaberg *et al.* 1981, Wachsmuth, 1985), and differentiation of bacterial strains (Mahony *et al.* 1987). In addition, the

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presence of plasmid DNA alone (the profile) is of diagnostic value (Wachsmuth, 1986). One or more plasmids may be unique to a particular pathogen and may be used for precise incrimination of a pathogen in an epidemic (Wachsmuth, 1985; 1986).

Although plasmids are non-essential for bacterial survival and may be transitory, under certain circumstances the profiles have proved to be sensitive indicators of a common source over time and under environmental stress (Wachsmuth, 1986). Debuono *et al.* (1988) reported the potential of plasmid analysis in the investigation of *B. cereus* food poisoning. However, plasmid profile alone is not often sufficient for strain identification and in most situations, analysis of restriction endonuclease cleavage pattern is also necessary (Wachsmuth *et al.* 1979; Wells *et al.* 1983; Wachsmuth, 1986).

Plasmid profile analysis is not without limitations. Sometimes a putative epidemic strain may contain no plasmid DNA, or the strains unrelated to the epidemic outbreak may have the same plasmid profile as the epidemic strain, while the presence of a particular plasmid in a strain does not provide evidence that the plasmid codes for a specific factor (Wachsmuth, 1986). This makes interpretation of the results of plasmid analysis difficult unless a large number of non epidemic plasmid containing control strains are available for comparison and phenotypic characteristics of the plasmid determined in a bacterial strain known to contain no plasmids (Wachsmuth, 1986).

2.3.3.2 Restriction endonuclease DNA cleavage pattern analysis.

Restriction endonuclease DNA analysis involves use of enzymes that recognize unique DNA sequences (target) to cleave the double stranded DNA at specific sites within the targets. These restriction enzymes (endonucleases) recognise specific palindromic, base sequences of four or more bases within the DNA molecule and cleave the DNA either within or close to these restriction sequences at defined positions (Roberts, 1978; Platt *et al.* 1986). The resulting fragments are then resolved electrophoretically in an agarose gel depending on their relative mobility, with smaller fragments moving faster compared to larger fragments (Roberts, 1978; Platt *et al.* 1986). The electrophoretic pattern of DNA that has been cut by a restriction enzyme differs from its uncut pattern in that more bands usually are visible after restriction digestion. By analysing the gel patterns, it is possible to identify related genomes (Roberts, 1978). Strains of the same bacteria with similar base pair arrangement produce similar restriction patterns with a particular restriction enzyme. Strains that produce pronounced differences in DNA patterns clearly have a different arrangement of base pairs within their chromosomes, and cannot be considered as having been derived from the same parent stock (Kakoyiannis *et al.* 1984). Sometimes the patterns may be similar when using one particular restriction enzyme. In such a case, a second or third enzyme may be used to differentiate the strains.

Both chromosomal and plasmid DNA can be digested by restriction endonucleases. The resulting fragment characterization will then depend on the presence of specific nucleotide sequences. Identical chromosomes and plasmids could have identical targets and would be cleaved

into identical DNA fragments or if they are different can yield different fragments (Roberts, 1977). The separation of these fragments by size in agarose gels produces a restriction endonuclease profile (Wachsmuth, 1986). This procedure which results in reproducible electrophoretic gel patterns is also known as bacterial restriction endonuclease DNA analysis (BRENDA) (Wachsmuth, 1986).

The selection for the appropriate endonuclease is based on the enzyme recognition sequence. Enzymes that recognise shorter sequences generate a larger number of fragments (Wachsmuth, 1986). To be useful, the number of fragments generated should be sufficient for specificity, but not so many that coincidental matching of bands occur (Roberts, 1978). A second consideration in the selection of an endonuclease is the guanine plus cytosine content of study DNA compared to that of the enzyme recognition site, and methylated bases of the bacterial DNA, such that only those enzymes recognizing the modified bases will cleave the DNA (Roberts, 1977). BRENDA is especially helpful in typing bacteria that lack plasmids (Brown *et al.* 1982).

BRENDA can be used either alone or in conjunction with serotyping as a means of identification of bacterial strains isolated from both animals and humans (Marshall *et al.* 1981; Kaper *et al.* 1982; Robinson *et al.* 1982; Collins and Ross, 1984; Kakoyiannis *et al.* 1984). Kakoyiannis *et al.* (1984) found *Campylobacter coli* isolates from poultry in the same environment to have identical restriction patterns, while isolates carried by suckling piglets were generally the same as those isolates recovered from their dams. Three human isolates in the same study had identical DNA patterns. Collins and Ross, (1984) found DNA fragments of *Campylobacter fetus, Campylobacter jejuni* and *Campylobacter coli* to be strikingly different

from each other. Kaper *et al.* (1982) used BRENDA to identify strains of *Vibrio cholera*, while, Marshall *et al.* (1981) and Robinson *et al.* (1982) were able to clearly distinguish *Leptospira interrogans* serova *hardjo* and *balcanica* from each other by restriction endonuclease analysis.

BRENDA typing could be made even more sensitive by applying different restriction enzymes to those organisms, which show identical restriction patterns after digestion with the initial enzyme. Minor differences between DNA electrophoretic patterns may be due to presence or absence of plasmids. To confirm for presence or absence of plasmids, a gel can be run with extracted plasmid DNA without subjecting it to restriction enzyme digestion. Plasmids with no cutting sites, which contribute to the BRENDA pattern, can be detected (Kakoyiannis *et al.* 1984).

2.3.3.3 DNA-DNA hybridization with DNA probes

This method has been used in bacterial identification. Labelled DNA probes consisting of fragments on the common DNA sequence are used in hybridization reaction with DNA fragments from an unknown culture (Denhardt, 1966). The method works on assumption that all members of the genus of bacteria share a given DNA sequence, and that this sequence is not shared by other organisms that might be associated with the group (Fitts, 1985). This technique (sometimes called genetic probing) is based on the unique physical properties of DNA, especially the fidelity of base pairing (Meinkoth and Wahl, 1984). The hydrogen bonds that join complementary bases can be broken with heat or alkali treatment, the double strand helix is denatured to form single strands of DNA (Meinkoth and Wahl, 1984). Single strands from one source are joined with single strands

from a second source under appropriate conditions of pH, temperature, and ionic strength (Meinkoth and Wahl, 1984). If the DNA from a known source is labelled and used as a probe, it is possible for one to measure the hybrids that are formed (Rigby *et al.* 1977). Hybridization reaction reaches its maximal rate at 25°C below the temperature at which half of the double stranded molecules dissociates (T^m).

Hybridization reactions can be carried out in three different formats: (1) on solid support either by southern blotting (Southern, 1975), dot (Meinkoth and Wahl, 1984) or Colony/plaque blotting (Hill *et al.* 1983a and 1983b; Benton and Davis, 1977), (2). In solution (Bryan *et al.* 1986; Kohne *et al*, 1986), and (3) in-situ (Brigatti *et al.* 1983, Myerson *et al.* 1984). Southern blotting involves transfer of DNA from the agarose gel onto the nylon membrane (solid support) by capillarity. The agarose is placed upside down on 3 mm buffer soaked whatman paper supported on a glass plate and a nitrocellulose or nylon filter membrane cut to the same size as the gel and placed on top of the gel. Another set of buffer soaked whatman paper also of the same size as the gel is then placed on top of the membrane, followed by sheets of paper towels. A weight is then placed on top of the paper towels for support and to provide enough pressure on the gel. The buffer in the agarose gel together with the DNA fragments are drawn through the agarose gel by capillarity to the filter membrane where they stick. The filter is removed after several hours and DNA linked to the filter by UV irradiation, or by baking at 80°C for 2 hours (Southern, 1975). The filter is prehybridized with a non homologous DNA (such as salmon sperm DNA or calf thymus DNA) to prevent the non-specific binding of probe DNA to the filter (Tenover, 1988). During prehybridization, sites on filter membranes that non-specifically bind to single or double

stranded DNA are blocked by unlabelled DNA. Hybridization of probe to target DNA is then allowed to take place followed by washing of filter at various temperatures to remove unbound and weakly binding probe (Southern, 1975; Platt *et al.* 1986) and hybridization is then revealed by autoradiography (Denhardt, 1966; Southern, 1975; Rigby *et al.* 1977; Hill, 1981; Platt *et al.* 1986), or enzyme detection systems (Ruth, 1984; Jablonski *et al.* 1986). Small fragments (500 nucleotides or less) diffuse during transfer and hybridize inefficiently and may be undetectable, while large fragments are difficult to transfer (Southern, 1975). This limitation is overcome by depurination with hydrochloric acid (Wahl *et al.* 1979).

Dot blotting involves spotting a DNA sample on a filter e.g nitrocellulose or synthetic nylon membranes aimed at concentrating the sample in a small area (Meinkoth and Wahl, 1984). Colony blotting involves placing a filter or membrane on plate inoculated with bacteria and incubated for 24 hours or more to allow colonies to develop on the membrane. The membrane can also be placed on an agar plate containing bacterial colonies or viral plaques for 2-5 minutes to allow colonies or viral particles in plaques to attach to the filter in a colony (Hill *et al.* 1983a and b) or plaque (Benton and Davis, 1977) lift procedure. The colony/viral particles in the filter are then lysed and DNA denatured by the addition of NaOH (Meinkoth and Wahl, 1984), or by steaming the filter above a beaker of ammonium acetate (Maas, 1983). Once denatured, the DNA may be fixed to the filter by either baking it in a vaccuum oven at 80°C for 2 hours (Meinkoth and Wahl, 1984) or exposing it to a UV light for 2-5 minutes. Hybridization and detection of hybrids is then done as described with southern blotting.

In solution hybridization takes place where both the target and probe nucleic acid are free to move, maximizing the chance that complementary sequences will align and bind (Bryan *et al.* 1986). Samples are incubated at high temperatures (72°C) in a waterbath to disrupt the cells and cause them to release their nucleic acids (Bryan *et al.* 1986). After the hybridization step, the nascent duplexes are removed from solution by addition of hydroxyapatite, which selectively binds duplex nucleic acids leaving single stranded nucleic acids in solution. The duplex DNA is then removed from hydroxyapatite, either by increasing the salt concentration or by heating (Bryan *et al.* 1986), or the hydroxyapatite bound double stranded nucleic acids are collected by centrifugation and the pellet washed and quantitated by scintillation counting or spectrophotometry (Kohne *et al.* 1986). Van Brunt and Klausner (1987) described an alternative technique where probe DNA is attached to micromagnetic beads and after hybridization in solution, the labelled duplex DNA is removed from solution by a magnet. Bryan *et al.* (1986) showed that in-solution hybridization reactions go to completion faster than those do on solid support. This technique is suitable for specimens like blood, cerebrospinal fluids, tissue homogenates, sputa, aspirates, and organisms taken directly from an agar medium (Bryan *et al.* 1986; Kohne *et al.* 1986, Tenover, 1988).

In-situ hybridization is carried out on formalin-fixed paraffin embedded tissues and is useful for detection of viral pathogens (Brigatti *et al.* 1983, Myerson, *et al.* 1984). The cytopathology seen by the hematoxylin and eosin staining can be correlated with the presence of infectious agents indicated by areas demonstrating homology to a horseradish peroxidase, or alkaline phosphatase labelled probes (Brigatti *et al.* 1983).

DNA probes have been employed for microbial diagnosis (Highfield and Dougan, 1985), such as *Salmonella* spp. in food (Fitts *et al.* 1983; Fitts, 1985), *Yersinia enterocolitica* (Hill *et al.* 1983b, Kapperud *et al.* 1990), enterotoxigenic *Escherichia coli* (Hill *et al.* 1983a; Hill *et al.* 1986), enterotoxigenic *Clostridium perfringens* (Marijke *et al.* 1990), enterotoxigenic *Staphylococcus aureus* (Roger *et al.* 1990). The method has been used to analyse DNA fragments of *B. cereus* transcribed during spore outgrowth (Silberstein and Cohen, 1978), bacterial strain identification (Tenover, 1985), identification of fastidious microorganisms such as Mycobacteria, many viruses and several parasites in clinical (Tenover, 1988) and histological specimens (Myerson *et al.* 1984) and antimicrobial resistance genes (Tenover, 1986).

High precision results are obtained by use of plasmid profiles and BRENDA in combination with hybridization and probing (Wachsmuth, 1986). However, DNA probes have various limitations. They include (a) detection of only those organisms that have probes, leaving out those that may be present in the sample and whose presence could otherwise be detected by culture methods, (b) majority of probe tests are less rapid compared to many latex agglutination kits, (c) the need to culture the organism even after detection with the probe to determine its antimicrobial susceptibility pattern (Tenover, 1988), although in certain circumstances DNA probes may be useful for susceptibility testing of organisms directly in patient's specimens (Perine *et al.* 1985) and, (d) DNA probe tests may give false negative results when low numbers of cells are present in clinical specimens, thus requiring an alternative method of specimen processing (Fung *et al.* 1985).

The polymerase chain reaction amplifies specific regions of DNA by utilizing DNA polymerase, a single stranded DNA, as template and a small section of double strand DNA to prime the synthesis of new complementary strands (Mullis and Faloona, 1987; Waston *et al.* 1992). Single strand DNA templates are produced by heating double stranded DNA to temperatures near boiling, and a repetitive series of cycles involving template denaturation, primer annealing, and extension of annealed primers by DNA polymerase results in an exponential accumulation of a specific DNA fragment whose termini are defined by the 5' ends of the primers (Saiki *et al.* 1985; Scharf *et al.* 1986). Because the primer extension products synthesized in a given cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle, thus 20 cycles of PCR yield about a million copies (2^{20}) of the target DNA. The elongation of the primers is catalysed by a heat stable DNA polymerase such as heat stable *Taq* DNA polymerase that is isolated from the thermophilic eubacterium *Thermus aquaticus* (Chien *et al.* 1976), or *Pfu* DNA polymerase isolated from a hyperthermophilic archae *Pyrococcus furiosus* (Lundberg *et al.* 1991).

Taq DNA polymerase has no proofreading activity and occassionally makes mutational errors during extension along the template as it does not have 3'-5'exonuclease activity (Saiki *et al.* 1988). This limited the fidelity of the PCR in the initial days of PCR technology (Saiki *et al.* 1988; Lundberg *et al.* 1991). Cline *et al.* (1996) reported a high fideliy amplification using a thermostable *Pfu* DNA polymerases isolated from *Pyrococcus furiosus*, while, Kong *et al.* (1993)

reported *Vent^{TM}* DNA polymerase isolated from *Thermococcus litoralis*. *Vent^{TM}* and *Pfu* DNA

polymerases have proofreading abilities due to their 3'- 5' exonuclease activity (Cline *et al.* 1996).

The use of *Pfu* DNA polymerase was reported to reduce to less than 10 % of mutational errors

made by *Taq* DNA polymerase (Lundberg *et al.* 1991; Cline *et al.* 1996). However, *Pfu* DNA

polymerase exhibits half the activity of *Taq* DNA polymerase at room temperature (Lundberg *et al.* 1991).

Some of the PCR applications include: direct cloning of genomic DNA and direct gene

sequencing (Scharf *et al.* 1986), gene detection and preparation of probes (Bugawan *et al.* 1988),

detection of bacterial (Brisson-Noel *et al.* 1989) and viral (Laure *et al.* 1988; Ou *et al.* 1988)

infections in patient's specimens. PCR technique was found to be rapid and more sensitive than

other molecular techniques (Mullis and Faloona, 1987). The PCR has the following limitations:

(a) some nucleotide sequences at both ends of the gene of interest must be known for use in

designing the primers (Saiki *et al.* 1988), (b) problem of sensitivity in identifying true positives, and

(c) presence of a single cell may falsely be identified as a case of food poisoning.

2.4   DEVELOPMENT OF DNA PROBES

2.4.1 Natural nucleic acids

The key to developing a nucleic acid probe (either DNA or RNA probe) is to isolate the

required unique sequences, reproduce them in large quantities and attach a reporter molecule to

them so that they can be incorporated into a hybridization reaction (Tenover, 1988). The unique sequence that can constitute the diagnostic probe may be a portion of a virulence gene (Moseley et al. 1982), or a restriction fragment cleaved at random from the organism's chromosomal DNA (Fitts, 1985; Fitts et al. 1983). The desired fragment can be isolated by cleaving in either side of DNA sequence with a restriction enzyme to liberate the fragment from the rest of the nucleic acid sequence. The isolated DNA fragment is recombined to either phage or plasmid DNA vectors using DNA ligase enzyme to produce hybrid DNA molecules. These hybrid DNA molecules are then introduced into E. coli by transformation process, where they are amplified making hundreds of copies (Tenover, 1988). The probes are re-isolated from plasmids or phages and labelled with a radioisotope or enzyme labels (Tenover, 1988). Alternatively, a PCR can be used to amplify the desired section of the gene to be used as a probe using primers that can attach at either side of the desired gene sequence. The products of amplification are electrophoresed on an agarose gel and the desired fragment isolated from the gel. The isolated DNA fragment is then purified and labelled (Bugawan et al. 1988; Waston et al. 1992).

2.4.2 Synthetic oligonucleotide probes

Oligonucleotide probes are small stretches (normally 14 to 40 base pairs long) of nucleotides that are synthesized de novo in the laboratory (Wallace et al. 1979; Suggs et al. 1981; Lathe, 1985). These probes display exquisite specificity in that under stringent conditions, they may be capable of detecting a change in a single base pair of a DNA or RNA sequence, which is

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enough to prevent binding of the probe to the target DNA (Wallace *et al.* 1979). These authors showed that, the dramatic decrease in thermal stability due to a single mismatch makes it possible to eliminate the formation of the mismatch duplexes by the appropriate choice of hybridization temperature. The specificity of short probes vary with the temperature and salt concentration of the hybridization buffer (Lathe, 1985). Oligonucleotide probes of mixed sequence were also shown to specifically identify complementary DNA sequences hybridized under high stringent (high temperature and low salt) conditions (Wallace *et al.* 1979).

Bryan *et al.* (1986) found oligonucleotide probes to be very stable over time, relatively simple to prepare and hybridize to target DNA at very rapid rates often with reaction times of less than 30 minutes due to their low complexity. This is in contrast to long probes that often require 4 to 16 hrs for complete hybridization even with rate enhancers such as dextran sulfate, which favours the formation of networks that amplifies the amount of labelled probe bound to the filter thereby increasing the sensitivity of the technique (Wahl *et al.* 1979).

The main disadvantage of short oligonucleotide probes is that they are less sensitive than long probes since they can be labelled with only one reporter molecule (Lathe, 1985). This problem can be overcome partially by using amplification systems to triple or quadruple the signal produced by each reporter molecule on each oligonucleotide probe (Bugawan *et al.* 1988, Tenover, 1988), or by a DNA tailing procedure (Deng and Wu, 1981; Roychoudhury *et al.* 1980; Schmitz *et al.* 1991) in which oligonucleotides are enzymatically labelled at their 3' end with terminal transferase by addition of a longer nucleotide tail (Schmitz *et al.* 1991). Oligonucleotides

from 14 -100 nucleotides in length tailed with DIG-dUTP showed increased sensitivity (Schmitz *et al.* 1991).

2.4.3 Oligonucleotide probes designed from known protein sequences

When natural nucleic acid probes are not available, and the nucleotide sequence of the gene of interest is not known, a synthetic oligonucleotide probe can be designed from known protein sequence (Lathe, 1985; Waston *et al.* 1992). For probe specificity, a minimum of 15 to 16 nucleotides are required and oligonucleotides that are 17 to 20 nucleotides long are commonly used (Tenover, 1988). To be able to design a probe of this length, knowledge of a minimum of six contiguous amino acids from the protein sequence is required (Waston *et al.* 1992). An oligonucleotide is synthesized carrying the codons known to encode the corresponding amino acid (Lathe, 1985). However, some amino acids are coded for by as many as six different codons, and therefore a stretch representing only six amino acids could be encoded by many different DNA sequences (Wallace *et al.* 1981; Waston *et al.* 1992). Oligonucleotide probes are for this reason often synthesized as mixtures, or pools that contain all possible combination of codons that translate into the protein sequence and within this pool will be one oligonucleotide that contains the correct sequence of the gene (Wallace *et al.* 1981; Waston *et al.* 1992).

A disadvantage of this approach is that the correct oligonucleotide is only a small fraction of the total population of molecules in the pool with a possibility of the remaining oligonucleotides hybridizing to undesired DNAs, leading to false positives (Tenover, 1988). An alternative strategy

if more than six contiguous amino acids are known is to synthesize a single rather longer probe of unique sequence with reduced homology (Lathe, 1985). The sequence is chosen on the basis of the frequency of codon usage, G-T base pairing, dinucleotide frequencies, confidence limits and choosing a region of the protein that contains the fewest degenerate codons (Lathe, 1985: Waston *et al.* 1992). Lathe, (1985) found the hybridization signal generated by a correct probe-target hybrid to be greatly reduced over that generated by a single optimal oligonucleotide probe and reported that non-specific affinity between the hybridization matrix is potentially capable of concealing weak positive signals obtained with multiple probes.

2.5 DETECTION SYSTEMS FOR DNA-DNA HYBRIDS

Several different methods for detecting the binding of probe to the target DNA have been described. These methods are based on attachment or incorporation of, a reporter molecule directly into the nucleic acid backbone of the probe (Jablonski *et al.* 1986), or by use of an indirect detection system in which the first component is added to the reaction mixture after hybridization reaction is completed (Al-Hakim and Hull, 1986). These methods include: radioactive labels (Rigby *et al.* 1977), Biotin-Avidin systems (Al-Hakim and Hull, 1986), enzyme conjugated probes (Ruth, 1984; Jablonski *et al.* 1986) and antibodies that recognise hybridization products (Young and Davis, 1983; Boguslawski *et al.* 1986).

Radioactive labelling can be done either by DNA 5' end labelling with T4 polynucleotide kinase (Silberklang *et al.* 1979; Maxam and Gilbert, 1980), DNA 3' end labelling (Roychoudhury

et al. 1980; Deng *et al.* 1981), nick translation (Rigby *et al.* 1977), random primed DNA labelling (Feinberg and Vogelstein, 1983; 1984) or by DNA tailing (Roychoudhury *et al.* 1980; Deng *et al.* 1981) procedures. After hybridization, the binding of the probe DNA to the target is detected by autoradiography. Radioisotope labelling has one of the highest sensitivities of all labelling methods, detecting less than one picrogram of DNA (Tenover, 1988). Unfortunately, radioisotopes have a short half-life and require frequent probe preparation, which increases the cost of the assay per test. There are also safety problems as radioisotopes require careful handling techniques, quality control, proper storage conditions and implementation and adherence to a written protocol for handling accidents and spills (Tenover, 1988).

Non-radioactive labelling systems were developed with a view of overcoming the problems associated with radioactive isotopes. Biotin- Avidin labelling system (Langer *et al.* 1981; Al-Hakim and Hull, 1986) involves incorporation of a primary label into the DNA probe by nick translation (Al-Hakim and Hull, 1986), end labelling or tailing reactions (Langer *et. al.* 1981). After hybridization, a second detector group usually consisting of an enzyme such as horseradish peroxidase attached to avidin or enzyme labelled antibiotin antibody is added to a filter or hybridization solution. The avidin or the antibody binds to the biotin moiety in the DNA hybrid and after addition of a substrate, a colour product is produced (Leary *et al.* 1983; Al-Hakim and Hull, 1986). This system was found to have problems of unspecific reaction with endogenous biotin and marked adherence of streptavidin even with blocked membranes (Wilchek and Bayer, 1988). Forster *et al.* (1985) developed a variation of this technique that uses a photoreactive substrate that could be measured by a fluorometer, while Chan *et al.* (1985) reported an amplification

system that couples several enzymes to each biotin moiety. Mathews *et al.* (1986) reported detection of less than one picrogram of DNA with a chemiluminescence system and Chollet and Kawashima (1985) reported detection of DNA using biotinylated oligonucleotide probes.

Enzyme conjugated probes have an enzyme e.g alkaline phosphatase attached directly to the DNA by using a 12 atom linker arm (Ruth, 1984: Jablonski *et al.* 1986), while monoclonal antibodies that recognize and bind directly to DNA-RNA hybrids can be labelled with either enzyme or other reporter molecule for detection of DNA hybrids (Boguslawski *et al.* 1986). Urdea *et al.* (1988) showed that the detection limits and non-specific binding of the probes lebelled with enzyme modified oligonucleotides to be significantly better than the fluorescent or chemiluminescent derivatives in sandwich hybridization assay with sensitivities comparable to radioisotope ^{32}P labelled probes. These authors reported that the significant loss of sensitivity of fluorescent and chemiluminescent probes during hybridization was due to high backgrounds they produce than do enzyme or radioisotope labels.

Schmitz *et al.* (1991) developed a nonradioactive labelling and detection system, which involves the incorporation of a nucleotide analog (digoxigenin-11-dUTP) into DNA by the random primed labelling technique. After hybridization of the digoxigenin labelled probe to immobilized DNA, detection of the labelled probe is effected with an antibody-enzyme conjugate, anti-digoxigenin-alkaline phosphatase. The system was reported to be suitable for detection of DNA in Southern blots, Northern blots, dot blots, colony hybridization, plaque lifts, slot blots, *in situ* hybridization of fixed cells, gene sequences on chromosomes and of mammalian tissue sections, with reliable specificity and sensitivity (Schmitz *et al.* 1991). These authors also reported DNA 3'

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end labelling and DNA tailing with digoxigenin-11-dUTP. Digoxigenin labelled probes were found to have very high stability of upto several years. They have possibilities of repeated usage of the hybridization solutions and do not have unspecific reactions experienced with biotin streptavidin systems since no other organisms produce endogenous digoxigenin except *digitalis* plants (Schmitz *et al.* 1991).

The objectives of this study were:

1. to determine the prevalence of food poisoning outbreaks in Kenya;

2. to identify and characterize toxigenic strains of *B. cereus* using plasmid profile, total bacterial restriction endonuclease analysis and antibiotic susceptibility tests;

3. to investigate and develop DNA based diagnostic techniques that can be used in the characterization of enterotoxigenic *B. cereus* strains isolated from foods and other sources;

4. to investigate the prevalence of enterotoxigenic strains of *B. cereus* in market milk by employing DNA based techniques developed in (3) above.

CHAPTER THREE

3 MATERIAL AND METHODS

3.1 SURVEY OF FOOD POISONING OUTBREAKS IN KENYA

A survey to determine the prevalence of foodborne disease outbreaks was carried out by use of Ministry of Health annual reports kept at the Ministry's Headquarters. Reports from all the old districts (before the subdivisions in 1993) in Kenya covering the period 1970 to 1994 were examined for reported food poisoning outbreaks. The nature of the outbreak, number of people and foods involved and results of any investigation carried out were recorded. Outpatient morbidity records kept at the district headquarters in corroboration with the Ministry's Health Information System (HIS) annual reports and statistical bulletins were examined for the number of poisoning cases treated in various health facilities. Outpatient morbidity records covering the period 1988 to 1991 only were available. The same records were examined for cases of specific diseases including typhoid fever, salmonellosis, cholera, and dysentery/shigellosis. Data was entered into a cricket graph in an Apple Macintosh SE Computer to perform descriptive statistics.

3.2 ISOLATION OF *B. CEREUS* FROM MILK

3.2.1 Reference strains

Bacillus cereus prototype strains NCTC 11143 from an emetic syndrome outbreak, and NCTC 11145 from a diarrhoeal syndrome outbreak (acquired from the National Collection of Type Cultures, London) and strain DSM 4384, which is the same strain as strain B-4ac used by Agata, *et al.* (1995) that was acquired from the Germany Type Culture were used as reference strains in all the experiments. They were preserved in nutrient broth containing 15 % glycerol at -20°C. Working stock was maintained in cooked meat medium kept at 4°C and renewed after every three months.

3.2.2 Culture Media and reagents

Composition and preparation of culture media, reagents and components of various kits used in this study are described in appendix 1.

3.2.3 Collection of milk samples

Raw milk samples were aseptically taken from various milk collection centres in Kiambu District. Pasteurised milk samples were bought from various retail shops in and around Nairobi. Samples were transported to the laboratory in cool boxes and analysed the same day.

3.2.4 Culture, isolation and confirmation

A portion of each milk sample was streaked onto *Bacillus cereus* selective agar (Holbrook and Anderson, 1980) and incubated at 37°C for 24 hrs. The rest of each milk sample was incubated at 37°C for 24 hours to enrich for *B. cereus*. After incubation, they were streaked onto *Bacillus cereus* selective agar and then incubated at 37°C for 24 hours. In this medium, *B. cereus* colonies are crenate, about 5 mm in diameter with distinctive turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same colour. Suspect colonies were purified by subculturing onto fresh *B. cereus* selective agar plates and subsequently streaked onto blood agar to check for hemolysis. Typical *B. cereus* colonies were stained with the rapid confirmatory staining procedure developed by Holbrook and Anderson (1980). In this procedure, a slide film was prepared from day old colonies growing on *Bacillus cereus* selective agar. The film was air dried and fixed with minimum flaming. The slide was then placed over boiling water and flooded with 5 % w/v malachite green. After 2 minutes, the slides were washed, blot dried and stained with 0.3 % w/v sudan black in 70 % ethyl alcohol for 15 minutes. The slides were then washed with xylene for 5 seconds, blot dried and counter stained with 0.5 % w/v safranin for 20 seconds. Excess safranin stain was washed off and the slide air dried.

The stained slides were microscopically examined for the characteristic appearance and presence of lipid globules in the vegetative cells. With this stain, vegetative cells usually are 4-5 μm long and 1.0 -1.5 μm wide with square ends and rounded corners. The spores stain pale green and

are either central or paracentral in position and do not swell the sporangium. Lipid granules stain black and the vegetative cytoplasm Red (Holbrook and Anderson, 1980). Further confirmation of *B. cereus* strains was done using the following biochemical tests.

3.2.4.1 Acid production from carbohydrates

The isolates were tested for their ability to ferment sugars with production of acid. Three mililitres of 10 % membrane filter sterilized glucose, xylose, arabinose, sucrose, inulin, maltose, raffinose, rhamnose, sorbitol, lactose, salicin, trehalose and mannitol sugar were added separately to 3 ml of sterilized ammonium salt-sugar medium in test tubes cooled to 50°C. The agar medium was allowed to solidify in slanting position. Inoculation with *B. cereus* was by stabbing of the butt with a straight wire and surface streaking. Incubation was done at 37°C for five days. Production of acid was indicated by a colour change of the bromothymol blue indicator to yellow (Sneath, 1986).

3.2.4.2 Production of acetyl methyl carbinol

Tubes of Voges-Proskauer broth were inoculated with *B. cereus* strains in triplicates and incubated for upto 7 days. Test for acetyl methyl carbinol production was done after incubation for 3, 5 and 7 days by mixing 3 ml of 40 % (w/v) sodium hydroxide with the broth culture and addition of 0.5 mg creatine. Production of acetyl methyl carbinol was indicated by development of a red colour after 30 to 60 minutes at room temperature (Sneath, 1986).

3.2.4.3 Citrate utilization

Bacillus cereus was inoculated onto slants of citrate medium according to Sneath (1986). The slants were incubated for upto 14 days at 37°C. After incubation, utilization of citrate was indicated by growth and a colour change to red due to production of an alkaline reaction.

3.2.4.4 Starch hydrolysis

Starch hydroysis test was performed as described by Shinagawa, (1990) by streaking the *B. cereus* strains onto nutrient agar (Oxoid, England) supplemented with 1 % soluble starch and incubated at 37°C for 24 hours. After incubation, the agar surface was flooded with lugol's iodine solution. A clear zone around the streak of bacterial growth was considered a positive reaction. The test was considered negative if the clear zone appeared only under the streak, or if the zone was absent altogether.

3.2.4.5 Production of indole

Bacillus cereus test organisms were inoculated into 5 ml of sterile 1.0 % w/v tryptone broth (Oxoid) and incubated at 37°C for 24 hours. After incubation, 2 ml of test solution (containing p-dimethylaminobenzaldehyde-5 g, iso-amyl alcohol 75 ml; and concentrated hydrochloric acid 25 ml) was added and mixed vigorously. Indole production was indicated by a pinkish red colour at the top alcohol layer that separated on standing (Cowan and Steel, 1974).

Cultures were maintained in nutrient broth containing 15 % glycerol at -20°C. They were subcultured temporarily into fresh cooked meat media and kept at 4°C after every three months.

3.3 TEST FOR ANTIBIOTIC SUSCEPTIBILITY

Antibiogram analysis of the *B. cereus* strains was assessed for its usefulness in differentiating diarrhoeal and emetic strains. The strains were tested for susceptibility to eight antimicrobial agents commonly used in the treatment of animal diseases, by the disc diffusion technique described by Bauer *et al.* (1966). Bacterial strains (including NCTC reference strains) were subcultured onto blood agar and incubated at 37°C for 24 hours. After incubation, 3 to 4 colonies were transferred into Mueller Hinton broth and incubated at 37°C for 24 hours. The inoculum was standardized by adjusting with sterile saline the turbidity of an actively growing broth culture to match that of barium standard. Mueller Hinton agar plates were then inoculated with sterile cotton wool swabs moistened in this bacterial suspension and spread as evenly as possible. One multidisk with ampicillin (25 µg) tetracycline (25 µg), cotrimoxazole (25 µg), streptomycin (10 µg), kanamycin (30 µg), gentamicin (10 µg), sulphamethoxazole (200 µg) and chloramphenicol (30 µg) was placed on the surface of each of the inoculated agar plates. All the plates were incubated at 37°C for 24 h, after which the diameter of the inhibition zone of each antimicrobial agent measured to the nearest millimeter. Isolates were considered resistant, intermediate resistant or sensitive based on the size of inhibition zones for individual antibiotics as reported by Bauer *et al.* (1966).

3.4 TEST FOR DIARRHOEAL ENTEROTOXIN PRODUCTION

Test for enterotoxin production by *B. cereus* strains was done using a reverse passive latex agglutination (RPLA) technique by employing BCET -RPLA test kit (Oxoid, Unipath, U.K). The kit is based on 48 kda protein, one of the three protein components of the enterotoxin complex (Granum *et al.* 1993) or L_2 component of hemolysin BL (Beecher and Wong, 1994a and 1994b). The kit detects the L2 component of hemolysin BL (Beecher and Wong, 1994b). Test organisms were inoculated into brain heart infusion broth and incubated on a roller drum at 37°C for 18 hours, after which the broth cultures were filtered through 0.45 µm diameter low protein binding membrane filters (HAWP, Molsheim, France).

Assay for enterotoxin was performed in v-well microtitre plates using the BCET-RPLA test kit according to the instructions of the manufacturer. The latex reagents (TD 951, 952) and diluent (TD 954) were provided in the kit ready for use. The latex reagents were thoroughly mixed before use to ensure a homogeneous suspension. To reconstitute the enterotoxin control reagent (TD 953), 0.5 ml of diluent (TD 954) was added to each vial, and shaken gently until the contents dissolved.

Microtitre plates were arranged so that each row consisted of 8 wells. Each sample needed the use of two such rows. Using a micropipette, 25 µl of diluent was dispensed into each well of the two rows (except the first well in each row). This was followed by addition of 25 µl of the test sample to the first and second wells of both rows. Contents of the second well in each row were mixed well, after which, 25 µl was picked and doubling dilutions performed along each of the

rows. The dilutions were stopped at the 7th well to leave the last well containing diluent only.

Twenty five microlitres of sensitized latex was then added to each well in the first row, while 25 μl of the latex control was added to each well in the second row. The contents of each well were mixed by rotating the plate using a micromixer. The plates were then covered with a lid to avoid evaporation of the contents and placed in a moisture box. The moisture box containing microtitre plates were incubated undisturbed on a vibration free surface at room temperature for 20 hrs. After incubation, each well was examined for agglutination using a Dynatech microtitre plate viewer.

If *B. cereus* diarrhoeal enterotoxin was present in the culture filtrate, agglutination occurred due to the formation of a lattice structure. Upon settling the agglutinate formed a diffuse layer on the base of the well. If *B. cereus* enterotoxin was absent, or was at low concetration below the assay detection level (2 ng/ml), no such lattice structure could be formed, instead a tight button was observed.

3.5 ANALYSIS FOR PRESENCE OF PLASMIDS

3.5.1 Isolation of plasmid DNA

Plasmid DNA was isolated from *B. cereus* strains as described by Birnboim and Doly (1979). Each *B. cereus* strain was inoculated into 3 ml tryptic soy broth and incubated overnight on a roller drum at 37°C. About 1.5 ml of each overnight broth culture were transferred into eppendorf tubes and centrifuged for one minute at 3000 g at room temperature. The supernatant

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was discarded and 100 μl of lysozyme solution (appendix 1.2.7) added to the pellet to disrupt the outer cell wall and cell membrane. Tubes were capped, vortexed and placed on ice for 30 minutes. Internal cell membranes were lysed by adding 100 μl of alkaline detergent solution (appendix 1.2.8). Tubes were inverted several times and then kept in the waterbath for 5 minutes. One hundred and fifty microlitres of 3M sodium acetate buffer (pH 4.8) was added and tubes inverted several times, then kept in ice cooled waterbath for at least 10 minutes to precipitate the denatured proteins and chromosomal DNA. Denatured proteins and chromosomal DNA were pelleted by centrifugation at 3000 g at room temperature for 5 minutes. The supernatant was transferred into new eppendorf tubes and 1.0 ml of 95 % cooled ethanol added to each tube. They were then kept at -20°C for 5 minutes to precipitate plasmid DNA. Precipitated plasmid DNA was pelleted by centrifugation at 3000 g for 3 minutes. The supernatant was discarded and the sediment containing plasmid DNA was suspended in 50 μl of 0.1 M sodium acetate/0.05 M Tris HCL buffer (pH 8.0).

3.5.2 Plasmid analysis

The contents of the DNA suspension from above were mixed gently, and 16 μl of loading dye 1 (appendix 1.2.10) added. Thirty-three microlitres (33 μl) of the contents was then loaded into wells of 1.0 % agarose gels. For each gel, plasmid DNA from molecular size markers (*E. coli* V517) was included. Electrophoresis was done at 25 mA overnight after which the gels were then stained with a solution containing 2 μl of 10 mg/ml ethidium bromide for 30 minutes, destained in distilled water for one hour and then viewed on a U/V transilluminator. Photographs of gels

were taken using a leicaflex SL-2 camera by exposing films for 90 seconds. Plasmid sizes were estimated from a standard graph of migration distance (x-axis) plotted against natural logarithm of molecular weights of the DNA marker (y-axis).

3.6 RESTRICTION ENDONUCLEASE ANALYSIS OF *B. CEREUS* GENOMIC DNA

3.6.1 Isolation of genomic DNA

Genomic DNA was isolated from the *B. cereus* strains according to Ausubel *et al.* (1990). The strains were inoculated each into 5 ml of brain heart infusion broth and incubated at 37°C for 24 hours on a roller drum. After incubation, 1.5 ml of the culture was transferred into an eppendorf tube and centrifuged for 5 minutes in a microcentrifuge to form a compact pellet of bacterial cells. The supernatant was discarded and pellet resuspended in 567 µl Tris-EDTA buffer (pH 8.0) by repeated pipetting. Bacterial cell walls were lysed by adding 30 µl of 10 % sodium deodsyl sulphate (SDS) and 3 µl of 20 mg/ml proteinase-K (to give a final concentration of 100 µg/ml proteinase K in 0.5 % SDS). The contents were thoroughly mixed and incubated in a waterbath at 37°C for one hour. One hundred microlitres of 5 M Nacl was then added, followed by 80 µl of cetyltriammomium bromide (CTAB/Nacl) solution. The contents were mixed thoroughly and incubated in the waterbath at 65°C for 10 minutes to precipitate cell wall debris, denatured proteins and polysaccharides complexed to CTAB. After incubation, an equal volume of

chloroform/ isoamyl alcohol was added, mixed by inverting the tube several times and centrifuged at 3000 g for 5 min to separate CTAB-protein/polysaccharide complexes, which form as a white interface. The supernatant was then transferred into a fresh microcentrifuge tube leaving the interface behind. An equal volume of phenol/chloroform/isoamyl alcohol was added to the supernatant to remove any remaining CTAB precipitate. The contents were mixed and then centrifuged for 5 min and supernatant transferred again to a fresh tube into which 600 μl isopropanol was added. The tube was inverted several times to precipitate the DNA. The DNA precipitate was pelleted by centrifugation at room temperature for 2 minutes. The supernatant was poured off and precipitated DNA washed with 1.0 ml of 70 % ethanol to remove residual CTAB. The DNA was again precipitated by centrifuging at room temperature for 2 min. Ethanol was then poured off and DNA precipitate briefly dried in a lyophilizer. The dry DNA was resuspended in 20 μl of TE buffer.

3.6.2 Digestion with Restriction Enzymes

Various restriction enzymes, which included *Hind111, EcoR1, Sau3A, Mbo1* and *Dra1* were used to digest *B. cereus* genomic DNA. To 18 μl of DNA /TE solution, 2.0 μl of appropriate 10 x digestion buffer and 10 units of restriction enzyme were added. The contents were mixed by repeated pipetting. The mixture was incubated at 37°C for one hour in a waterbath after which the reaction was stopped by adding 8.0 μl of the loading dye (stop mix). The contents were briefly mixed and loaded into wells in 1 % agarose gel and electrophoresis carried out at 25 mA

overnight. The gels were stained with a solution containing 2 mg/ml ethidium bromide for 30 minutes and destained with distilled water for one hour and viewed on a UV transilluminator. Gels were photographed with a Leicaflex SL-2 camera. Films were exposed for 90 seconds.

3.7 DETECTION OF *BCET* GENE IN *B. CEREUS* STRAINS BY POLYMERASE CHAIN REACTION

Sixty *B. cereus* strains from various sources were tested for presence of *bceT* gene. Two DNA primers were designed from the published *bceT* sequence (Agata *et al.* 1995) for amplification of the *bceT* gene. The forward primer 5'-TTA GTT TCA ACA GCG CGT ATC GGT -3' contained 24 bases and was coded bceT 5' (1). The reverse primer 5'-ATA CAC ATG CAA ATG CTC CGG AC -3' contained 23 bases and was coded bceT 3' (2). The two primers were 694 bases apart in the *bceT* gene and products of amplification were expected to be 741 base pairs long. The designed primers were then ordered from a commercial DNA synthesizing company (MWG BIOTECH Gmbh, Germany).

The reagents for the PCR reaction were added to the reaction mixture in a sterile microcentrifuge tube. A master mix was made that contained distilled water, 1x PCR reaction buffer, 0.2 mM nucleotide mixture (dNTPs), 1.0 μM primer 1, 1.0 μM primer 2 and 4.0 Units /100 μl Taq DNA polymerase according to the number of samples analysed. Equal amounts (usually 45 μl) of the master mix was dispensed into each reaction tube containing 5 μl *B. cereus* chromosomal DNA template. The reagents were then thoroughly mixed and 50 μl of mineral oil

overlayed in each reaction tube to reduce evaporation of the contents during the amplification process. The reaction tubes were placed in a PCR thermocycler (HYBAID OmniGene). Cycling conditions were programmed to as follows:

a). Initial denaturation at 95°C for 5 min. -1 cycle.

b). Annealing (51°C, for 30 sec.); extension (72°C, 1 min.); denaturation (95°C, 1 min) - 30 cycles.

c). Final annealing at 72°C for 5 min - 1 cycle.

The PCR reaction was usually complete in 2.5 hours. Products of the PCR reaction were analysed for presence of a 741 bp DNA band by electrophoresis on an agarose gel. A 1.0 Kilobase (kb) DNA ladder was included on the gel to estimate the size of the PCR products.

3.8 PREPARATION OF DNA PROBE FOR DETECTION OF *BCET* GENE

3.8.1 Isolation of the 741 bp DNA fragment

The 741 bp PCR fragment from the reference strain DSM 4384 was used as a probe to screen for the *bceT* gene in other *B. cereus* strains. This was to determine if the *bceT* gene could be detected with the same sensitivity (as with the PCR) by a simpler dot blot hybridization technique and be used adequately to test for presence of *bceT* gene in unknown *B. cereus* strains. Genomic DNA from *B. cereus* strain DSM 4384 from which *bceT* gene was cloned by Agata *et*

al. (1995) was isolated and used as template for the amplification reaction. After amplification the PCR product was run in an agarose gel to verify amplification of the right DNA fragment. The PCR DNA product was then isolated and purified from agarose using QIAEX II Gel Extraction Kit (GIAGEN Gmbh, Germany) as recommended by the manufacturer.

The 741 bp band was excised from agarose gel with a clean sharp scapel and placed in a preweighed microcentrifuge tube and then weighed. Three volumes of buffer QXI to one volume of the gel was added to the tube and vortexed for 30 seconds. Ten microlitres (10 µl) of QIAEX II silica particle suspension was added and the mixture incubated at 50°C for 10 min, while mixing after every 2 min to keep QIAEX particles in suspension. The pH was checked and maintained at below 7.5 by adding 3 M sodium acetate, pH 5.0 for efficient adsorption of DNA to QIAEX II silica particles. The sample was then centrifuged for 30 sec and the supernatant carefully removed with a pipette. The DNA pellet was washed with 500 µl of buffer QXI and centrifuged to remove residual agarose, ethidium bromide and protein contaminants. The pellet was again washed twice with buffer PE and centrifuged for 30 min to remove residual salt contaminants. The pellet was air dried for 10-15 min to remove traces of buffer PE and then resuspended in 20 µl of distilled water to elute DNA from QIAEX II particles. The suspension was incubated at room temperature for 5 min to allow DNA to dissolve. The DNA solution was centrifuged for 30 sec and the supernatant containing purified DNA was carefully pipetted into a clean tube, leaving QIAEX II silica gel particles behind.

3.8.2 Labelling of purified DNA for use as probe

The purified DNA was labelled with the non-radioactive digoxigenin-11-dUTP labelling kit (Schmitz *et al.* 1991) using a random primed DNA labelling procedure (Feinberg and Vogelstein, 1983; 1984). The method is based on the hybridization of a mixture of all possible hexanucleotide to the DNA to be labelled. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme (labelling grade). Digoxigenin labelled deoxyribonucleoside triphosphates (dNTPs) present in the reaction mixture are incorporated in the newly synthesized complementary DNA strand at a rate of one molecule for every 20-25 nucleotides (Schmitz *et al.* 1991).

The labelling procedure involved denaturing 14 µl of DNA in a microcentrifuge tube by heating it at 95°C for 10 min and cooling on ice for 3 min. While on ice, the following reagents were added: 2 µl hexanucleotide mixture, 2 µl dNTP labelling mixture, 2 µl klenow enzyme (4 units), to make a total volume of 20 µl. The contents were then centrifuged briefly and incubated at 37°C overnight. After incubation, 2 µl of 0.2 M EDTA (pH 8.0) was added to stop the reaction. DNA was then precipitated by adding 2.5 µl of 4 M LiCl and 75 µl pre-chilled absolute ethanol (-20°C), and kept at -70°C for at least 30 min. After 30 min, the contents were centrifuged at 4,000 g for 10 min, pelleted DNA washed with 40 µl cold 70 % ethanol and centrifuged again. The supernatant was pipetted carefully and discarded. The DNA pellet was dried under vacuum to remove traces of ethanol. DNA was then dissolved in 50 µl of Tris EDTA (pH 8.0) for 30 min at 37°C.

3.8.3 Detection of the *bceT* gene in *B. cereus* strains

Dot-blot hybridization (Kafatos *et al.* 1979) was used to identify *bceT* gene sequence in genomic DNA from *B. cereus* isolates. *Bacillus cereus* strain DSM 4384 and NCTC 11145 were used as positive and negative controls respectively. Genomic DNA was denatured at 95°C for 10 min and immediately chilled for 3 min. About 4 µl of the denatured DNA was dot blotted onto nylon membranes and left to dry. The membranes were wetted in a denaturing solution for one minute and then transferred to a neutralizing solution for 2 minutes. The membranes were blot dried with a filter paper and then air-dried. The DNA was fixed to the nylon membranes by cross-linking with UV autolinker (Strategene 1800, Strategene, Germany) for 3 min. After the UV cross-linking, the membranes were rinsed briefly in water and allowed to air dry.

The membranes were prehybridized in sealed glass bottles containing 10 ml of hybridization solution without probe for at least one hour at 42°C. This was done in a mini oven with a rotating device to redistribute the solution over the membranes from time to time. After one hour, prehybridization solution was discarded and replaced with a hybridization solution containing DNA probe denatured by heating at 95°C for 10 minutes. The filters were re-incubated overnight at 42°C in the mini oven (Meinkoth and Wahl, 1984). After hybridization, the probe was removed and kept for reuse, and the membranes washed two times for 5 min in wash solution 1, and two times for 15 min in wash solution 2 at 42°C (Meinkoth and Wahl, 1984; Tenover, 1988).

The membranes were removed from the hybridization bottles and prepared for immunological detection. They were washed at room temperature in buffer 1 for 1 min, and then incubated in 100 ml of buffer 2 for 30 min. and once again washed briefly in buffer 1. They were then incubated for 30 min with 20 ml of antibody conjugate solution containing 150 mU/ml in buffer 1. The unbound antibody conjugate was removed by washing 2 times for 15 min with 100 ml of buffer 1. The membranes were then equilibrated for 2 min with 20 ml of buffer 3, and incubated in the dark with 10 ml of freshly prepared 5-bromo-4-chloro-3-indolyl phosphate (BCIP) /nitroblue tetrazolium chloride (NBT) colour-substrate without shaking (Schmitz *et al.* 1991). A blue precipitate formed after simultaneous oxidation of BCIP and reduction of NBT (Schmitz *et al.* 1991). The colour precipitate started to form within a few minutes and the reaction was usually complete after 1 day. When the desired spots were detected, the reaction was stopped by washing the membranes for 5 minutes with 50 ml of buffer 4. Results were documented by photography.

3.9 DETECTION OF *BACILLUS CEREUS hblA* GENE

3.9.1 Preparation of *hblA* gene probe

The probe was prepared by PCR amplification of the DNA fragment internal to *hblA* gene coding sequence. Two DNA primers were designed from the published *hblA* sequence (Heinrichs *et al.* 1993). The forward primer 5'-TGC AGA TGT TGA TGC CG-3' corresponding to positions 672 to 688 in the *hblA* gene contained 17 bases and was coded primer (1). The reverse primer 5'- CCC GCA AGA TCC CGT AT -3', which corresponded to the complement of positions 1011 to 1027 in the *hblA* gene also contained 17 bases and

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was coded primer (2). The two primers were 311 bases apart in the *hblA* gene. The product of amplification was expected to be 355 base pairs. The designed primers were then ordered from a commercial DNA synthesizing company (MWG BIOTECH Gmbh, Germany). After amplification the PCR product was run on an agarose gel, isolated from the gel, purified and then labelled with a random primed DNA labelling procedure as described in section 3.8.1.

3.9.2 Detection of *hblA* gene in *B. cereus* isolates

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Dot blotting, hybridization and detection of *hblA* gene was done as described in sections 3.8.3, but using *hblA* probe.

3.10 Statistical analysis

Pearson's chi-square test of significance was used to test for the difference in the isolation rates of *B. cereus* from raw and pasteurized milk and the correlation between (a) starch hydrolysis and enterotoxin production, and (b) enterotoxin production and presence of *bceT* gene.

CHAPTER FOUR

4 RESULTS

4.1 FOOD POISONING OUTBREAKS IN KENYA

Information from records at the Ministry of health indicated that few cases of foodborne disease outbreaks were reported from around the country between 1970 and 1994. Only 37 food poisoning outbreaks were reported to the Ministry and 13 of these involving a total of 926 people, were confirmed to be due to particular aetiological agents (Table 3). Twenty outbreaks involving 518 people were tentatively diagnosed as *Staphylococcus aureus*, *Bacillus cereus*, salmonellosis, Anthrax, chemical poisoning, aflatoxicosis, plant poisoning and heavy metal poisoning (Table 4). Three outbreaks of unknown aetiology involving 29 people and suspected to be due to consumption of maize flour and other unknown foodstuffs were reported.

Foods that were involved in the outbreaks included milk and milk products, meat and meat products, maize flour, bread scones and other wheat products, vegetables, and lemon pie pudding. Anthrax was suspected after consumption of uninspected meat. Chemical poisoning was suspected after consumption of maize flour products contaminated with diazinon and other organophosphorus insecticides. These chemicals were being transported in the same vehicle with

maize flour at which time there was spillage and contamination of bags containing maize flour. Plant poisoning involved consumption of vegetables, cassava and wheat flour contaminated with *Datura stramonium.*

The number of reported outbreaks was thought to be a gross underestimate of the actual situation in the country especially when compared with the number of individuals treated for poisoning at out-patient clinics in various health facilities. For example, the national figures for poisoning cases treated at out-patient clinics in various health facilities countrywide between 1988 and 1991 ranged between 28,973 and 207, 941 per year with Central Province reporting the highest number of cases, followed by Rift Valley (Table 5). In Central Province, the highest number of cases were reported in 1990, with Nyandarua district leading, followed by Kirinyaga, Muranga, Nyeri, Embu and Kisii districts in that order. In 1991, Kiambu district recorded the highest, followed by Kirinyaga and Muranga districts respectively (Figs. 1-8). A variety of causes were responsible including food poisoning, herbal medicine, drug poisoning, petrol and kerosine poisoning, battery poisoning, chemical poisoning, and alcohol poisoning among many others.

Twenty four individual cases of plant poisoning were reported in Isiolo and 298, 142, 91 and 111 cases of chemical poisoning were reported in Nyeri, Nyandarua, Meru and Isiolo districts respectively. Six cases of heavy metal poisoning were reported in Muranga district, two in Meru district and two in Isiolo district in the same period. It was evident that proper diagnosis of food poisoning disease outbreaks was severely hampered by lack of adequate laboratory facilities, lack of reporting and failure to obtain the right sample at the right time.

Table 3: Confirmed food poisoning outbreaks reported to the Ministry of Health between 1970-1993

Aetiological agent	No. of confirmed outbreaks	Total number of people involved.
Staphylococcus aureus	5	883
Clostridium perfringens	1	7
Plant poisoning	3	16
Chemical poisoning	2	5
Botulism	2	15
Total	13	926

Table 4: Tentative diagnoses of twenty unconfirmed food poisoning outbreaks reported to the Ministry of Health between 1970 and 1993.

Tentative diagnosis	No. of unconfirmed outbreaks	Total number of people involved.
Staphylococcus aureus	2	192
Bacillus cereus	1	3
Salmonellosis	3	46
Anthrax	5	230
Chemical poisoning	2	5
Aflatoxicosis	2	24
Plant poisoning	1	11
Heavy metal poisoning	4	7
Total	20	518

Table 5: Number of poisoning cases treated as outpatients in various health facilities in Kenya between 1988 and 1991 by province.

Province	1988	1989	1990	1991
Central	29,426	13,850	195,269	19,254
Coast	2,251	1,539	1,256	770
Eastern	651	5,011	3,263	316
N/Eastern	616	396	1,005	30
Nyanza	460	4,317	2,461	1,043
Western	151	452	809	1,697
R/Valley	7,075	6,005	3,289	5,300
Nairobi	-	77	586	563
Total	40,630	31,647	207,941	28,973

Source: *Health Information System statstical Bulletin, 1993*

Note: The figures given in this table are totals from the reporting health centres throughout each province. Not all health centres were reporting to their district headquarters. The high number of cases in 1990 for Central province was due to the high number of health centres that reported that year.

Figure 1: Poisoning cases reported in Central province in 1990 and 1991.
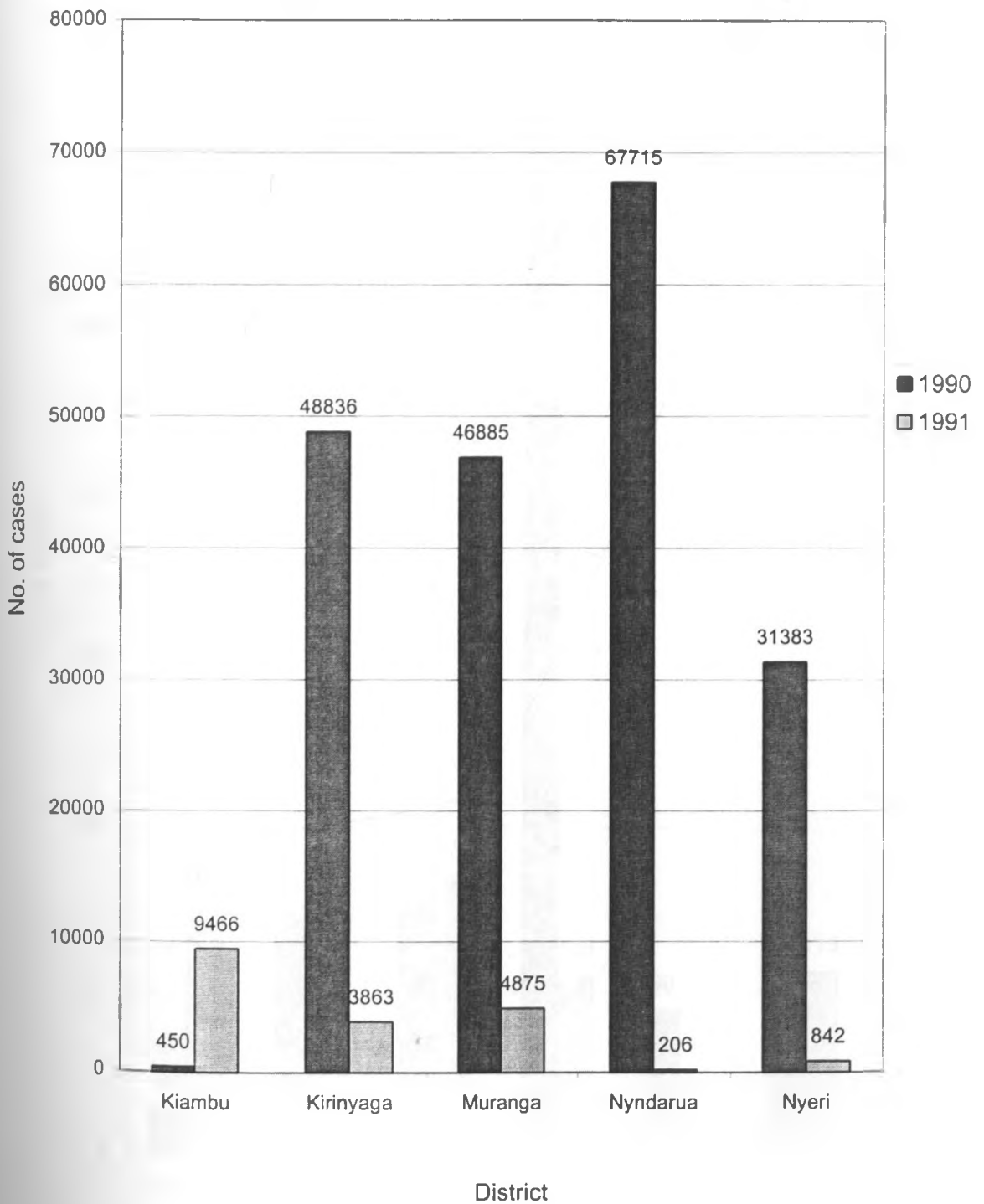
Figure 2: Poisoning cases reported in Coast Province in 1990 and 1991.
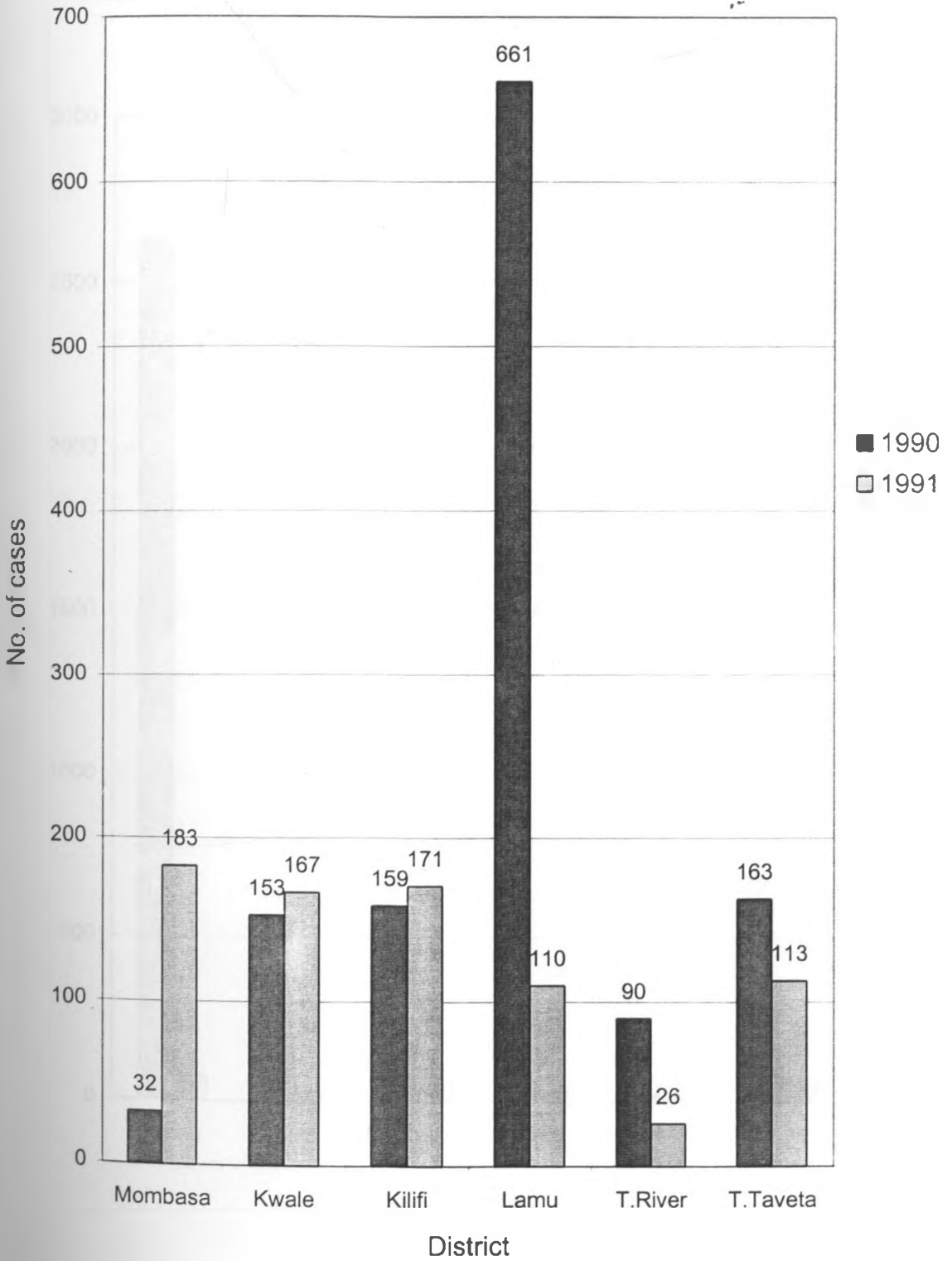
Figure 3: Poisoning cases reported in Eastern Province in 1990 and 1991.
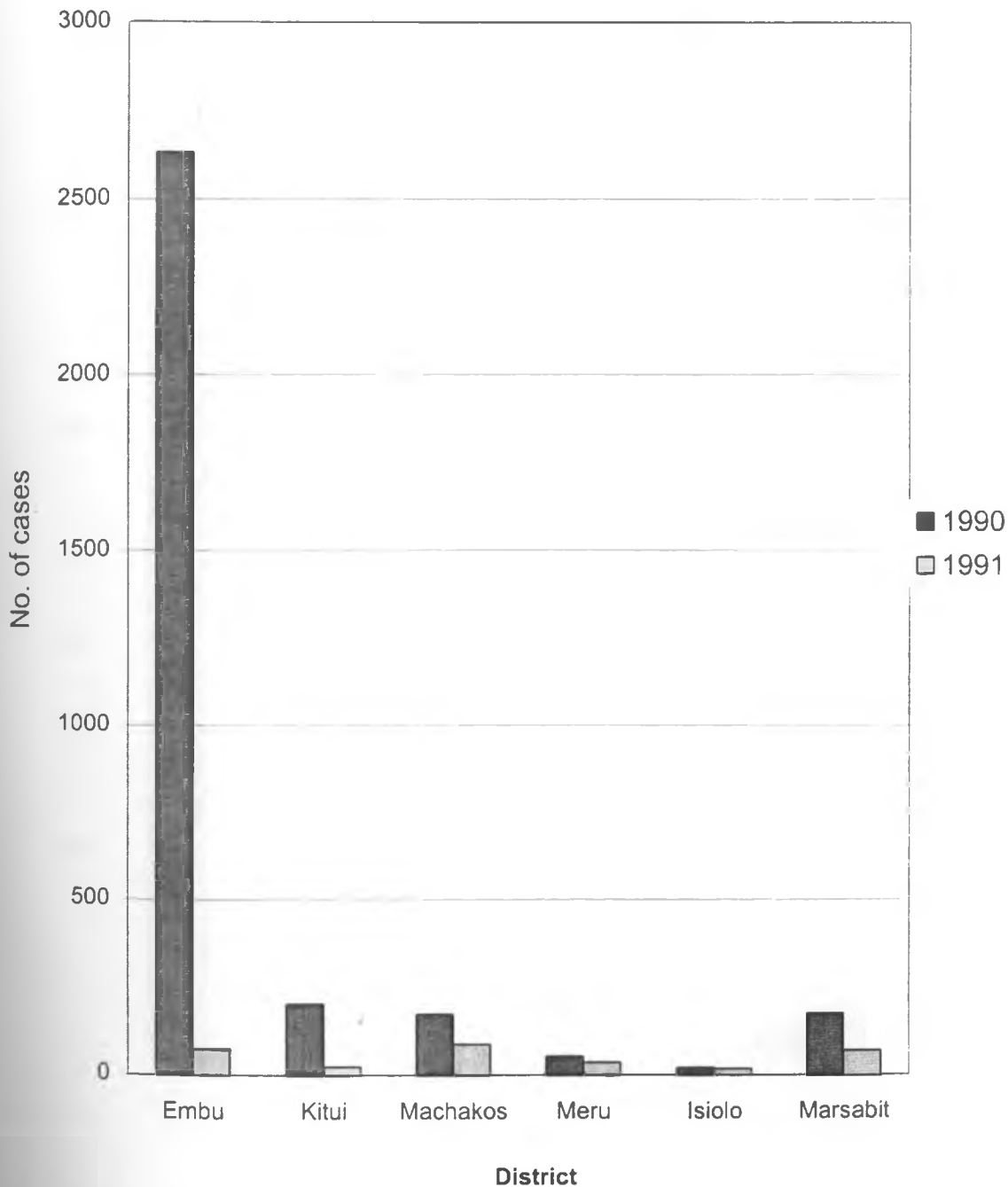
Figure 4: Poisoning cases reported in Nyanza Province in 1990 and 1991.
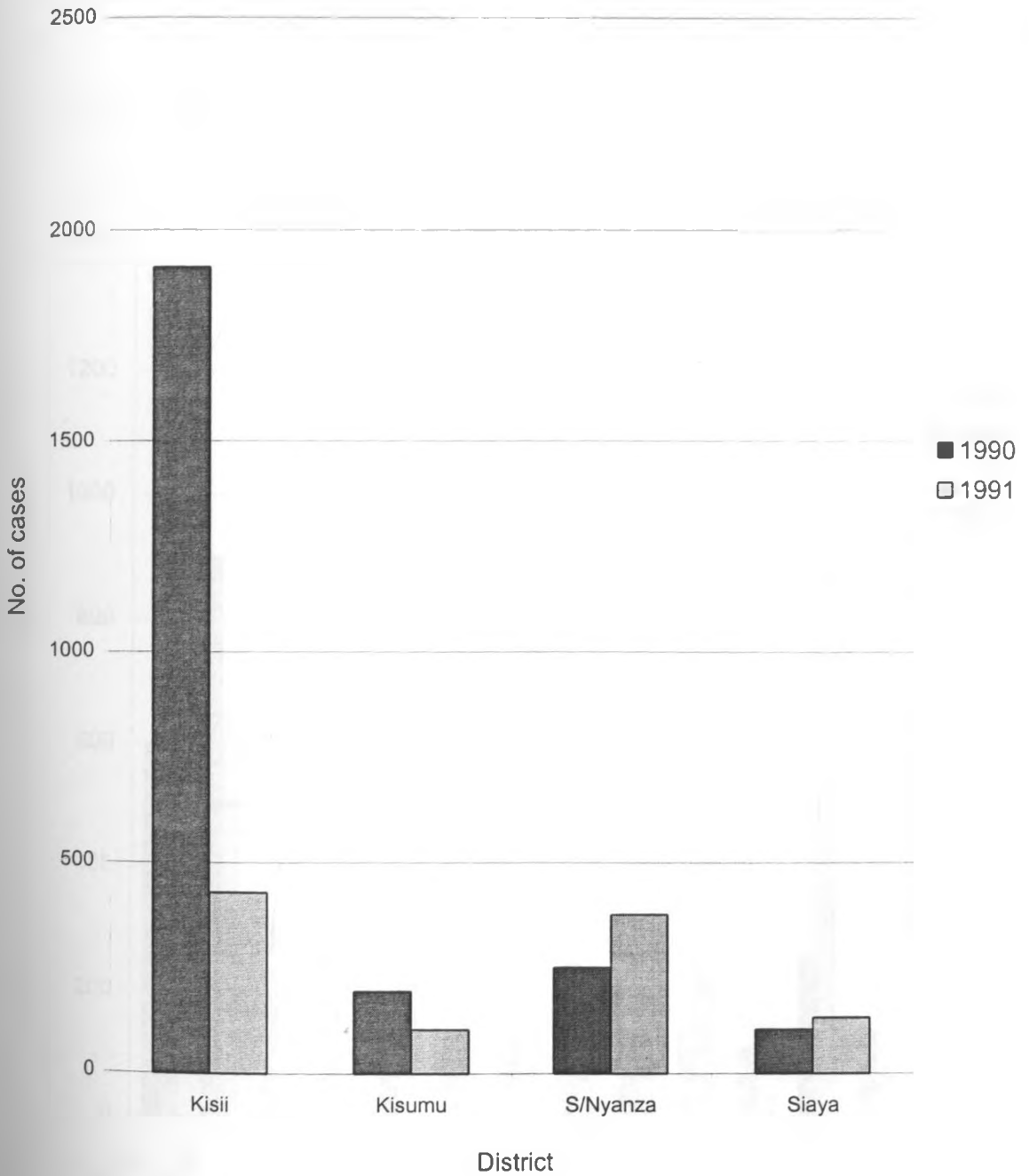
Figure 5: Poisoning cases reported in Riff Valley Province in 1990 and 1991.
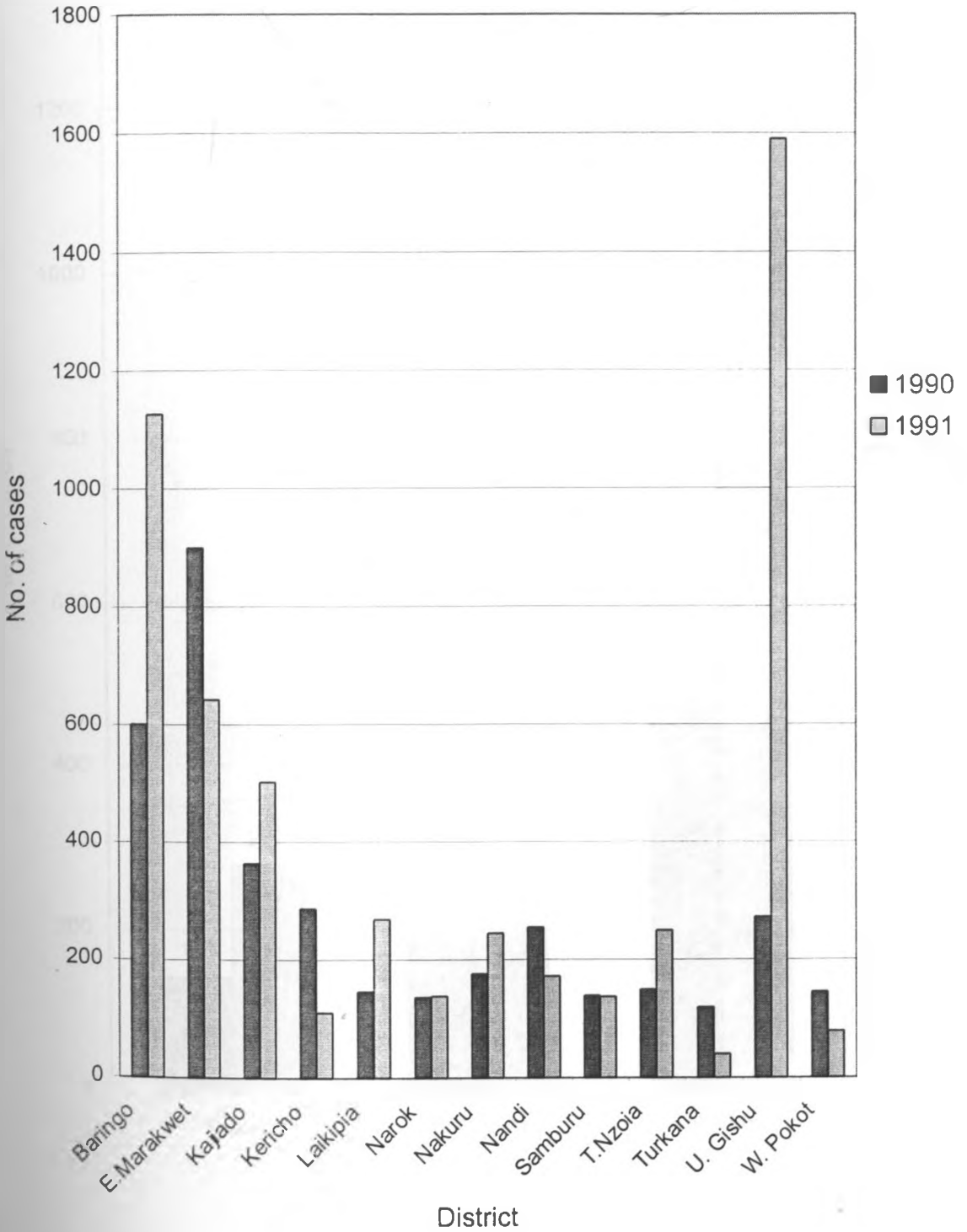
Figure 6: Poisoning cases reported in Western Province in 1990 and 1991.
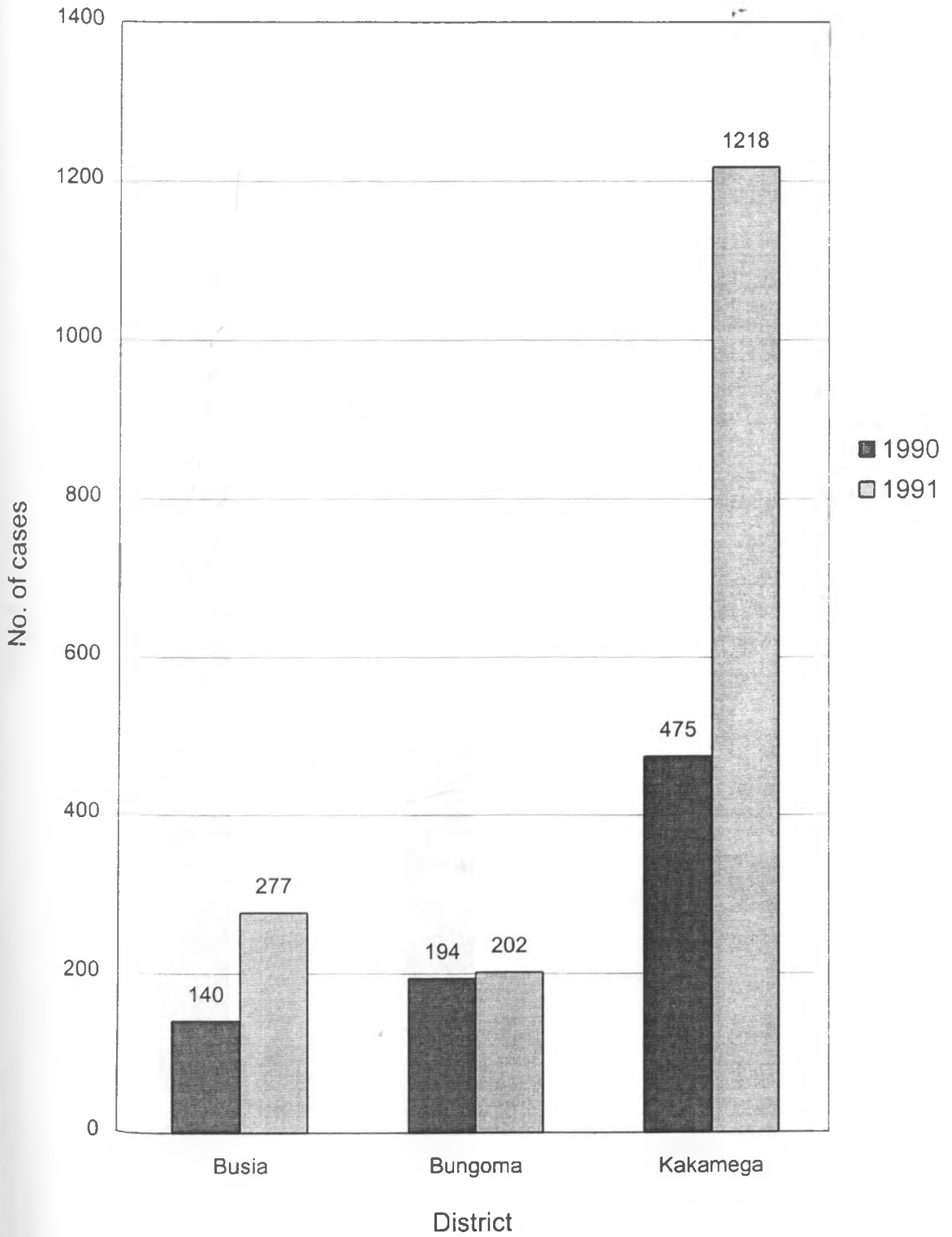
Figure 7: Poisoning cases reported in North Eastern Province in 1990 and 1991.
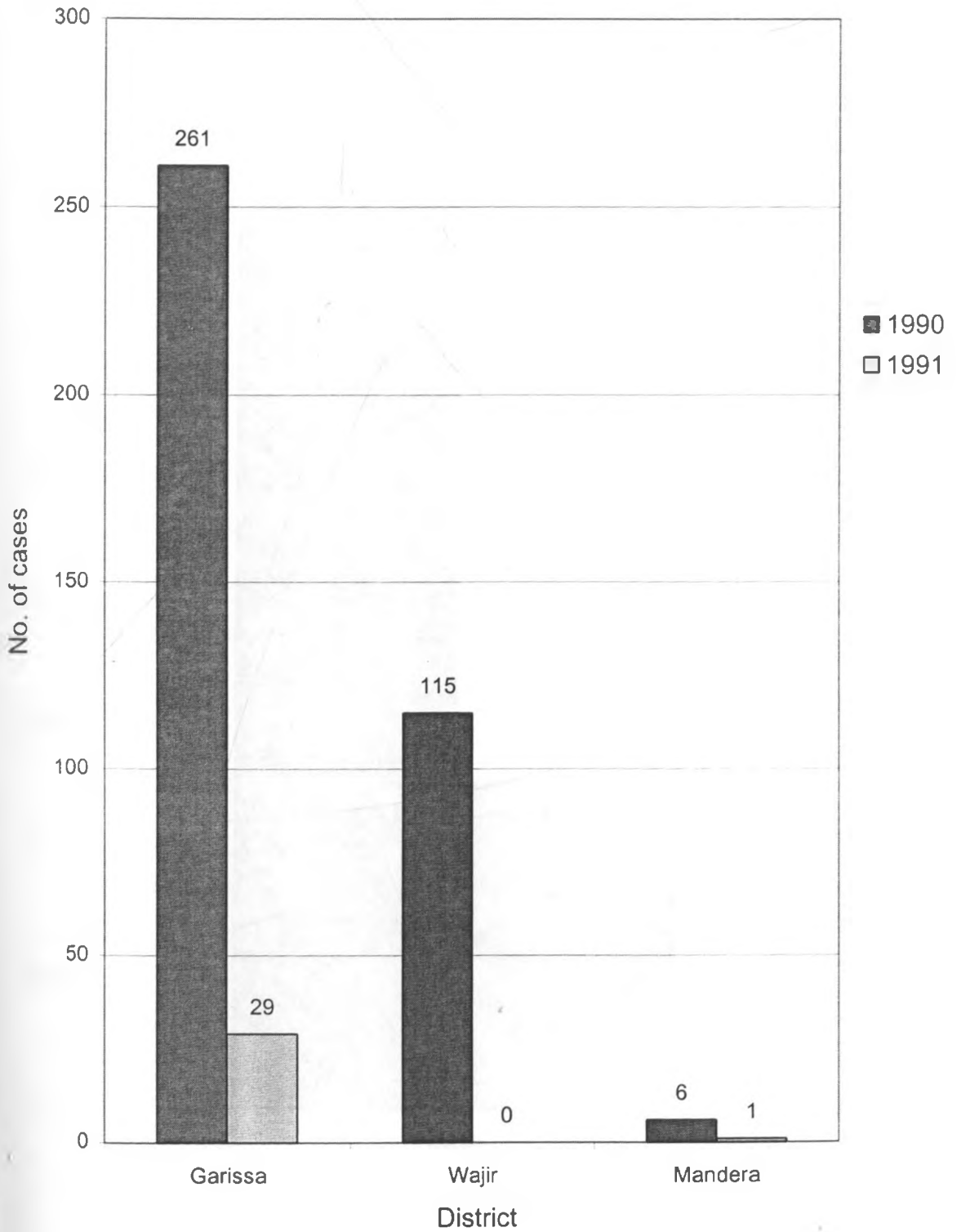
Figure 8: Poisoning cases reported in Nairobi in 1990 and 1991.
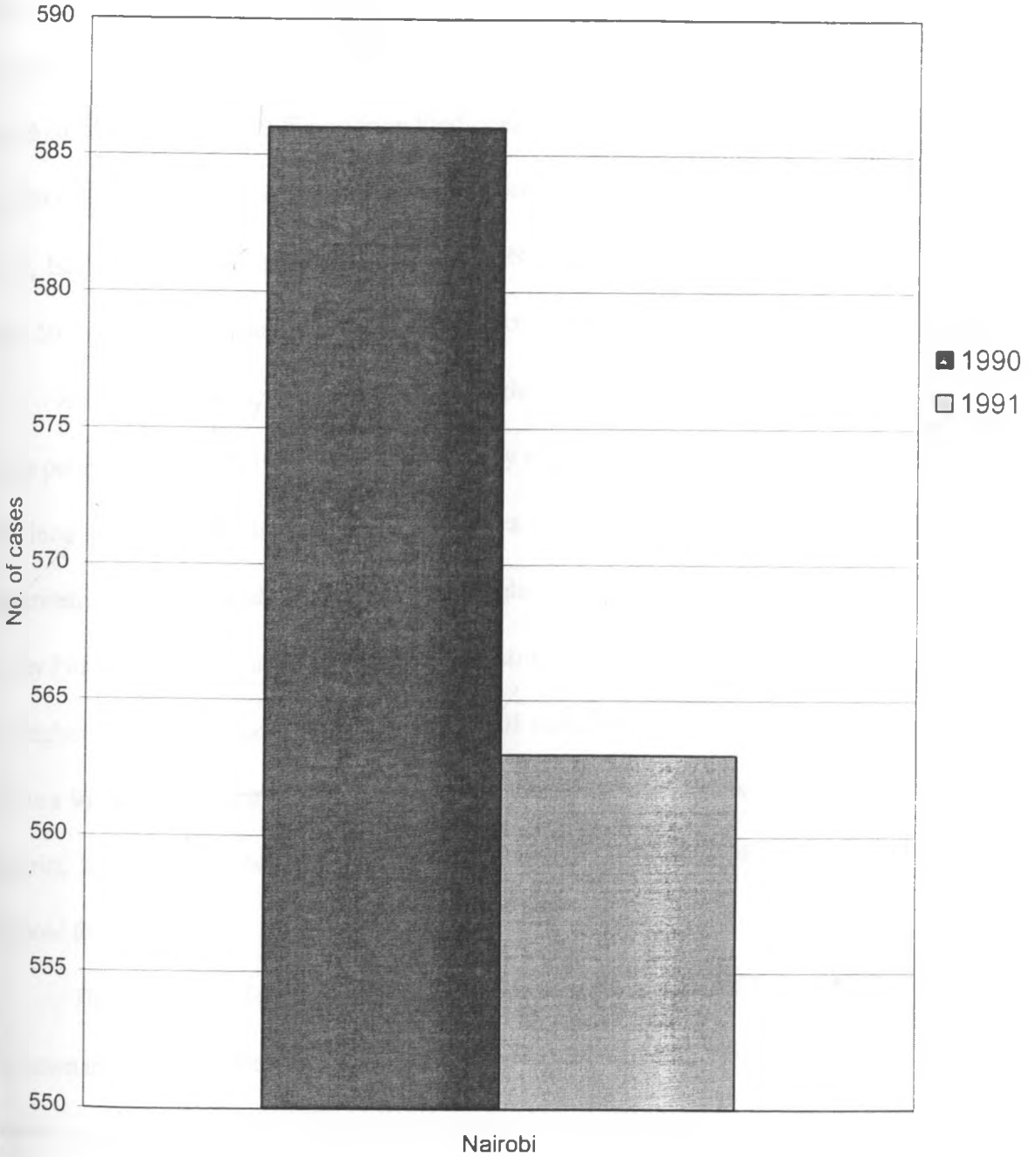
4.1.1 Bacterial foodborne infections

Most of foodborne disease outbreaks with confirmed diagnosis in this study were due to *Staphylococcus aureus* (5 outbreaks, 883 people*)*. A number of other foodborne bacteria including *E. coli, Clostridium perfringens* and *Clostridium botulinum* were diagnosed from individuals suspected to be suffering from food poisoning illnesses. For example, between 1984 and 1993, 420, 181, 29 and 41 cases of foodborne bacterial pathogens were diagnosed in Embu, Meru, Isiolo, and Machakos districts repectively. Nyeri district diagnosed 24 individual cases, while 50 cases were diagnosed in Nyandarua district.

As shown in table 6, Nyanza province led in the number of cases of typhoid fever reported in the period 1984 to 1993 followed by Rift Valley and Central Provinces respectively. Nyanza Province also led in the number of cholera cases followed by Coast and Eastern Provinces repectively. Dysentery/shigellosis generally had a higher incidence than cholera in Central and Rift Valley Provinces. The prevalence of typhoid fever showed an increasing trend over the years, with the highest number of cases being reported in 1991 from Nyanza, and in 1993 from Rift Valley (Figure 9). The incidence of typhoid fever was nearly constant throughout 1991 in Kisumu District. Salmonellosis showed the same trend although the prevalence was low compared to typhoid fever (figure 10).

The prevalence of dysentery/shigellosis in the period between 1984 and 1993 by province is shown in figure 11. The bulk of the cases were reported between 1984 and 1986. The highest number of cases of dysentery/shigellosis in the period between 1984 and 1993 were reported from

Central Province (33,189) with majority of the cases reported from Kiambu (14,904), Nyeri (9983) and Nyandarua (5,757) districts. Central Province was followed by Nyanza Province with 27,192 cases, majority of which reported from Kisumu (18,681) and Siaya (6976) distrcts. The prevalence of dysentery/shigellosis in the period between 1984 and 1993 by districts is shown in figure 12 (a-g). In Kiambu district, the bulk (65.1 %) of the cases were reported in 1985 and the rest in 1992 (10.6 %) and in 1993 (24.3 %) with no cases reported in other years. This indicates an outbreak of the disease in those years. However, Nyeri reported cases of dysentery/shigellosis throughout the period 1984 to 1990 with a peak in 1990. Nyandarua reported the most (87 %) of cases in 1984 with few cases in subsequent years. Ninety seven percent of the cases reported from Coast Province occurred in Mombasa District and only in 1984 and 1985. In Western Province dysentery/shigellosis was reported from all the three districts, and mainly between 1984 and 1988. Few cases were reported in 1992 and 1993 and mainly in Busia District. Ninety three percent of the cases of dysentery/shigellosis recorded in Eastern Province were reported in Marsabit District between 1984 and 1986.

Cholera showed a decreasing trend with most of the cases being reported between 1884 and 1986 (figure 13). Fifty one percent of the total cases were reported in Nyanza province, 95 % of them between 1984 and 1987. Seventy five percent of the cases reported in Nyanza province occurred in Kisumu District, 17 % in South Nyanza and 7.4 % in Siaya Diatrict (Figure 14). Outbreaks occurred in the months of February to July with peak cases being reported in April (Figure 15).

Table 6: Number of cases of typhoid fever, cholera and dysentery/shigellosis reported

in various provinces of Kenya between 1984 and 1993.

Province	Typhoid	Cholera	Dysentery/ Shigellosis
Nyanza	7,317 (85)	4,086 (124)	27,192
Rift Valley	4,708 (2)	78	7,277
Central	1683	243	33,189
Western	599 (21)	295 (7)	6,226
Coast	287	2110	3,059
Eastern	347	1190 (10)	2,391
North Eastern	1	71	1,631
Total	14,942 (108)	8,073 (141)	80,965

Note: Figures in parentesses indicate number of deaths due to the disease.

Figure 9: Prevalence of typhoid fever in the period 1984-1993 by province
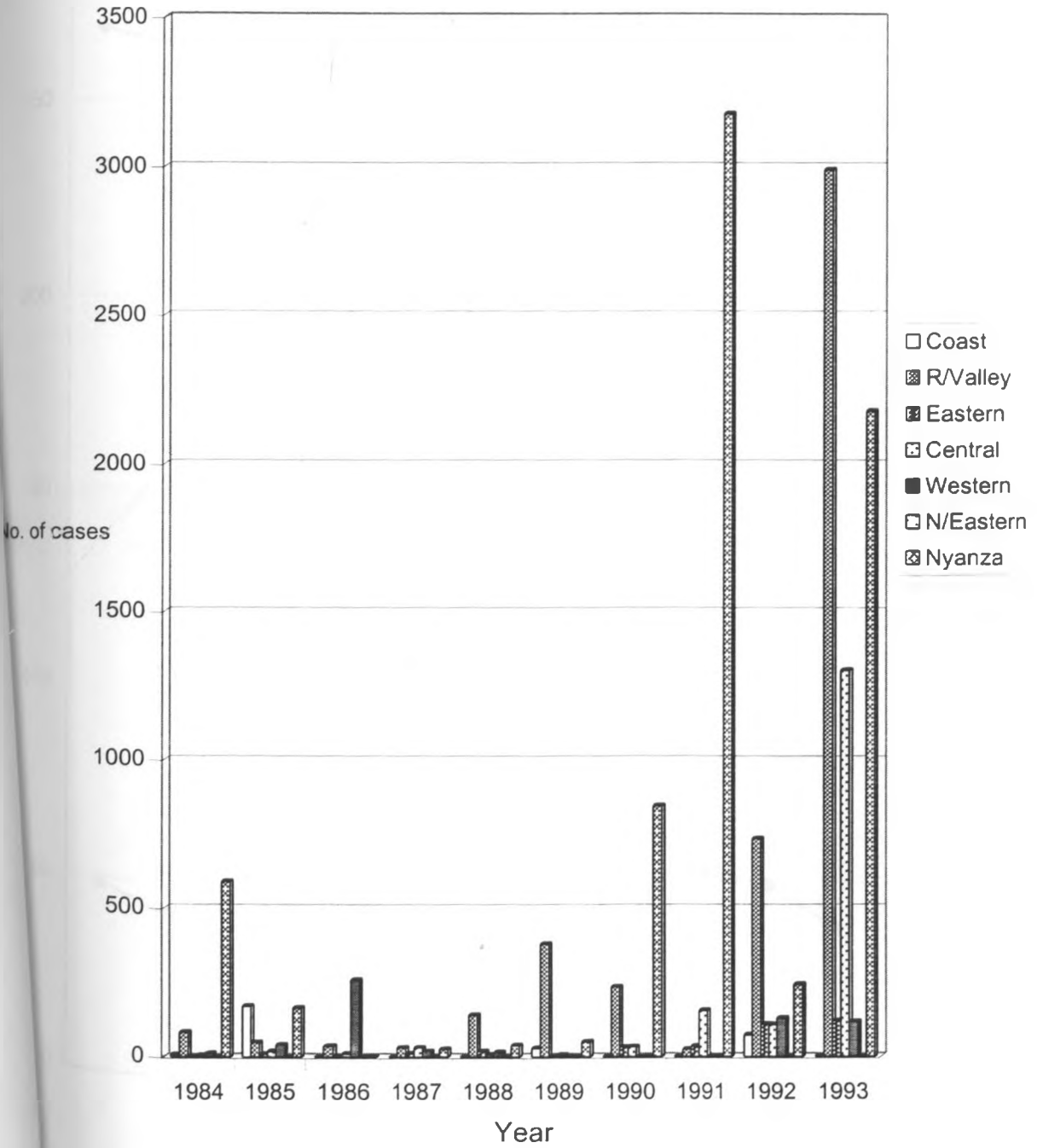
Figure 10: Incidence of typhoid fever and salmonellosis in Kisumu District in 1991.
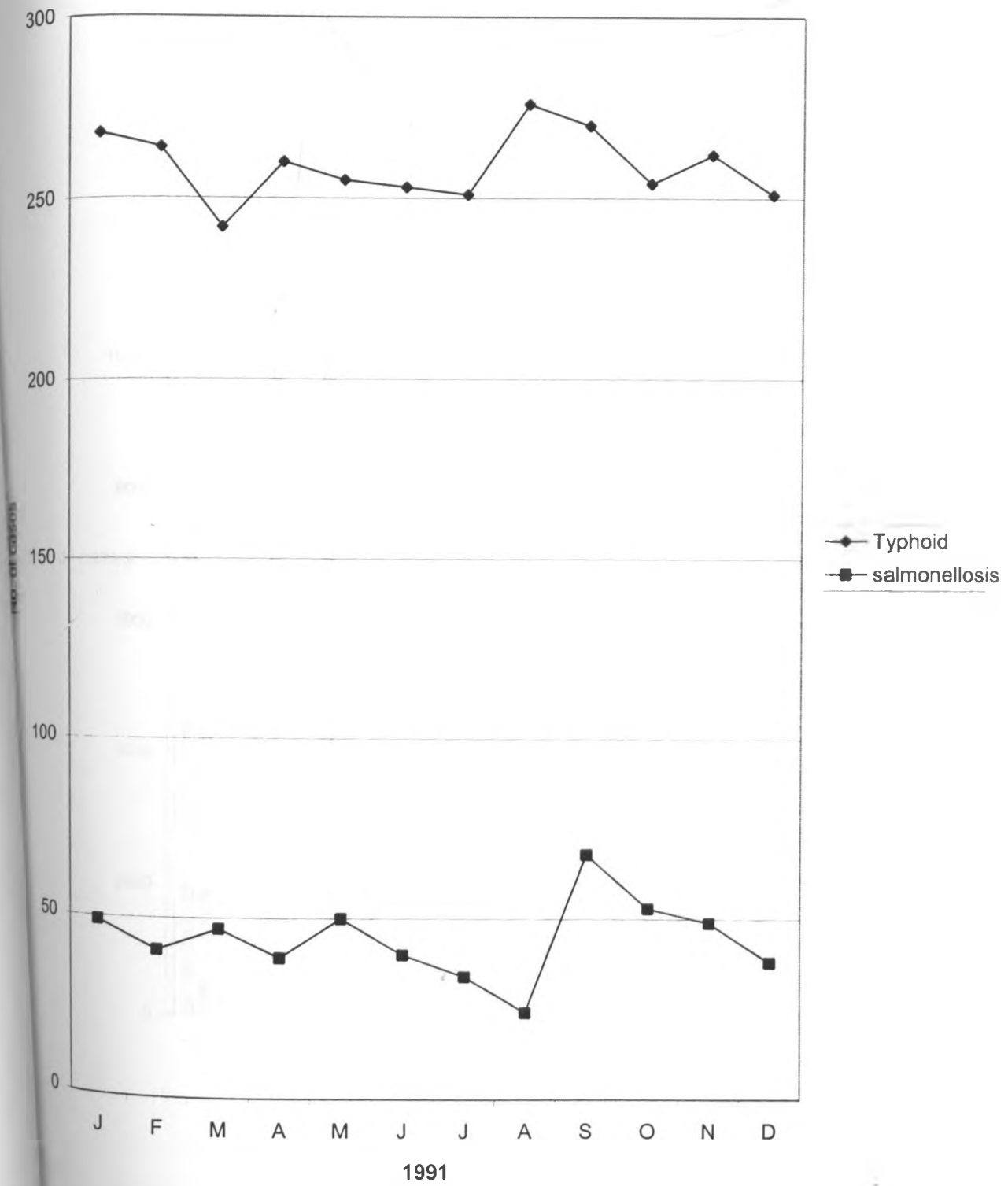
Figure 11: Prevalence of dysentery/shigellosis in the period 1984-1993 by province
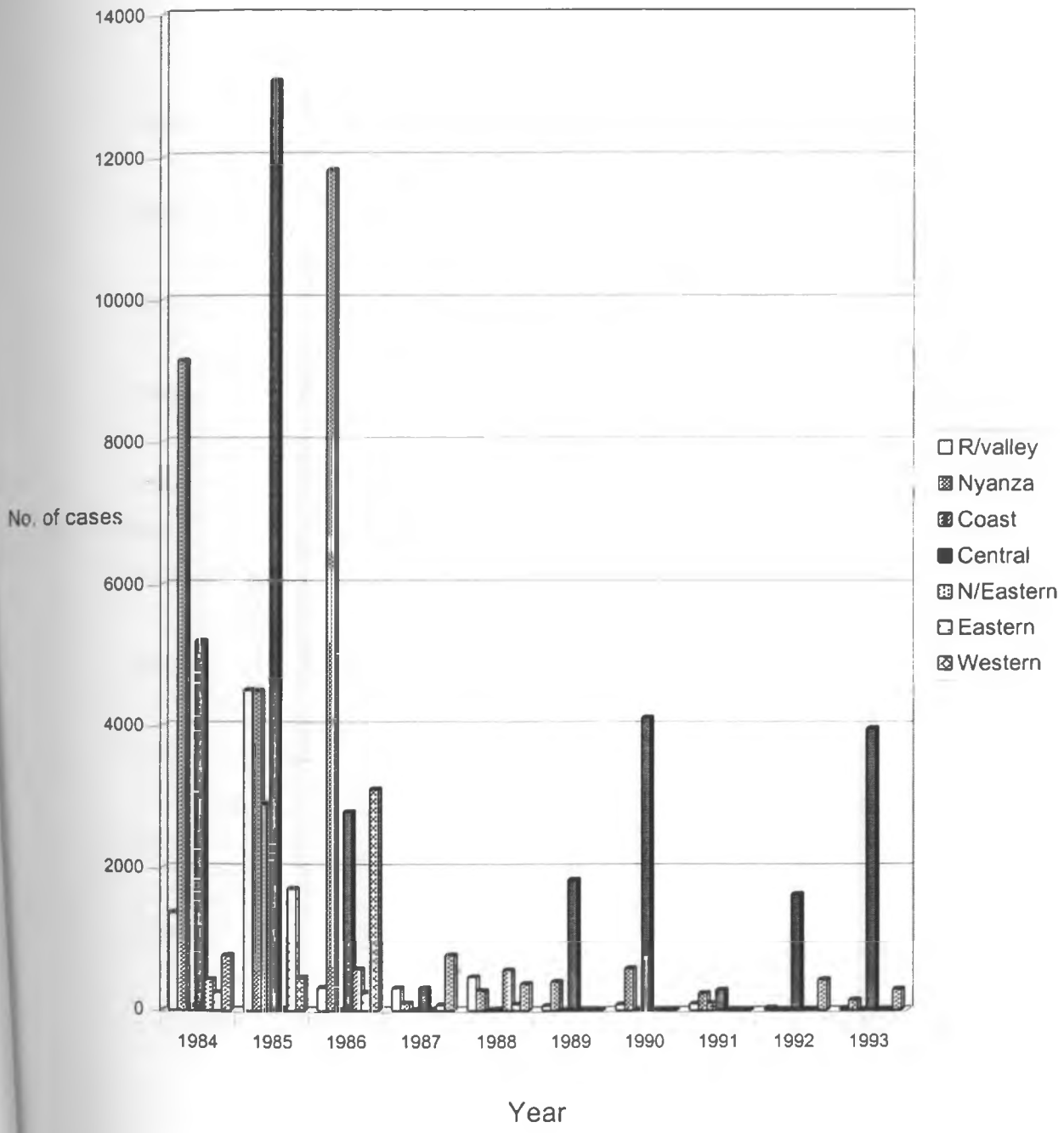
Figure 12: Prevalence of dysentery/shigellosis by Province in the period 1984 to 1993

Figure 12 a: Central Province
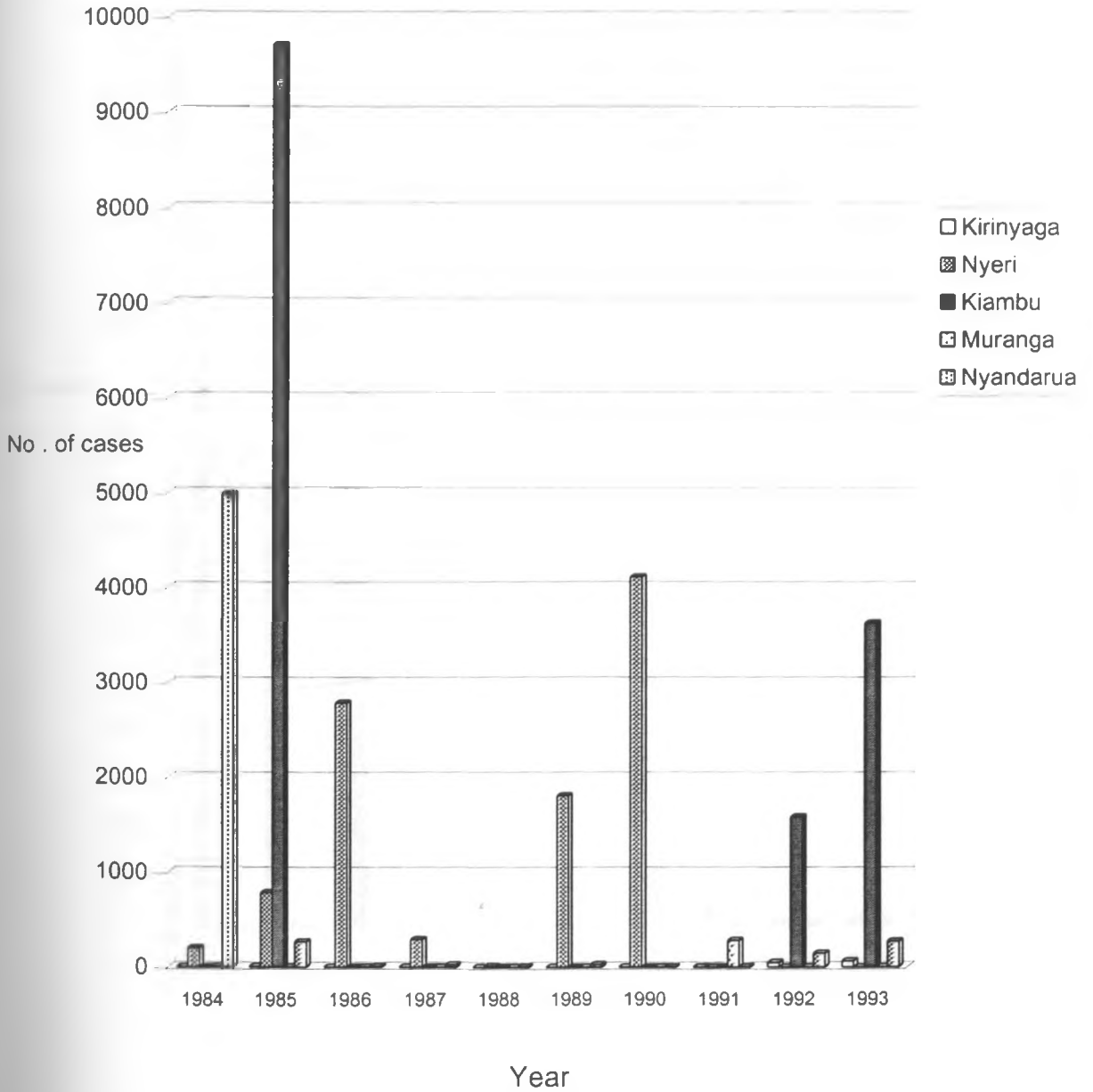
Figure 12b: Nyanza Province

Legend:
- Kisumu
- Siaya
- S/Nyanza
- Kisii

Y-axis: No. of cases (0 to 10000)
X-axis: Year (1984–1993)
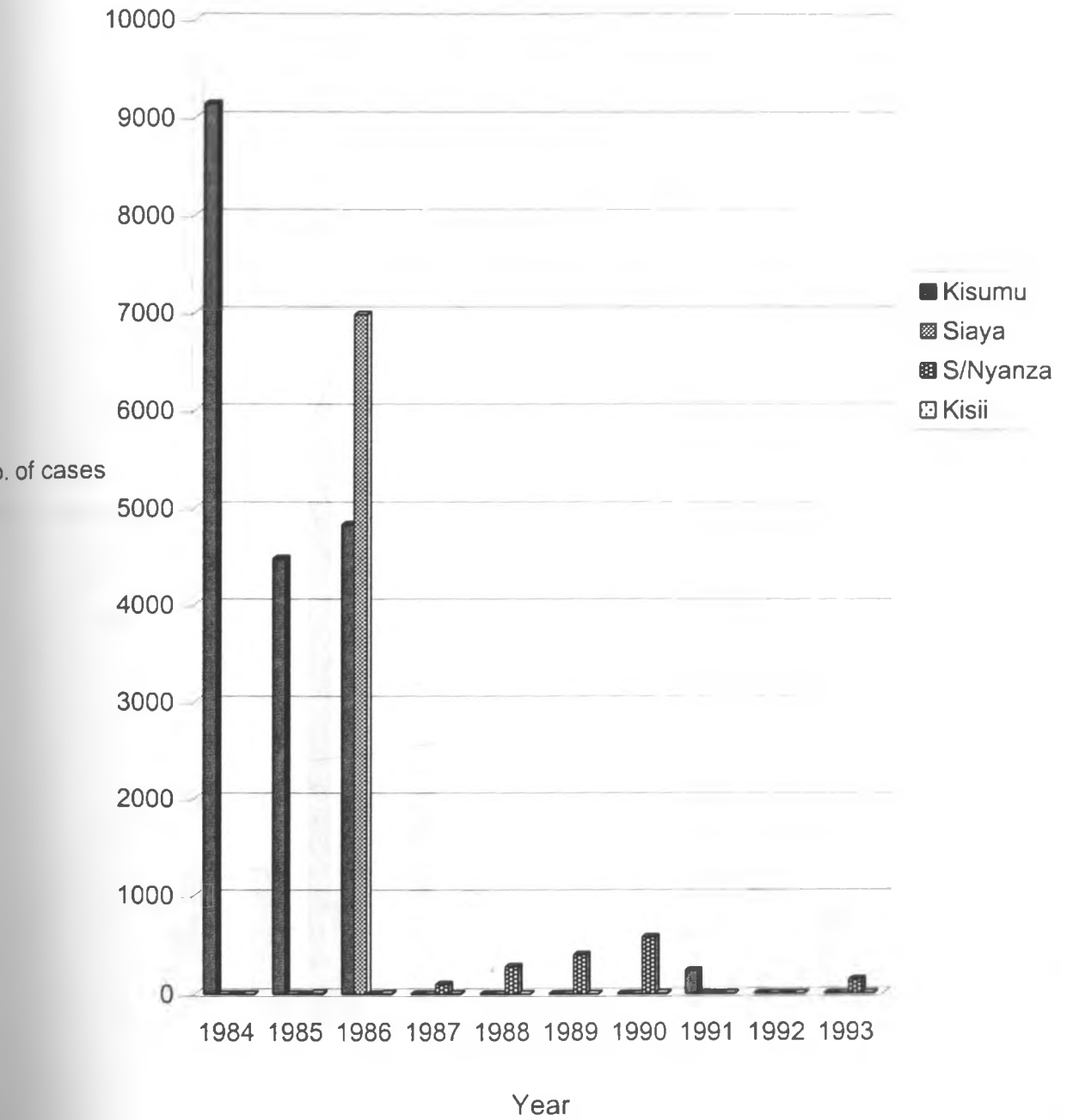
Figure 12c: Western Province
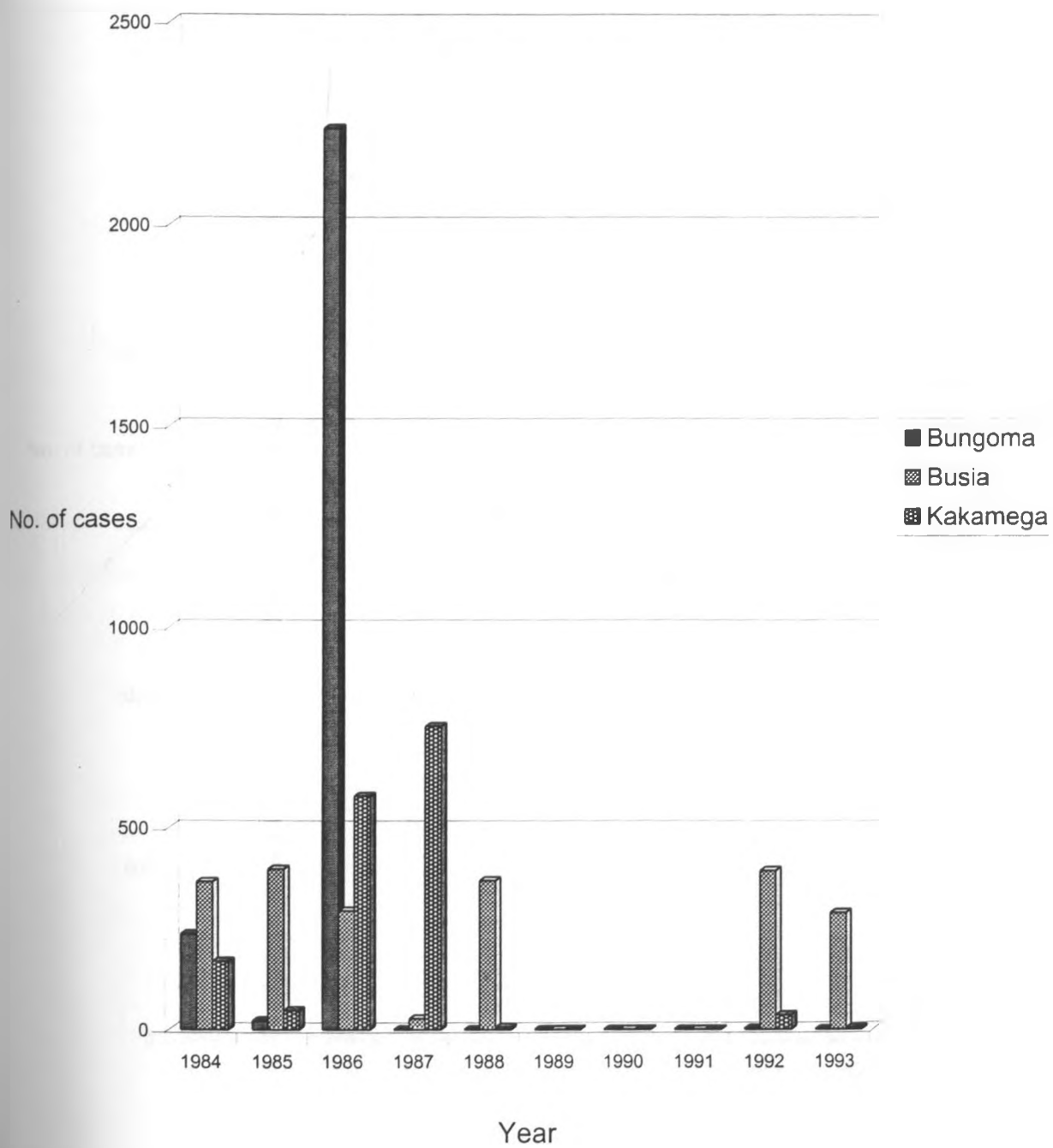
Figure 12d: Coast Province

Legend:
- ☐ Kwale
- ▨ Mombasa
- ▨ Kilifi
- ▣ Lamu
- ▨ T/River

Y-axis: No. of cases (0, 500, 1000, 1500, 2000, 2500, 3000)

X-axis: Year (1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993)

Figure 12e: Rift Valley Province

Legend:
- ▢ Laikipia
- ▨ W/Pokot
- ▨ T/Nzoia
- ▢ Nakuru
- ▥ E/Marak
- ▢ Nandi
- ▨ Kericho
- ▨ Samburu
- ▨ Turkana
- ▨ Narok
- ▢ Kajiado
- ▨ Baringo
- ▤ U/Gishu

No. of cases

Year

Figure 12e: Rift Valley Province

Legend:
- ☐ Laikipia
- ▦ W/Pokot
- ▨ T/Nzoia
- ☒ Nakuru
- ⊞ E/Marak
- ☐ Nandi
- ⊠ Kericho
- ▣ Samburu
- ⊠ Turkana
- ▨ Narok
- ☐ Kajiado
- ▧ Baringo
- ☰ U/Gishu

No. of cases (y-axis)

Year (x-axis)
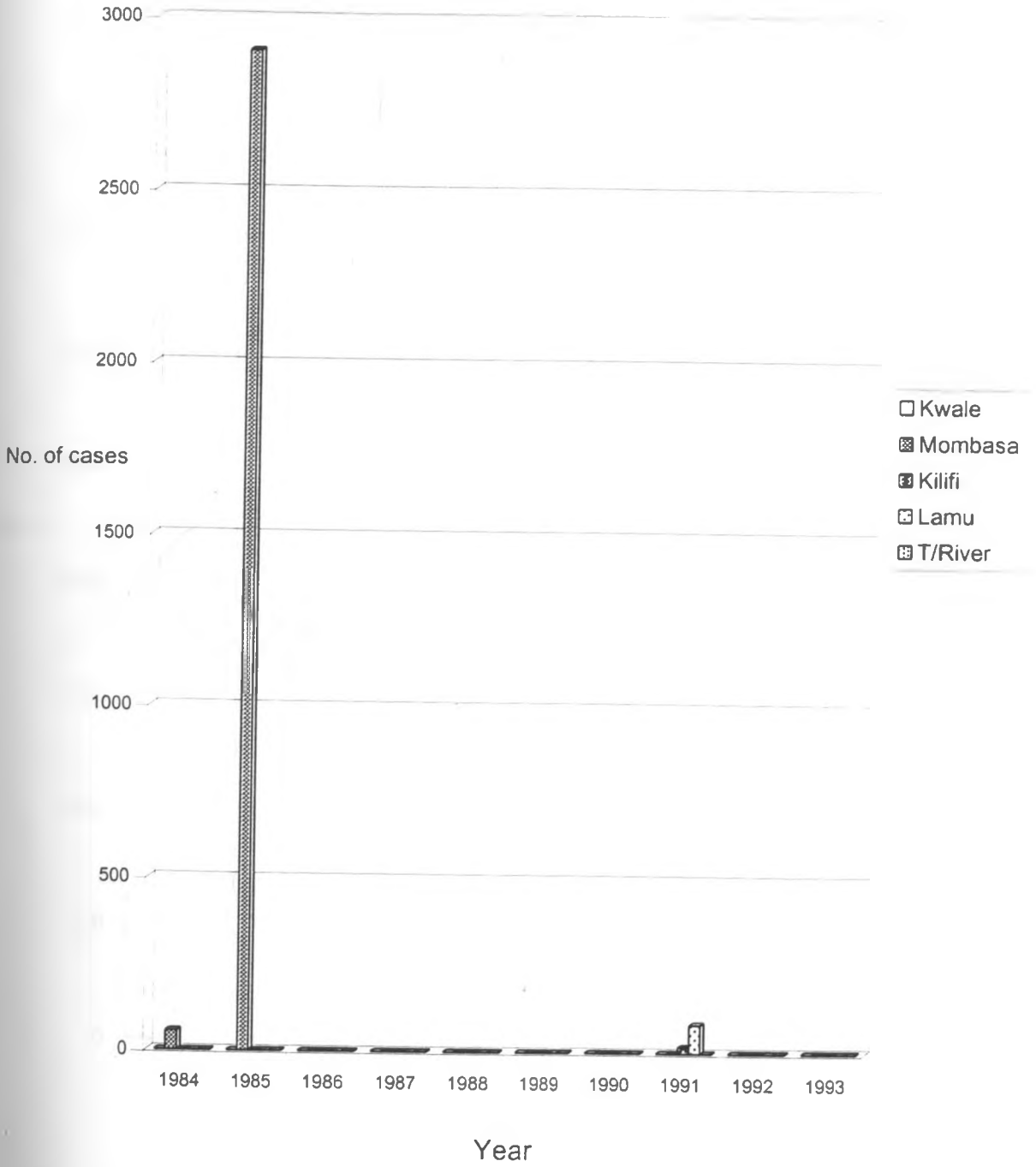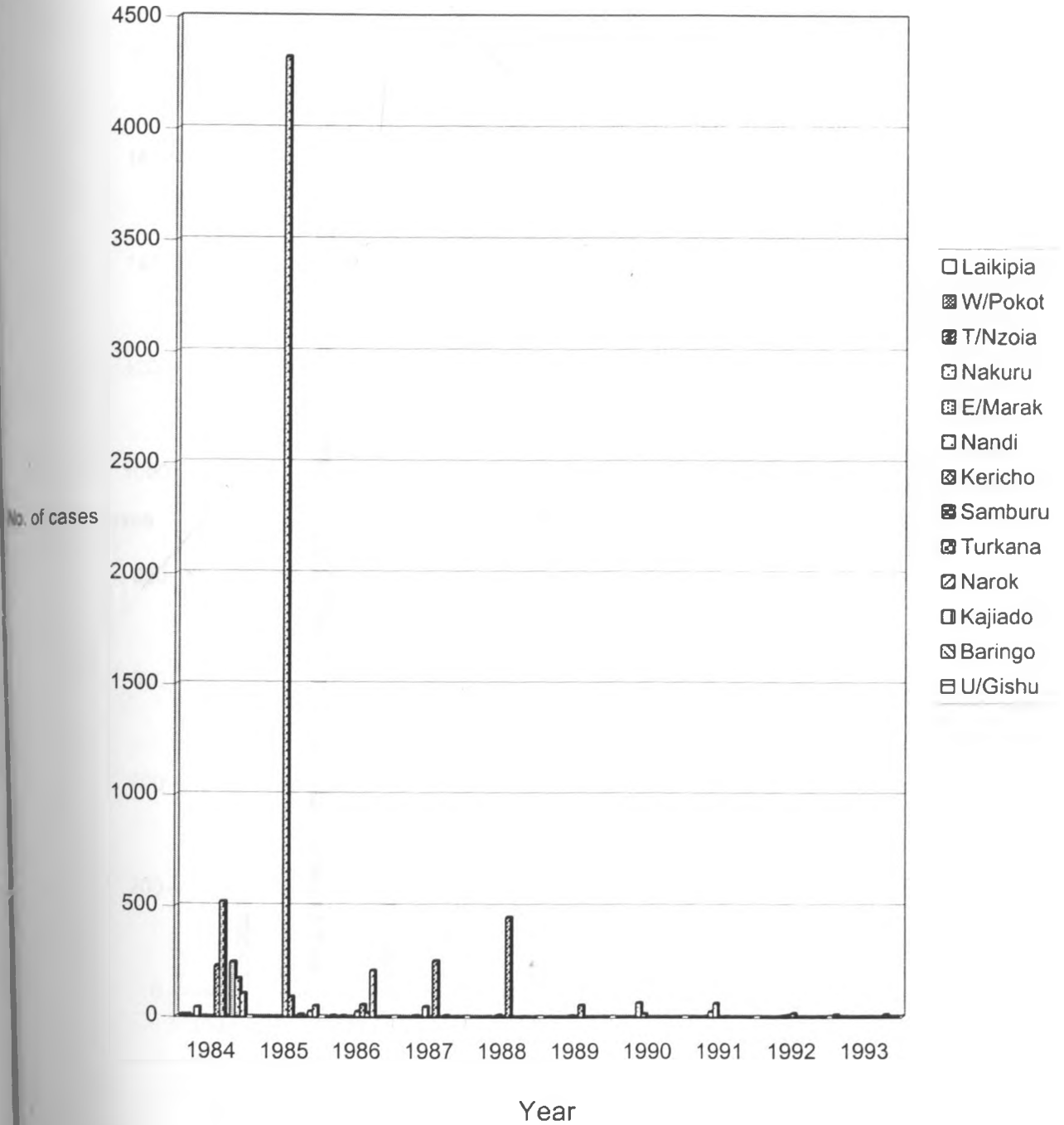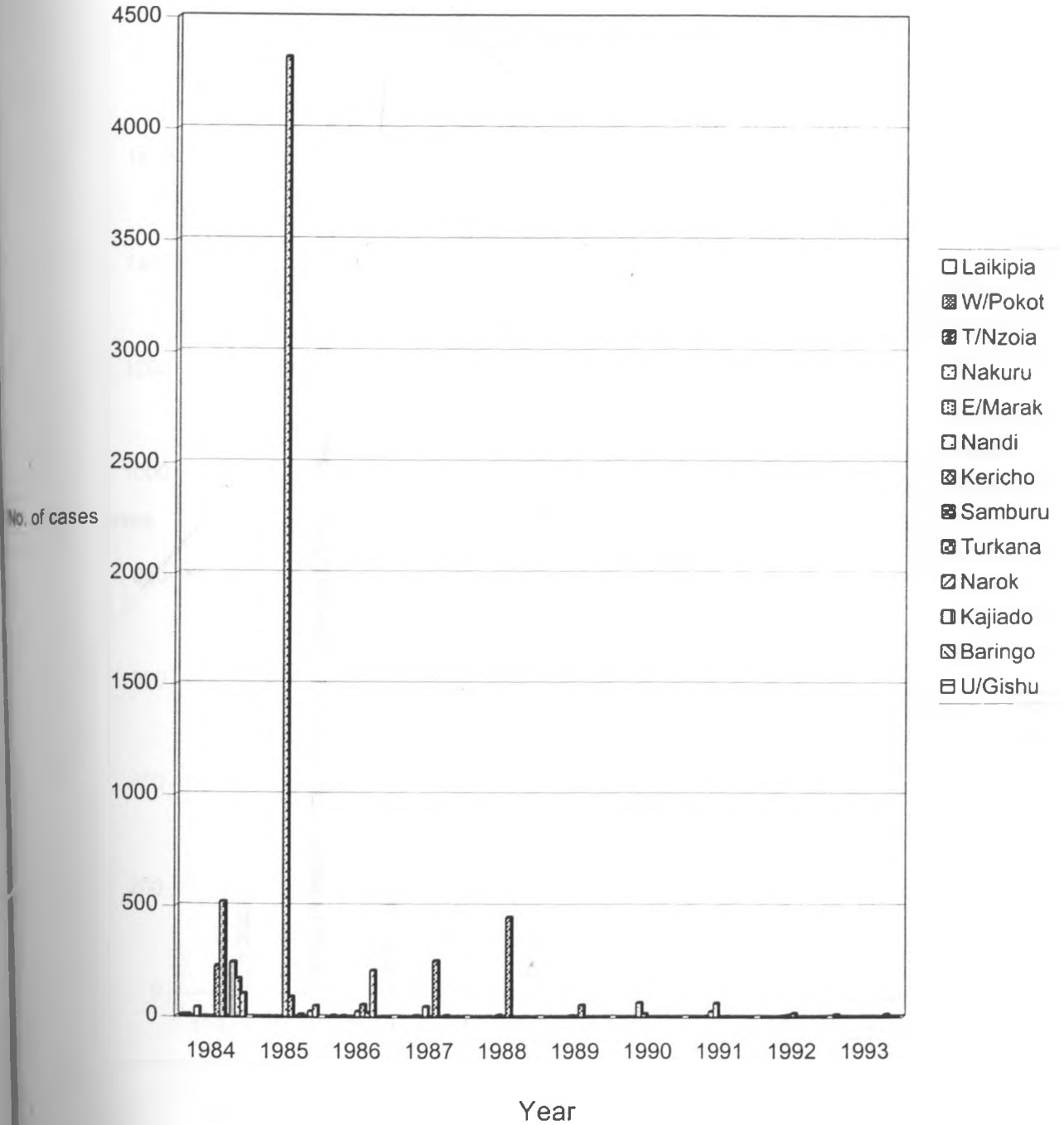
Figure 12f: Eastern Province
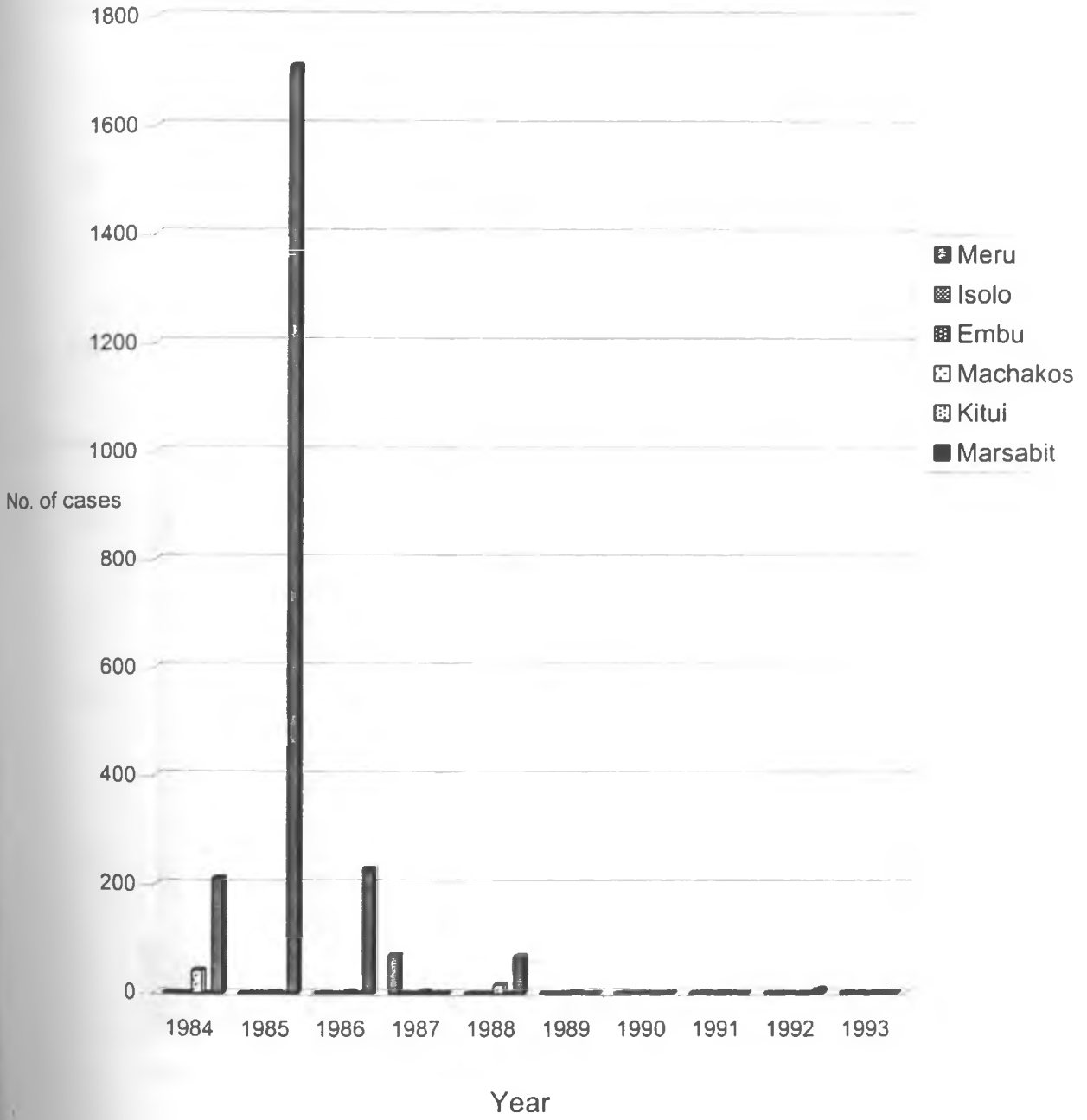
Figure 12g: North Eastern Province
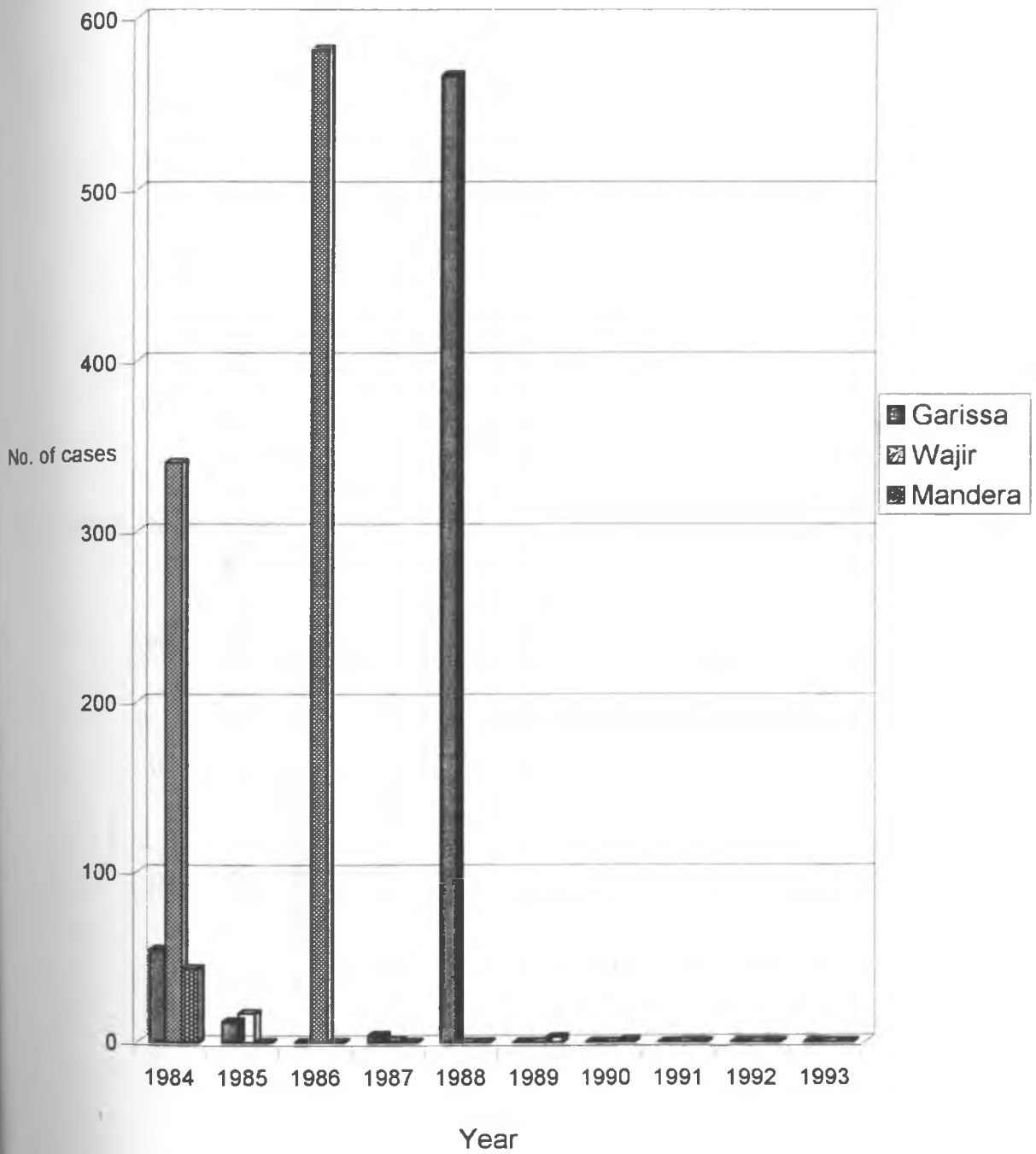
Figure 13: Prevalence of cholera in the period 1984 to 1993 by province.
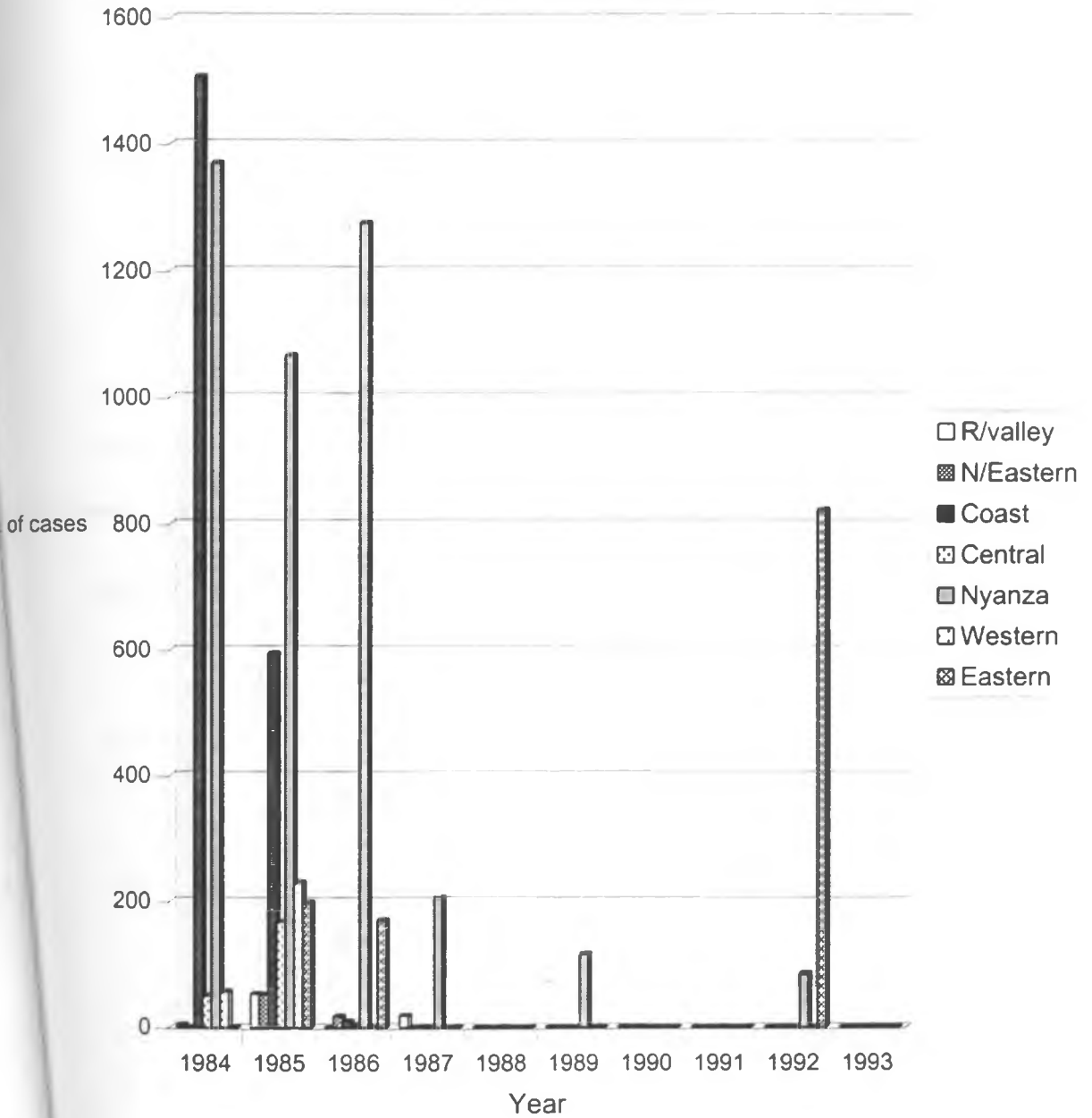
Figure 14: Prevalence of Cholera in Nyanza province in the period 1984 to 1993 by district
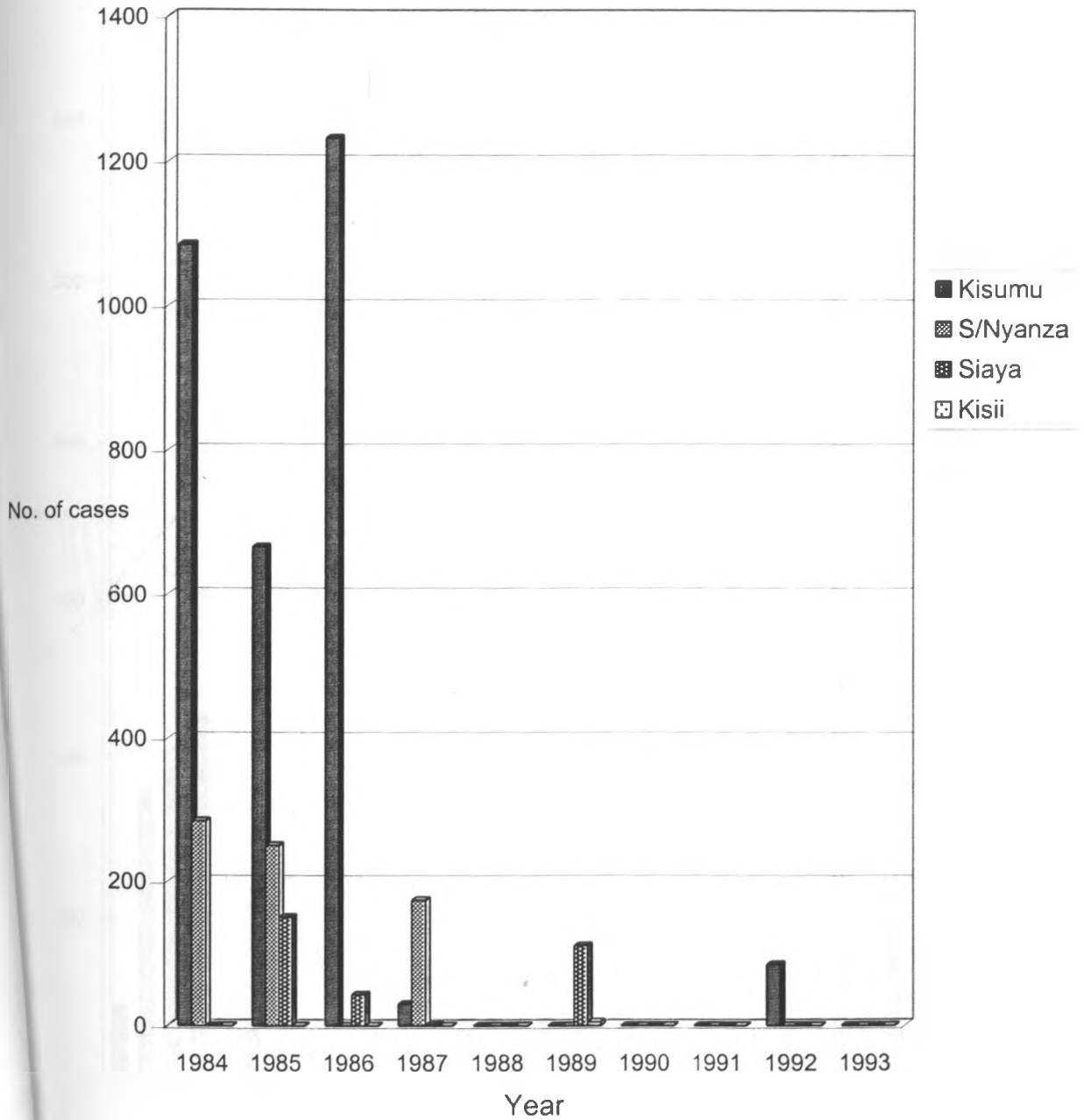
Figure 14: Prevalence of Cholera in Nyanza province in the period 1984 to 1993 by district
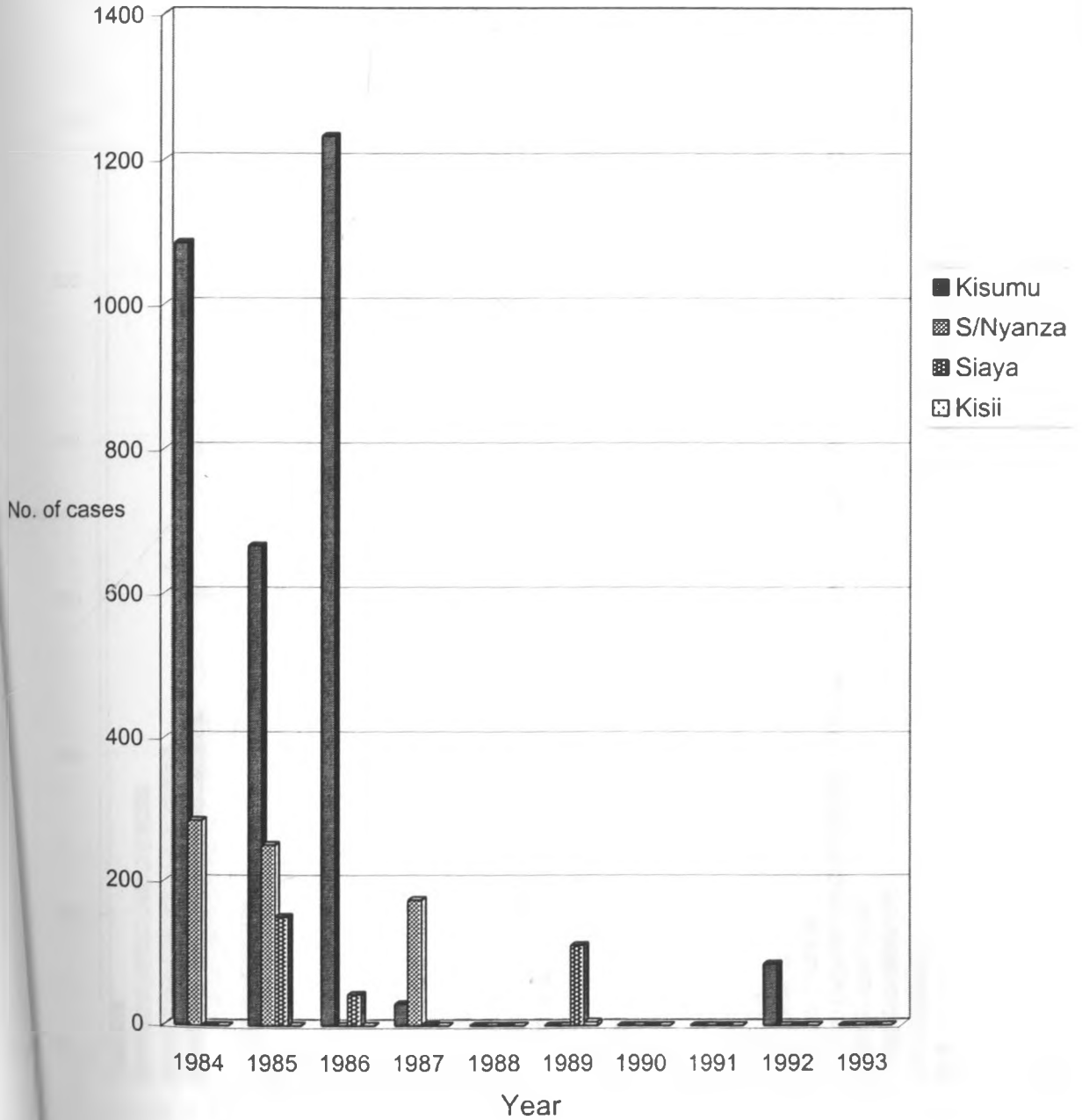
Figure 15: Prevalence of cholera in Kisumu District between 1984 and 1986

No. of cases

Month(1984-1986)
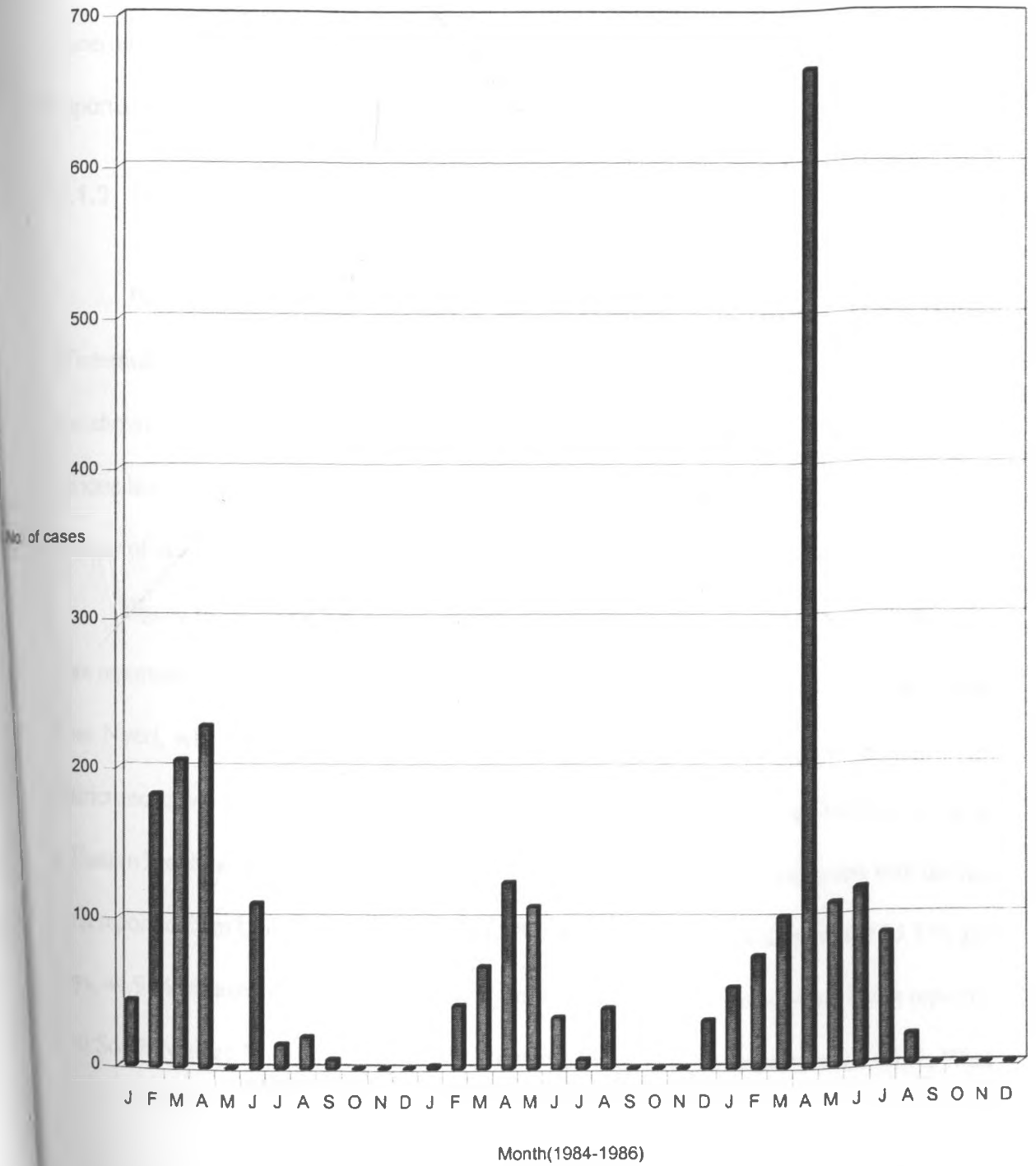
4.1.2 Viral foodborne infections

Infectious hepatitis A and poliomyelitis are viral infections that sometimes are transmitted through food. The prevalence of foodborne hepatitis A and poliomyelitis infection is difficult to ascertain, but records from the Ministry of Health indicate that cases of these diseases were reported from around the country in the period 1988 to 1991 (Table 7).

4.1.3 Foodborne parasitic infections

Parasitic infections may sometimes be transmitted through food. The total number of cases of intestinal worms diagnosed in various health facilities in the country in the period 1988 to 1991 are shown in table 7. Table 8 shows the number of specific groups of parasites diagnosed in various health facilities in 1989 and 1990. It was not possible to ascertain from these figures the number of cases that were foodborne.

Figure 16 shows the prevalence of taenia infection in various provinces. Most of the cases were reported between 1984 and 1990. Over 70 % of the cases reported in Central Province were from Nyeri, while Nyandarua reported 24.2 % of all the cases in Central province. Kakamega District accounted for 79.6 % and Bungoma 20.4 % of the cases reported from Western Province. In Eastern Province, Marsabit District accounted for 94.6 % of all the cases reported with the rest being reported from Isiolo (4.2 %) and Meru (1.2 %) Districts. Kisumu accounted for 53.3 % and Siaya, 44.9 % of taeniasis cases reported in Nyanza province, with the remaining being reported from South Nyanza District. In Rift Valley Province, Narok District accounted for 66 %, West

Pokot 13.6 % and Samburu 11.0 % of the cases reported. Kericho and Kajiado Districts reported

5.0 and 2.6 % of the cases respectively. No cases were reported from the rest of the districts.

Table 7: Annual outpatient morbidity statistics for some diseases between 1988-1991.

Disease	1988	1989	1990	1991
Diarrhoeal diseases	Na	888,694	778,823	710,058
Intestinal worms	788,455	997,866	792,630	713,045
Infectious hepatitis	23,034	8,705	25,147	41,501
Poliomyelitis	1,668	1,559	1,752	2,441

Na - figures were not available.

Table 8: The number of various groups of parasites diagnosed at various health facilities in Kenya in 1989 and 1990.

Parasites	1989	1990
Tapeworms	5,100	2,256
Roundworms	18,667	18,274
Hookworms	15,262	14,825
Amoeba	43,023	37,086
Other parasites	8,941	7,536

Figure 16: Number of cases of taeniasis reported in the period 1984-1993
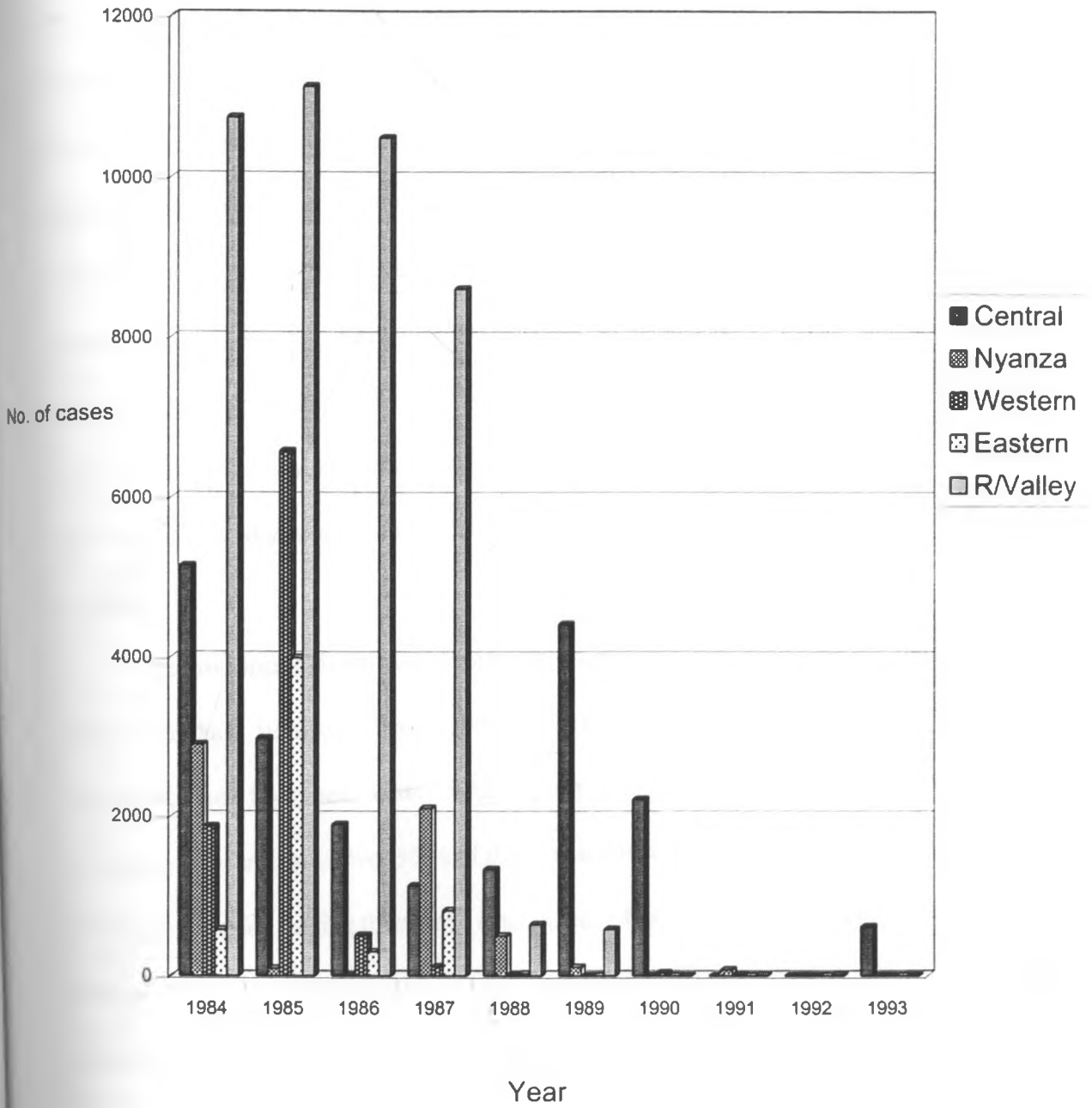
4.1.4　Routine analysis of food samples

A total of 2141 food samples were analysed for total viable bacterial counts, coliform counts, *Escherichia coli, Staphylococcus aureus, Clostridium perfringens, salmonella spp., shigella spp,* and aerobic bacilli by the National public health Laboratories and Nairobi city commission, public health department, between 1986 and 1993, as routine quality control of foodstuffs. Forty two percent (900/2141 samples) were free of coliforms and other pathogens and were therefore found to be of satisfactory quality. Coliforms and *E. coli* were found to be the most common (upto 80 %) bacteria contaminating food products. Other isolates included *staphylococcus aureus* and *streptococcus spp. Bacillus spp.* yeasts and moulds were detected in only one sample. A variety of foodstuffs from factories, shops and restaurants, which included milk and milk products, meat and meat products, juices, sauce salads, bread, water etc. were analysed.

Aflatoxin analysis is routinely done by the government chemist for quality control of grains and grain products. Between the years 1989 and 1993, 528 grain samples including maize, beans, rice, wimbi, maize flour, peas, barley, wheat, green grams, groundnuts etc, from various parts of the country were analysed. Over 30 % of the samples were found to contain aflatoxins B_1 and B_2 in various quantities ranging from trace amounts to 200 ppb. Of the 161 samples with aflatoxins detected, 82 % contained aflatoxin B_1, 12.5 % contained aflatoxin B_2 while 5.5 % contained both aflatoxin B_1 and B_2 in various amounts.

4.2 ISOLATION OF *BACILLUS CEREUS* FROM MILK

Bacillus cereus was isolated from 37% and 41.2 % of raw and pasteurized milk samples respectively. A total of 60 isolates (27 from raw milk and 33 from pasteurised milk) were recovered from the 153 samples analysed.

4.3 BIOCHEMICAL TESTS FOR CONFIRMATION OF B. CEREUS ISOLATES

4.3.1 Carbohydrate fermentation.

All *Bacillus cereus* isolates fermented glucose, maltose, trehalose and salicin with production of acid, but did not ferment mannitol, arabinose, inulin, sorbitol, raffinose, lactose, xylose and rhamnose. Sucrose was fermented by only 70 % of the isolates with production of acid. *Bacillus cereus* prototype strain NCTC 11145 produced acid from sucrose, while the prototype emetic strain NCTC 11143 did not. However, some enterotoxin positive strains produced acid from sucrose as were enterotoxin negative strains.

4.3.2 IMViC reaction.

All *B. cereus* isolates, including both the diarrhoeal strain NCTC 11145 and emetic strain NCTC 11143, were negative for acetyl methyl carbinol (VP) and indole production. Methyl red and citrate were positive for all the strains. Results of the biochemical reactions are shown in appendix 2.

4.3.3   Starch hydrolysis

About 65 % of 58 strains tested including the prototype strain NCTC 11145 hydrolysed starch, while the emetic strain NCTC 11143 did not.

4.4   PRODUCTION OF DIARRHOEAL ENTEROTOXIN

Fifty percent of *58 B. cereus* strains isolated from milk produced enterotoxin in broth culture as detected by reverse passive latex agglutination technique. Seventy three percent of the 29 enterotoxin positive strains were from raw milk, while 27 % were from pasteurised milk. Results of enterotoxin production are shown in appendix 3. No relationship was apparent between sucrose fermentation and production or non-production of enterotoxins by *B. cereus*, as fermentation could be seen in both enterotoxin positive and enterotoxin negative strains. At the same time, lack of sucrose fermentation was observed in both cases. A significantly higher proportion (96 %; p=0.000) of enterotoxin positive isolates (29) hydrolysed starch compared to 36 % of enterotoxin negative (29) isolates. Hydrolysis of starch was strongly associated (Odds ratio=53.2) with enterotoxin production (table 9).

Table 9: Correlation of starch hydrolysis and enterotoxin production by 58 *B. cereus* strains.

		Enterotoxin production (n = 58)	
		+	-
Starch hydrolysis	+	28 (48.3 %)	10 (17.9 %)
	-	1 (1.7 %)	19 (33.9 %)

Chi-square test value = 24.73.

P - value = 0.000

Odds Ratio (OR) = 53.2

4.5   RESISTANCE TO ANTIBIOTICS

Table 10a shows the frequency of antimicrobial resistance among the 60 *B. cereus* isolated from raw and pasteurised milk. All the isolates were resistant to at least one or more antimicrobial agents including ampicillin, while all were susceptible to streptomycin. Over eighty one percent (81.5 %) of isolates from raw milk and 100 % from pasteurised milk showed multiple (2 or more drugs) resistance. Table 10b shows the number and percentage of isolates resistant to a specific number of drugs. *Bacillus cereus* strains NCTC 11145 and NCTC 11143 showed similar antibiotic resistance patterns with both strains being resistant to ampicillin, cotrimoxazole amd sulphamethoxazole and sensitive to all the other antibiotics used. However, isolates that produced diarrhoeal enterotoxin positive had different antibiotic resistance patterns (appendix 3). Ninety one percent of the isolates from pasteurized milk were resistant to both cotrimoxazole and sulphamethoxazole. However, 70 % and 37 % of the isolates from raw milk were resistant to cotrimoxazole and sulphamethoxazole respectively. The difference was attributed to the high frequency of use of cotrimoxazole compared to sulphamethoxazole and a possibility that cotrimoxazole had a different sulphonamide other than sulphamethoxazole.

Table 10a: Frequency of antimicrobial resistance among 60 *B. cereus* milk isolates

Antimicrobial agent	Frequency of resistance (%)	
	Raw milk isolates n = 27	Pasteurised milk isolates n = 33
Ampicillin(25µg)	100	100
Tetracycline(25µg)	33	33
Cotrimoxazole(25µg)	70	91
Sulphamethoxazole(200µg)	37	91
Kanamycin(30µ)	4	3
Gentamycin(10µg)	4	0
Chloramphenicol (30µg)	4	0
Streptomycin(10µg)	0	0

Table 10b: Number (%) of 60 *B. cereus* isolates resistant to specific number of drugs

No. of resistant drugs	1	2	3	4	5
No. (%) isolates	5(8.33)	12(20)	29(48.33)	12(20)	2(3.3)

4.6   PLASMIDS IN *B. CEREUS*

Plasmids were isolated from 71.7 % of all *B. cereus* milk isolates. The diarrhoeal prototype strain NCTC 11145 had no plasmids, while the emetic strain NCTC 11143 had two plasmids of molecular weight 3.9 and 3.7 megadaltons. Figures 17a and 17b shows the plasmid profiles of some isolates. The isolates were grouped into 20 plasmid profile groups according to the molecular weights of plasmids they carried with those that had no plasmid in one profile group. The number of plasmids per isolate ranged between 1 and 5 with most of the isolates containing between 1 and 3 plasmids. Table 11 shows the plasmid profiles of 60 *B. cereus* isolates. A variety of plasmids were found with molecular weights ranging from 0.1 (by extrapolation from graph) to 60 megadaltons. The most common plasmid sizes encountered were 60 and 3.7. Results of this study indicated no apparent relationship between ability of *B. cereus* to produce enterotoxin and presence of plasmids. This is because enterotoxin production was observed in strains that contained plasmids as well as those that had no plasmids (appendix 4).
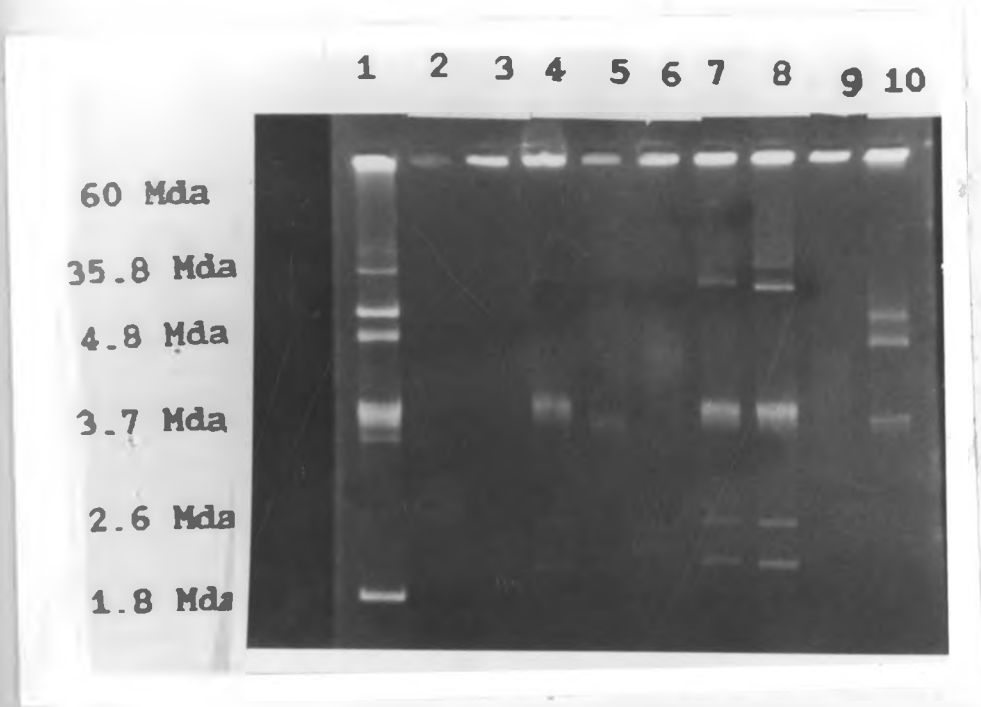
Fig. 17a: Plasmids in some *B. cereus* strains isolated from milk.

Lane 1: Molecular weight marker E.coli V517.

Lanes 2-10: *B. cereus* milk isolates
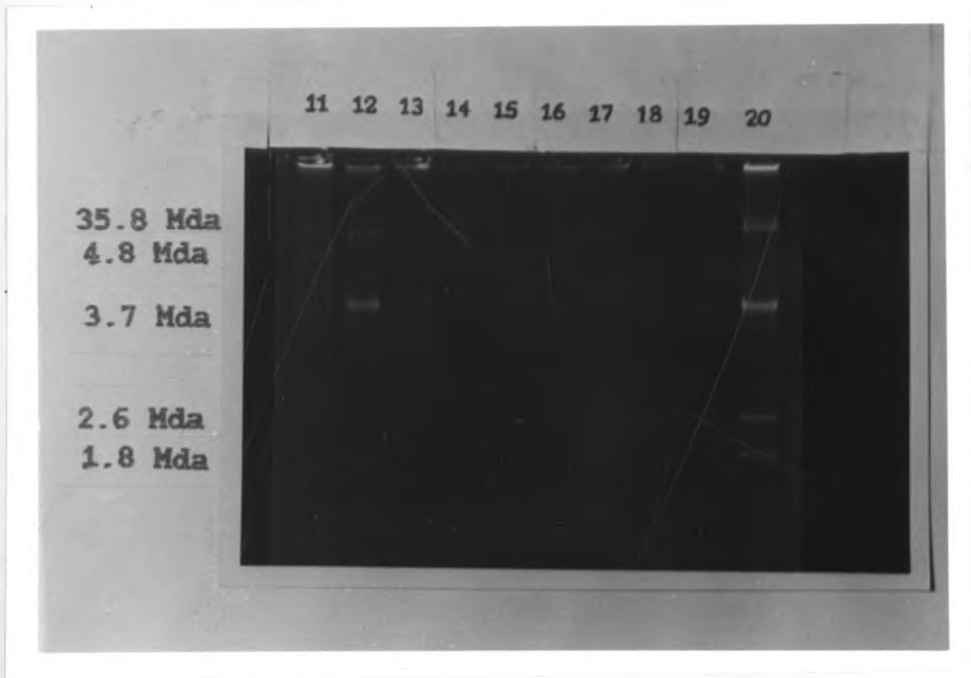
Fig. 17b: Plasmids in some *B. cereus* strains isolated from milk.

Lanes 11-19: *B. cereus* milk isolates

Lane 20: Molecular weight marker *E. coli* V517

Table 11: Plasmid profiles of 60 *B. cereus* isolates.

Plasmid profile group	No. of isolates	Molecular weights of plasmids (in MDa)
1	17	None
2	1	0.1
3	1	3.4
4	6	3.7
5	2	3.9
6	3	35.8
7	11	60
8	1	1.5, 0.1
9	1	3.7, 2.5
10	1	4.8, 3.7
11	2	3.9, 3.7
12	1	60, 35.8
13	3	35.8, 3.7
14	3	60, 3.7
15	2	60, 4.8, 3.7
16	1	60, 4.8, 3.9
17	1	35.8, 4.8, 3.7
18	1	35.8, 4.8, 3.9
19	1	35.8, 4.8, 4.5, 3.7
20	1	2.6, 3.7, 4.6, 58, 60

Table 11: Plasmid profiles of 60 *B. cereus* isolates.

Plasmid profile group	No. of isolates	Molecular weights of plasmids (in MDa)
1	17	None
2	1	0.1
3	1	3.4
4	6	3.7
5	2	3.9
6	3	35.8
7	11	60
8	1	1.5, 0.1
9	1	3.7, 2.5
10	1	4.8, 3.7
11	2	3.9, 3.7
12	1	60, 35.8
13	3	35.8, 3.7
14	3	60, 3.7
15	2	60, 4.8, 3.7
16	1	60, 4.8, 3.9
17	1	35.8, 4.8, 3.7
18	1	35.8, 4.8, 3.9
19	1	35.8, 4.8, 4.5, 3.7
20	1	2.6, 3.7, 4.6, 58, 60

4.7 RESTRICTION ENDONUCLEASE DNA ANALYSIS OF *B. CEREUS* GENOMIC DNA

Restriction enzymes including *EcoR1*, *Sau 3A*, Mbo1 and *Dra1* were used to cut genomic DNA from *B. cereus* isolates. Restriction patterns for both enterotoxin positive and enterotoxin negative strains differed among themselves for all the restriction enzymes used. Strains NCTC 11143 and NCTC 11145 restriction patterns were also different with all the restriction enzymes.

4.8 DETECTION OF *bceT* GENE

The *bceT* gene was detected by PCR in 41.1 % of *B. cereus* strains. Both diarrhoeal prototye strain NCTC 11145 and the emetic strain NCTC 11143 did not contain the *bceT* gene. However, strain DSM 4384 contained the gene. Figures 18a and 18b show the PCR product corresponding to the 741 base pair fragment of the *bceT* gene for strain DSM 4384 and some milk isolates. A dot hybridization with the 741 bp DNA probe detected the *bceT* gene in 44.6 % of the isolates including all that were *bceT* positive by PCR. The higher detection rate by probe compared to PCR was because some *bceT* negative strains (by PCR) that showed weak reactions to the DNA probe. Figure 19 shows results of dot blot hybridization reactions of some *B. cereus* strains with the 741 base pair probe. As shown in table 12, there was no correlation between enterotoxin production and the presence of the *bceT* gene (p=0.2561)

Table 12: Correlation between enterotoxin production and presence of the *bceT* gene among 56 *B. cereus* strains.

BCET-RPLA test	Presence of *bceT* gene	Absence of *bceT* gene
Enterotoxin production	9 (16.1 %)	18 (32.1 %)
No enterotoxin production	14 (25 %)	15 (26.8 %)

There was no correlation between enterotoxin production and presence of *bceT* gene.

Chi-square test value = 1.29; p = 0.2561.
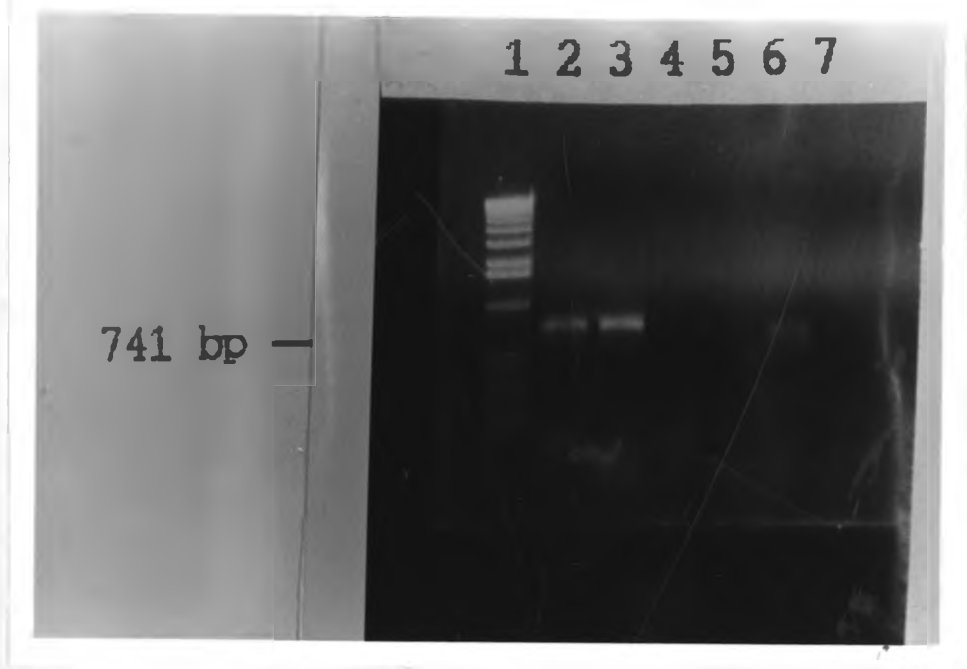
Figure 18a: An agarose gel analysis of the 741 base pair PCR product after amplification of *bceT* gene in *B. cereus* strains. From left to right; lanes (1) Mol wt. marker, (2) DSM 4348, (3) W 22 (4) NCTC 1145 (5) NCTC 11143 (6) N 10 (7) BC 38. Strains W22, N10 and BC 38 were milk isolates.
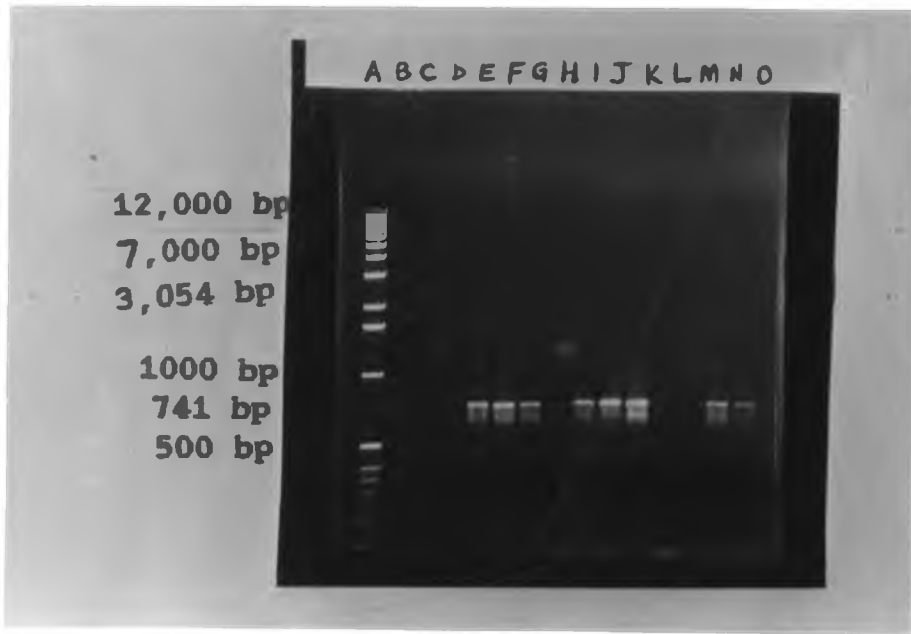
Figure 18b: A 741 base pair gene product after PCR amplification of some *B. cereus* isolates. (A) 1 0 kb molecular weight marker (B) BC 4 (C) BC 7 (D) BC 12 (E) BC 28 (F) BC 31 (G) BC 33 (H) BC 37 (I) BC 44 (J) N 15 (K) L 15 (L) W 29 (M) L 13 (N) W 32 (O) W 22

Figure 19: Dot blot hybridization of some *B. cereus* strains with the 741 base pair probe. Positive strains are: BC 33, K1, BC 13, N8, BC 34, N18, DMS, L11, BC 4, L12, N6, W29, W22, N9, W38, N10, L15, N7, W25, BC44, BC28, N4, W32, and DMS. The positive control strain was DSM 4384 (DMS) while NCTC 11145 (145) was the negative control strain.

4.9   DETECTION OF h*blA* GENE

A polymerase chain reaction was used to detect the presence of *hblA* gene, and *B. cereus* strain DSM 4384 and NCTC 1145 gave one clear band characteristic for *hblA* at the correct position, while *B. cereus* strain NCTC 11143 did not. Two *Escherichia coli* strains included in the test produced several bands of different intensities, none of them at the position characteristic for *hblA* (figure 20). A probe prepared by PCR from the nucleotide sequence of the *hblA* gene detected *hblA* gene in all *B. cereus* isolates including the *B. cereus* diarrhoeal and emetic prototype strains NCTC 11145 and NCTC 11143 respectively (figure 21).

Figure 20: Results of a PCR test for presence of *hblA* gene in *B. cereus* and *E. coli* strains. Only strains DSM 4384 and NCTC 1145 had the correct (355 base pair) band representing the *hblA* gene.
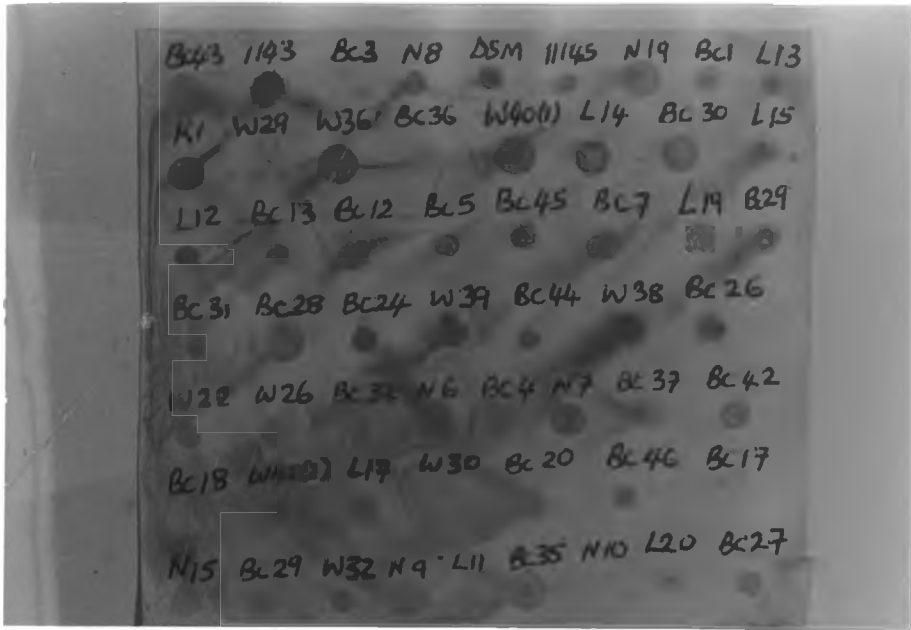
Figure 21: Dot blot hybridization of some *B. cereus* strains with *hblA* gene probe.

CHAPTER FIVE

5   DISCUSSION

Foodborne diseases occur worldwide. In Kenya, few cases of food poisoning outbreaks were reported to the Ministry of Health between 1970 and 1994. The low rate of food poisoning outbreaks was thought to be due to lack of reporting, inadequate diagnostic facilities in various health centres, and failure to obtain the right samples at the right time. Confirmation of bacterial food poisoning mainly relied on the isolation of pure organisms from the suspected food or patient stool or vomitus (Ministry of Health, Personal communication). No analysis for toxin was attempted. Bacterial diseases like cholera, typhoid fever and shigellosis are under constant surveillance by the Ministry of Health. The diseases are transmitted mainly by consumption of water contaminated with *Vibrio cholera, Salmonella typhi,* and *Shigella dysenteriae*. However, the diseases can also result from consumption of foods, which are contaminated with these microorganisms. Available records at the Ministry of Health indicate that outbreaks of these diseases occur frequently in various parts of the country, with some parts reporting very high number of cases compared to others (Table 6). Plant poisoning was suspected or confirmed mainly on the knowledge of consumption of the toxic food plant. Heavy metal poisoning was diagnosed

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CHAPTER FIVE

5 DISCUSSION

Foodborne diseases occur worldwide. In Kenya, few cases of food poisoning outbreaks were reported to the Ministry of Health between 1970 and 1994. The low rate of food poisoning outbreaks was thought to be due to lack of reporting, inadequate diagnostic facilities in various health centres, and failure to obtain the right samples at the right time. Confirmation of bacterial food poisoning mainly relied on the isolation of pure organisms from the suspected food or patient stool or vomitus (Ministry of Health, Personal communication). No analysis for toxin was attempted. Bacterial diseases like cholera, typhoid fever and shigellosis are under constant surveillance by the Ministry of Health. The diseases are transmitted mainly by consumption of water contaminated with *Vibrio cholera, Salmonella typhi,* and *Shigella dysenteriae.* However, the diseases can also result from consumption of foods, which are contaminated with these microorganisms. Available records at the Ministry of Health indicate that outbreaks of these diseases occur frequently in various parts of the country, with some parts reporting very high number of cases compared to others (Table 6). Plant poisoning was suspected or confirmed mainly on the knowledge of consumption of the toxic food plant. Heavy metal poisoning was diagnosed

after detection of high levels of mercury, copper, or lead in serum of patients and was thought to have resulted from contamination of food by agrochemicals.

The high number of reported cases of intestinal worms gives a rough indication of the extent of the potential problem of foodborne parasitic infections. However, it is difficult to distinguish between foodborne and non-foodborne parasitic infections. Amoeba, roundworms and hookworms were the most prevalent groups of parasites diagnosed in various health facilities in the period 1989 to 1990 (table 8). The high rate of amoeba infection could be attributed to drinking of contaminated water, while nematode infection was attributed to various factors including poor hygiene, farming practices involving use of untreated sewage, dietary habits particularly eating raw or undercooked vegetables and walking bare feet, a practise that is common in rural areas. Nematode eggs or larvae enter man by various routes including direct contamination of hands, eating soil contaminated vegetables, penetration of the skin by walking bare feet on infected soil, ingestion of eggs or larvae from animal sources and drinking contaminated water. Tapeworm infestations (taeniasis) occur particularly where meat is eaten raw or undercooked and its presence in man is almost entirely due to poor dietary habits. Taeniasis was common in some districts.

The worldwide occurrence of foodborne diseases calls for an urgent need to improve food preparation processes and to educate those responsible for the provision of food particularly in mass catering situations. Bacterial agents are reported as the main cause of foodborne diseases, and milk, meat and poultry as the most common foods involved in outbreaks (Roberts, 1986). Cooked rice is also commonly implicated in *B. cereus* food poisoning. Most incidents of food

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poisoning involve foods prepared in restaurants, hotels, clubs, hospitals, institutions, schools and canteens, and few prepared at home mainly for consumption and sometimes for external catering (Roberts, 1986).

The control of foodborne diseases in Kenya is the responsibility of Public Health Department of the Ministry of Health and the Veterinary Department of the Ministry of Agriculture and Livestock Development. In order to execute this responsibility, the Public Health Department of the Ministry of Health regularly carries out food quality control activities. These activities include inspection of food outlets such as general retail shops, butcheries, meat roasting premises, wholesale shops, food kiosks, food processing factories, cereals stores, hotels, supermarkets and bakeries. Premises are inspected for general cleanliness and quality of foodstuffs. All foods found unfit for human consumption are condemned and their disposal supervised by the public health personnel. The Veterinary Public Health Department provides meat inspection services aimed at preventing transmission of food poisoning pathogens from animal food products to the consumers.

Bacillus cereus was isolated from 37 % of raw and 41.2 % of pasteurized milk. The isolation rate was similar (p=0.5895) for both raw and pasteurized milk. This study found a higher recovery of *B. cereus* from preincubated milk compared to milk that was streaked directly onto blood agar. Preincubation allowed the few organisms contaminating milk to multiply to detectable levels. Crielly *et al.* (1994) also found *B. cereus* to dominate the bacillus population of pasteurized milks and reconstituted milk powders after preincubation at ambient temperatures, reaching levels associated with enterotoxin production.

Bacillus cereus has been found to contaminate dairy products. Wong *et al.* (1988) found *B. cereus* to occur in 17 % of fermented milks and 52 % of ice cream. Granum *et al.* 1993 reported isolation of *B. cereus* from at least 75 % of pasteurized milk products and (Coghill and Juffs, 1979) reported isolation of *B. cereus* from refrigerated products. These refrigerated products are contaminated with psychrotrophic *B. cereus*. These pyschrotrophic strains have also been reported to produce enterotoxin at refrigeration temperatures (Van Netten *et al.* 1990). These authors found 25 % of pyschrotrophic *B. cereus* strains isolated from pasteurized milk to be enterotoxigenic. In this study, 50 % of *B. cereus* isolates were enterotoxigenic, including 26 % strains from pasteurized milk. The presence of these organisms in pasteurized milk products present a potential hazard to the dairy industry and human health. Temperatures below 4°C and pH below 5.0 could prevent growth and enterotoxin production by psychrotrophic *B. cereus* strains (Van Netten *et al.* 1990).

The study found 65 % of all the strains from milk to hydrolyse starch. This finding compared well with those of other workers who have found *B. cereus* isolates from wholesome foods to vary in their ability to hydrolyse starch. Shinagawa *et al.* (1979) reported 83 % of *B. cereus* isolates from uncooked rice and 38 % from cooked rice to hydrolyse starch, while Shinagawa *et al.* (1984) found 71 % of the isolates from meat products, raw meat and meat additives to be able to hydrolyse starch. A significantly higher proportion (96 %; p=0.000) of the enterotoxigenic strains in this study hydrolysed starch, compared to 36 % of enterotoxin negative strains. Enterotoxigenic strains in this study could represent both emetic and atoxigenic strains, and

those strains that hydrolysed starch were most probably atoxigenic. Therefore, ability of a *B. cereus* strain to hydrolyse starch is not indicative of its ability to produce enterotoxin.

A comparison of enterotoxin production and drug resistance did not reveal any apparent relationship between enterotoxin production and drug resistance patterns of various *B. cereus* strains tested. Enterotoxin positive strains had differing antibiotic resistance patterns, while the diarrhoeal prototype strain NCTC 11145 and emetic prototype strain NCTC 11143 had the same antibiotic resistance pattern (appendix 3). Antibiogram analysis therefore cannot be used to differentiate diarrhoeal enterotoxin strains from emetic strains.

Presence of plasmids in *B. cereus* may be important in the epidemiological investigation of *B. cereus* food poisoning outbreaks. A plasmid bearing strain causing a food poisoning outbreak can be traced to various outbreak victims and vehicle foods causing that particular outbreak. This study found a variety of plasmids in many of *B. cereus* isolates. However, some isolates had no plasmids. The *B. cereus* isolates harboured between 1 and 5 plasmids of different molecular weights ranging between 0.1 and 60 megadaltons. DeBuono *et al.* (1988) found all *B. cereus* strains isolated from stools of outbreak victims to be of the same biotype and serotype designated H26, and all had identical plasmid profiles, which differed from those of *B. cereus* strains selected randomly from other outbreaks. Different food poisoning outbreaks may therefore be caused by *B. cereus* strains with differing plasmid profiles. Isolation of large numbers of *B. cereus* strains from suspect food and/or stool and vomitus of outbreak victims with the same plasmid profile could indicate a common source infection.

Plasmids have been shown to code for production of Staphylococcal enterotoxin D (Bayles and Iandolo, 1989) and *Clostridium botulinum* enterotoxin type G (Eklund, et al. 1988). However, this study found no relationship between carriage of plasmids and enterotoxin production in *B. cereus* isolates. This was attributed to the fact that the *B. cereus* strains were isolated from milk drawn from different sources and the fact that these strains were not from an outbreak episode(s). In addition, no particular plasmid could be used to indicate enterotoxigenicity of *B. cereus* as enterotoxin production was observed in plasmid bearing strains as well as in those without plasmids. These findings compare well with those of Debuono *et al.* (1988) who found enterotoxin production not to correlate with plasmid composition of strains in the investigated outbreak since serotypically identical *B. cereus* isolates with identical plasmid profiles produced diarrhoeal enterotoxin in various degrees. This, therefore, means that although no particular plasmid profile can be used to indicate the potential enterotoxigenicity of *B. cereus* strains, plasmid analysis can be a useful tool in investigating outbreaks of *B. cereus* food poisoning. In addition, the prototype *B. cereus* emetic strain NCTC 11143 carried two plasmids of molecular weights 3.9 and 3.7 megadaltons respectively. These same plasmids were carried by some diarrhoeal enterotoxin producing strains. Therefore, plasmid analysis may not be useful in the differentiation of diarrhoeal enterotoxin and emetic toxin producing strains of *B. cereus*.

Similar analyses have proven useful in foodborne outbreaks of *Clostridium perfringens* (Mahony *et al.* 1987). Absence of plasmids in all strains in a given outbreak will also indicate some similarity of the strains, although one may not be sure that they are the same, or they are different only that they just do not contain the plasmids. Such a case will require other tests like biotyping,

serotyping or restriction endonuclease DNA analysis to confirm if the strains involved are truely the same.

Restriction endonucleases are enzymes used by bacteria to defend themselves against invasion by foreign DNA. Bacterial restriction endonuclease DNA analysis (BRENDA) has been shown to be useful for identification of bacterial species in epidemiological investigation. Similar bacterial strains have been shown to produce similar BRENDA patterns when digested with the same restriction enzyme. During investigation of *B. cereus* food poisoning outbreak, one would expect all the strains isolated from ill patients and incriminated food to have similar restriction patterns using any particular restriction enzyme. However, if different strains are involved, then BRENDA patterns will differ.

The results of bacterial restriction endonuclease DNA analysis of 60 *B. cereus* strains in this study found no unique or common restriction pattern (profile) by any of the enzymes used for either enterotoxin positive or enterotoxin negative strains. However, some produced same restriction pattern with one particular enzyme but differed when digested with a different restriction enzyme. This technique cannot be used to differentiate *B. cereus* diarrhoeal enterotoxin producing from non-enterotoxin producing strains since even among, for example the enterotoxin producing strains, there were widely differing restriction patterns. This shows that although these *B. cereus* strains produce enterotoxins, their base pair arrangement in their genomic DNA are quite different. The fact that there are many different strains producing diarrhoeal enterotoxin or emetic toxin makes it impossible to have a common restriction pattern that can be used to identify one group from the other. Various *B. cereus* serotypes are involved in food poisoning (Gilbert, 1979),

indicating that diarrhoeal enterotoxin or emetic toxin production is not limited to one particular serotype.

Analysis for diarrhoeal enterotoxin production and detection of the *bceT* gene found the *bceT* gene in both enterotoxin positive and negative strains. The gene was also absent in some enterotoxin positive and some negative strains, indicating that presence of *bceT* gene has no correlation to production of the three components of the enterotoxin complex. The results of this study indicated a likelihood of two or more enterotoxins being produced by *B. cereus* strains with a possibility of strains producing one type or more than one type of diarrhoeal enterotoxins (Ombui *et al.* 1997). In addition, the enterotoxin complex and single component enterotoxin described by various authors in the literature could be two distinct enterotoxins produced by different strains of *B. cereus*. Agata *et al.* (1995) probably isolated one of the diarrhoeal enterotoxin genes, while Beecher and Macmillian, (1990; 1991) and Bitsaev and Ezepchuk, (1987) most likely described another type of diarrhoeal enterotoxin that may be encoded by different gene(s). The confusion as to whether *B. cereus* diarrhoeal enterotoxin is composed of one protein (Shinagawa *et al.* 1991a: 1992: Agata *et al.* 1995), or is a three protein complex may be due to differences in particular *B. cereus* strains that were used in the respective studies. There is a possibility that Agata *et al.* (1995) used strains that did not contain the gene for the three proteins of the enterotoxin complex, while the other workers used *B. cereus* strains that contained only the gene(s) responsible for production of the three proteins that comprise the enterotoxin complex. Since the strains used in this study represented both categories, it was concluded that two or more enterotoxins might be involved in *B. cereus* diarrhoeal food poisoning. Three types of enterotoxins

have since been reported, including two types of multicomponent enterotoxin and a single component enterotoxin (Lund and Granum, 1997). They include the hemolysin BL (Beecher *et al.* 1995), a non-hemolytic enterotoxin (Lund and Granum, 1996) and a single componet enterotoxin (Shinagawa *et al.* 1992).

Several (26.4 %) strains in this study that were found to have the *bceT* gene, tested negative with the BCET-RPLA (Oxoid) test kit. This means that an enterotoxin negative test with BCET-RPLA kit will not conclusively exonerate a *B. cereus* strain from being involved in a food poisoning outbreak, such a strain may produce the single component enterotoxin encoded by the *bceT* gene or the non-hemolytic enterotoxin. A probe prepared from the *bceT* gene will be limited to the identification of those *B. cereus* strains that contain the *bceT* gene and not the multicomponent enterotoxin coding genes (Ombui *et al.* 1997).

A *hblA* probe detected *hblA* gene in all *B. cereus* strains, including strains DSM 4384, NCTC 1145 and emetic producing prototype strain NCTC 11143. A direct PCR on the other hand correctly detected the *hblA* gene only in *B. cereus* strains DSM 4384 and NCTC 11145. However, there was amplification of non-specific sequences in strain NCTC 11143 and other *B. cereus* and *E. coli* strains, which may explain why the probe could detect all the strains as having the *hblA* gene in a dot blot hybridization procedure. Results of diarrhoeal enterotoxin assay using BCET-RPLA Oxoid test Kit showed that, not all *B. cereus* strains produce the L_2 or 48 kda protein component of the enterotoxin complex. It has previously been shown that some *B. cereus* strains do not produce L2 protein (Granum, 1994; Lund and Granum, 1996; Granum *et al.* 1996), while hemolysin BL has been found in some *B. cereus* strains and not others. PCR tests need to

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be performed to amplify sequences within the *hblA* gene that would be specific to all hemolysin positive strains. PCR tests should also be done using amplified *hblC* and *hblD* genes (Ryan *et al.* 1997) that code for the L2 and L1 proteins of hemolysin BL respectively. The tests should involve direct detection of *hblA*, *hblC* and *hblD* by (1) PCR and (2) probe prepared from the PCR products on a number of *B. cereus* strains representing both enterotoxin positive and negative strains. This approach will most likely reveal the gene that is specific enough to characterize enterotoxigenic strains of *B. cereus*.

6 CONCLUSIONS AND RECOMMENDATIONS

1. Foodborne disease outbreaks occur in Kenya and the problem is much more than is recorded by the Ministry of Health. This is so because there are diagnostic problems and under-reporting of cases. Surveillance and investigation of all food poisonings should be stepped up, while the public should be educated and encouraged to report food poisoning cases. The Ministry of Health should endeavour to revamp and improve diagnostic facilities in district hospitals to enable detection of microbial toxins, poisonous chemicals and emerging food pathogens.

2. Presence of enterotoxigenic *B. cereus* in pasteurized milk products poses a potential public health hazard. Post pasteurization contamination, which is commonly blamed for spoilage of milk products by *B. cereus* is therefore not necessarily the most important source of this organism.

3. Majority of *B. cereus* diarrhoeal enterotoxin producing strains hydrolyse starch, however, starch hydrolysis is not a definite indicator of the ability of the strain to produce diarrhoeal enterotoxin(s).

4. Antibiogram analysis data is not useful in distiguishing between enterotoxigenic strains of *Bacillus cereus*.

5. Plasmid profile analysis and bacterial restriction endonuclease DNA analysis cannot differentiate between diarrhoeal enterotoxin and non-enterotoxin producing strains of *B. cereus*.

6. Two or more diarrhoeal enterotoxins may be involved in *B. cereus* diarrhoeal food poisoning. Upto three different enterotoxins have now been characterized, including two multicomponent enterotoxins i.e hemolysin BL and non-hemolytic enterotoxin, and one single component enterotoxin (Lund and Granum, 1997).

7. Some *B. cereus* strains may contain both the *bceT* and enterotoxin complex genes, and thus produce more than one independent diarrhoeal enterotoxins.

8. A polymerase chain reaction and *bceT* 741 base pair probe are alternative methods for screening *B. cereus* for presence of *bceT* gene that will show the potential ability of the strains to produce a diarrhoeal enterotoxin.

9. During investigation of a suspected *B. cereus* food poisoning outbreak, it will be important to screen for the two multicomponent enterotoxins, and for the presence of the *bceT* gene.

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APPENDICES

APPENDIX 1: COMPOSITION AND PREPARATION OF CULTURE MEDIA

1.1.1 Ammonium salt-sugar agar medium (Sneath, 1986)

Preparation

Ammonium salt-sugar basal medium was prepared by adding

diammomium hydrogen phosphate - 1.0 g; potassium chloride- 0.2 g; Magnesium sulphate- 0.2

g, yeast extract- 0.2 g; Agar- 15.0 g to 985 ml of distilled water. The pH was adjusted to 7.0 and

15 ml of a 0.004 % (w/v) of bromocresol purple added. Three millilitres of basal medium was

dispensed into 10 ml screw capped tubes and then sterilized by autoclaving at 121°C for 20 min

after which they were allowed to cool to 50°C. Three mililitres of 10 % of each test sugar

(glucose, manitol, xylose, rhamnose, etc) sterilized by membrane filtration were aseptically added

to each tube and mixed gently.

1.1.2 *Bacillus cereus* selective agar (Oxoid)

Formula	gm/litre
Peptone	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium chloride	0.1
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromothymol blue	0.12
Sodium pyruvate	10.0
Agar	14.0

PH 7.2 ± 0.2

This was prepared by suspending 20.5 g of *Bacillus cereus* selective agar base (Oxoid, Unipath, Hampshire, England) in 475 ml of distilled water and boiled to dissolve completely. Sterilization was done at 121°C for 15 min. The sterile base was cooled to 50°C and the contents of 1 vial of *Bacillus cereus* selective supplement (Oxoid SR99) containing 50,000 IU polymyxin B reconstituted by 2 ml of distilled water and 25 ml of sterile egg-yolk emulsion (Oxoid, SR47) were aseptically added. The contents were mixed and poured into sterile petri dishes.

1.1.3 Blood Agar (Oxoid)

Blood agar base formula	grams per litre
Lab-lemco powder	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	15.0

Blood agar plates were prepared by dissolving 37 grammes of blood agar base (Oxoid: Basingstoke, Hampshire, England) in one litre of water and sterilized at 121°C for 15 min. The molten blood agar base was cooled to 50°C and 50 ml of sterile defibrinated bovine blood prewarmed to 37°C was added to give a final concentration of 5 % (v/v) blood.

1.1.4   Brain Heart Infusion Broth (Lab M)

Formula	grams/litre
Brain heart infusion solids	17.5
Tryptose	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium hydrogen phosphate	2.5

pH 7.4

The broth was prepared by suspending 37 g of brain heart infusion (Lab M, Burry, Lancs, London, U.K) in 1.0 litre of distilled water, soaked for 10 min and dissolved with gentle heating before distribution into screw capped test tubes and then sterilized by autoclaving at 121°C for 15 minutes.

1.1.5 Citrate Medium (Sneath, 1986).

Composition

Trisodium citrate. $2H_2O$	1.0 g
Magnesium sulphate. $7H_2O$	1.2 g
Diammonium hydrogen phosphate	0.5 g
Potassium chloride	1.0 g
Agar	15 g
Distilled water	980 ml
0.04 % w/v solution of phenol	20 ml

The above reagents were mixed and dissolved by boiling. The pH was then adjusted to 6.8 and 3 ml of the medium dispensed into screw capped bijou bottles. They were then sterilized at 121°C for 20 min and prepared into slants.

1.1.6 Cooked meat medium (Lab M)

The medium wa prepared by adding 15 grammes of cooked meat pellets to 20 ml nutrient broth (Oxoid) in universal bottles and sterilized by autoclaving at 121°C for 15 minutes.

1.1.7. Mueller- Hinton Broth (Oxoid CM 405)

Formula	grams/llitre
Beef dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5

This was prepared by dissolving 21 g of dehydrated medium in one litre of distilled water and sterilized by autoclaving at 121°C for 15 min.

1.1.8. Mueller-Hinton Agar (Oxoid, CM 337)

Formula	grams/litre
Beef dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

pH 7.4 ± 0.2

1.1.7. Mueller- Hinton Broth (Oxoid CM 405)

Formula	grams/llitre
Beef dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5

This was prepared by dissolving 21 g of dehydrated medium in one litre of distilled water and sterilized by autoclaving at 121°C for 15 min.

1.1.8. Mueller-Hinton Agar (Oxoid, CM 337)

Formula	grams/litre
Beef dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

pH 7.4 ± 0.2

This was prepared by dissolveing 35 g of Mueller Hinton powder CM 337 (Oxoid, Unipath, England) in 1.0 litre of distilled water and boiled to dissolve completely. Sterilization was then done at 121°C for 15 minutes.

1.1.9 Nutrient broth (Oxoid)

The broth was prepared by adding 25 grams of dehydrated medium to 1.0 litre of distilled water, boiled to dissolve and then distributed into final containers. This was then sterilized by autoclaving at 121°C for 15 minutes.

1.1.10   1.0 % w/v Tryptone broth (lab M)

Typical formula	(%)
Total nitrogen	12.7
Amino acids	5.1
Ash	6.1
Sodium chloride	0.5

The broth was prepared by dissolving 10 grammes of dehrdrated broth in one litre of distilled water and sterilized by autoclaving at 121°C for 15 minutes (final pH 7.2).

1.1.11. Tryptone soya broth (TSB) (Oxoid CM 131)

Typical formula	grams/litre
Tryptone	15.0
Soya peptone	5.0
NaCl	5.0

Tryptic soy broth (Oxoid) was rehydrated by dissolving 30 g of the dehydrated powder in one litre of distilled water, and then 3.0 ml amounts dispensed into screw capped test tubes and then sterilized at 121°C for 15 min.

1.1.12. Tryptone soya Agar (TSA) (Oxoid CM 131)

Typical formula	grams/litre
Tryptone	15.0
Soya peptone	5.0
NaCl	5.0
Agar	15.0

Tryptone soy agar was prepared by suspending 40 g of powder (Oxoid, Unipath, Hampshire, England), in one litre of distilled water and boiled to dissolve completely. This was the sterilized at 121°C for 15 minutes.

1.1.13. Voges-proskauer broth (Sneath, 1986)

Formula	grams/litre
Proteose peptone	7.0 g
Glucose	5.0 g
Sodium chloride	5.0 g
pH - 6.5	

The medium was prepared by dissolving the above substances in one litre of distilled water, and the pH adjusted to 6.5. The medium was distributed in 5 ml amounts into screw capped tubes and sterilized by autoclaving at 121°C for 15 minutes.

1.2 PREPARATION OF SOLUTIONS

1.2.1 Phenol: chloroform : isoamylalcohol (25:24:1)

100 ml saturated phenol, 96 ml chloroform and 4 ml isoamyl alcohol were combined in a dark bottle in a fume hood. The bottle was capped and inverted several times to mix.

1.2.2 Chloroform : isoamylalcohol (24:1)

480 ml chloroform, and 20 ml isoamyl alcohol were combined in a dark bottle. The bottle capped and inverted several times to mix.

1.2.3 50 X TAE buffer (Tris Acetate EDTA)

57.1 ml glacial acetic acid, 242 g tris base, 100 ml 0.5 M EDTA(pH 8.0), were mixed and dissolved in 1.0 litre of water. The solution was then diluted to 1X working concentration with distilled water (e.g 20 ml of 50 x TAE per litre of working strength buffer).

1.2.4   10 X TBE Buffer (Tris borate EDTA buffer)

108 g tris base, 55 g boric acid and 40 ml 0.05 M EDTA (pH 8.0) were dissolved in one litre of water.

1.2.5   1.0 % Agarose gel in TBE

1.25 g agarose electrophoresis grade was added to 1.25 ml distilled water and 12.5 ml of 10 X TBE added. The mixture was heated to dissolve, then cooled to 60°C and 7.5 µl ethidium bromide (10 mg/ml) added and shaken to mix.

1.2.6   Tris EDTA buffer, (TE)

10 ml of 1 M Tris HCL (pH, 8.0) and 100 ml of 0.1 M ETDA, (pH 8.0) were mixed and made upto 1.0 litre with distilled water. The solution was then autoclaved at 121°C for 15 minutes.

1.2.4   10 X TBE Buffer (Tris borate EDTA buffer)

108 g tris base, 55 g boric acid and 40 ml 0.05 M EDTA (pH 8.0) were dissolved in one litre of water.

1.2.5 1.0 % Agarose gel in TBE

1.25 g agarose electrophoresis grade was added to 1.25 ml distilled water and 12.5 ml of 10 X TBE added. The mixture was heated to dissolve, then cooled to 60°C and 7.5 μl ethidium bromide (10 mg/ml) added and shaken to mix.

1.2.6 Tris EDTA buffer, (TE)

10 ml of 1 M Tris HCL (pH, 8.0) and 100 ml of 0.1 M ETDA, (pH 8.0) were mixed and made upto 1.0 litre with distilled water. The solution was then autoclaved at 121°C for 15 minutes.

1.2.7 Lysozyme solution

This was prepared by addiding 0.04 g of lysozyme powder (4 mg/ml) to 10 ml of lysis buffer (0.5 M glucose, 0.1 EDTA, 0.25 M Tris HCL, pH 8.0 made up to 10 ml with distilled water) and mixed. The solution was kept in ice until use.

1.2.8 Alkaline detergent solution

Sodium hydroxide pellets (0.8 g) and 1.0 g of sodium deodecyl sulphate (SDS) were dissolved in 100 ml distilled water in a polystyrene bottle.

1.2.9 Ethidium bromide stock solution, (10 mg/ml)

1.0 g Ethidium bromide, was added to 100 ml distilled water, and then stirred with a magnetic stirer until ethidium bromide dissolved. The solution was then transferred to a bottle and wrapped in aluminium foil and stored at 4°C. (**Caution:** Handlers should wear a face mask and hand gloves as ethidium bromide is carcinogenic).

1.2.10 Loading dye 1 (Stop mix)

2.5 g Sodium deodecyl sulphate (SDS), 25 ml glycerol and 0.0125 g bromothymol blue were mixed in 50 ml distilled water and warmed to 37°C to dissolve the SDS.

1.2.11 10 % Sodium Deodecyl Sulphate (SDS)

10 g of SDS was dissolved in 100 ml of distilled water.

1.2.12 Sodium Acetate (3 M)

Sodium acetate (26.6 g) was dissolved in 45 ml distilled water, pH adjusted to 4.8 with glacial acetic acid, and final volume brought to 100 ml with distilled water. The solution was then autoclaved at 121°C for 15 minutes.

1.2.13 CTAB/NaCL Solution (10 % CTAB in 0.7 % M NaCl)

4.1 g sodium chloride was dissolved in 80 ml distilled water and 10 g of CTAB (hexadecyltrimethylammonium bromide) slowly added, while heating and stirring. Final volume was then adjusted to 100 ml.

1.2.14 Proteinase-K

Proteinase-K (20 mg/ml) was prepared in appropriate amounts of sterile distilled water, and stored in 1.0 ml aliquots at -20°C.

1.2.15 Preparation of 10 mg/ml DNase free RNAse

RNAse (10 mg), 10 µl of 10 mM Tris HCl pH 7.5 and 3.0 µl 15 mM NaCl were dissolved and made to 1.0 ml with distilled water. This was then boiled at 100°C for 20 min. cooled slowly and stored in 1.0 ml aliquots at -20°C.

1.2.16 DNA Denaturing solution

1.5 M NaCl, 0.5 M Na0H

1.2.17 Neutralization solution

1.5 M NaCl,

0.5 M Tris-HCL, pH 7.2

0.001 M Na_2EDTA.

1.2.18 Solutions for hybridization of labelled DNA

20 x SSC;   3M NaCl; 0.3 M Na-citrate; pH 7.0 (20°C)

Hybridization solution:

     5 x SSC

     50 % formamide

     0.1 % (w/v) N-laurosarcosine, Na-salt.

     0.02 % (w/v) SDS

     Add to a freshly prepared solution 5.0 % (w/v) blocking reagent

     (NB: Hybridization performed at 42°C)

Wash solution 1   2x SSC; 0.1 % (w/v) SDS.

Wash solution 2   0.1 x SSC; 0.1 % (w/v) SDS.

1.2.19 Solutions for detecting probe DNA

Buffer 1:   100 mM Tris-HCl, 150 mM NaCl, pH 7.5(20°C)

Buffer 2:   0.5 % (w/v) blocking reagent in buffer 1

                  The solution is prepared 1 hour in advance

                  by dissolving at 50-70°C.

Buffer 3:   100 mM Tris-HCl: 100 mM NaCl; 50 mM

MgCl2; pH 9.5 (20°C)

Buffer 4:   10 mM Tris-HCl: 1 mM EDTA, pH 8.0 (20°C)

Colour solution;   45 µl NBT solution {1.25 ml nitroblue tetrazolium salt, 75 mg/ml in

dimethylformamide, 70 % (v/v)}and 35 µl X-phosphate solution are

added to 10 ml of buffer 3.

NB: X- phosphate contains 0.9 ml of 5-bromo-4-chloro-3-indolyl phosphate and 50 mg/ml

toluidinium salt, in dimethylformamide.

1.3 COMPONENTS OF VARIOUS KITS

1.3.1 BCET-RPLA Toxin Detection Kit (Oxoid, Unipath, U.K)

A kit for detection of *B. cereus* diarrhoeal enterotoxin in foods and culture filtrates by a commercially available reverse passive latex agglutination technique. The kit is based on 48 kda protein , one of the three protein components of the enterotoxin complex (Granum *et al.* 1993) or L_2 component of hemolysin BL (Beecher and Wong, 1994b).

Kit Components

(i). Sensitized latex

Latex sensitized with specific *B. cereus* anti- enterotoxin (rabbit IgG).

(ii). Latex control

Latex suspension sensitized with non- immune rabbit globulins.

(iii). Enterotoxin control (lyophilized)

Lyophilized *B. cereus* enterotoxin.

(iv). Diluent

Phoshate buffered saline containing bovine serum albumin.

1.3.2 DIG DNA labelling and detection kit components

a). Unlabelled Control-DNA 1

One vial with 20 µl pBR328 DNA, 100 µg/ml. pBR328 was digested separately with *BamHI, Bgl* and *Hinf I* and mixed in ratio of 2;3;3. Sizes of the sixteen fragments: 4907, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298 (2x), 234 (2x), 220 and 154 (2x) base pairs.

b). Unlabelled control DNA 2

One vial with 20 Ul pBR328, 200 µg/ml, linearized with BamHI.

c). DNA dilution Buffer

Two vials with 1 ml herring sperm DNA, 50 µg/ml, in Tris-HCl, 10 mmol/l; EDTA, 1 mmol/l; pH 8.0 (20 °C), each.

d). Labelled control DNA

One vial with 50 µl linearized pBR328 DNA, labeled with digoxigenin according to the standard protocol containing 20 µg template DNA and approximately 5 µg synthesized labeled DNA per ml.

e). Hexanucloetide mixture

One vial with 50 µl of 10 x concentrated hexanucleotide reaction mixture.

f). dNTP labelling mixture

One vial with 50 μl of 10 x concentrated dNTP labelling mixture containing dATP, 1 mmol/l; dCTP, 1 mmol/l; dGTP, 1 mmol/l; dTTP, 0.65 mmol/l; Dig-dUTP, 0.35 mmol/l; pH 6.5 (20°C).

g). Klenow enzyme labelling Grade

One vial with 25 μl Klenow enzyme, labelling grade, 2 U/μl.

h). DIG-AP-conjugate

One vial with 200 μl polyclonal sheep anti-digexigenin Fab fragments, conjugated to alkaline phosphatase, 750 U/ml.

Preparation of DIG-AP conjugate

After immunization with digoxigenin, the sheep IgG was purified by ion exchange chromatography and the specific IgG was isolated by immunoabsorption. The Fab fragments obtained by papain digestion were conjugated with alkaline phosphatase (AP) and stabilized in 50 mM triethanolamine buffer, 3mM NaCl, 1mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 % bovine serum albumin (w/v), pH 7.6. For detection of digoxigenin labelled nucleic acids on membranes as in southern and dot blots a dilution of 1:5000 = 150 mU/ml was made.

h). Nitroblue tetrazolium dimethylformamide (NBT)

Two vials with 1.25 ml nitroblue tetrazolium salt, 75 mg/ml in dimethylformamide, 70 % (v/v) each.

i). X-phosphate

Two vials with 0.9 ml of 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/ml, in dimethylformamide each.

j). Blocking reagent

Two bottles with 50 g powder each.

1.3.3 PCR core kit componnets

a). Taq DNA polymerase

One vial containing 250 units, 5 units/µl in storage buffer: 20 mM Tris/HCl, 100 mM dithiothreitol, 0.1 mM EDTA, Nonidet P40, 0.5 5 (v/v), Tween 20, (0.5 % v/v), glycerol, 50 % (v/v), pH 8.0 (4 °C).

b). dNTP stock solution

One 200 µl vial containing 10 mM dATP, dCTP, dGTP, dTTP each in sterile distilled water, pH 7.0.

c). PCR reaction buffer, 10x concentration

2 vials with 1.0 ml 100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 (20°C).

e). $MgCl_2$ stock solution

One 1.0 ml vial with 25 mM $MgCl_2$.

f). PCR-reaction buffer without $MgCl$, 10x concentration

One 1.0 ml vial with 100 mM Tris/HCl, 500 mM KCl, pH 8.3 (20°C).

1.3.4   QIAEX 11 Gel Extraction Kit

Components

1.5 ml QIAEX II silica particles suspension,

500 ml solubilization buffer QXI and

100 ml concentrated wash buffer PE.

APPENDIX 2: Results of the biochemical tests and enterotoxin production.

Biochemical test	NCTC 11145	NCTC 11143	Other *B. cereus* strains
Glucose	+	+	+
Maltose	+	+	+
Trehalose	+	+	+
Salicin	+	+	+
Mannitol	-	-	-
Arabinose	-	-	-
Inulin	-	-	-
Sorbitol	-	-	-
Raffinose	-	-	-
Lactose	-	-	-
Rhamnose	-	-	-
Xylose	-	-	-
Sucrose	-	+	+/-
indole	-	-	-
Methyl red	+	+	+
VP reaction	-	-	-
Citrate	+	+	+
Starch hydrolysis	+	-	+/-
Enterotoxin prod.	+	-	+/-

APPENDIX 3: Enterotoxin production and resistance of various _B. cereus_ isolates to various antibiotics.

Isolate No.	Enterotoxin Prod.	Resistant drug(s)
DSM 4384	+	NT
NCTC 11145	+	amp, cot, smx
NCTC 1143	-	amp, cot, smx
BC 1	+	amp, cot, ka, gen, smx
BC 2	-	amp, cot
BC 3	+	amp
BC 4	+	amp, cot
BC 7	+	amp
BC 12	+	amp, tet,
BC 13	+	amp, cot, smx
BC 20	+	amp, cot
BC 24	-	amp, cot
BC 26	+	amp, tet, cot, smx
BC 27	+	amp, cot
BC 28	+	amp, cot, smx
BC 29	_	amp, tet, cot, chl, smx
BC 30	-	amp, tet, cot, smx
BC 31	-	amp, tet, cot, smx
BC 32	-	amp, cot cot
BC 33	+	amp, cot smx
BC 35	+	amp
BC 36	+	amp, cot, smx
BC 37	+	amp, cot, smx
BC 38	+	amp, tet
BC 39	+	amp, cot, smx
BC 40	-	Amp, smx

BC 42	+	amp, tet, cot
BC 43	+	amp
BC 44	+	amp, tet
BC 45	+	amp
BC 46	-	amp, cot
N 15	-	amp, cot
N 19(1)	-	amp, cot, smx
N 19(2)	-	amp, cot, smx
N 19 (3)	-	amp, cot, smx
L 15	-	amp, cot, smx
L17	-	amp, tet, cot, smx
K3	-	Amp, tet, cot, smx
W 38	-	amp, cot
N 7	-	amp, cot, smx
W 36	+	amp, tet, cot, smx
N 8	-	amp, cot, smx
L 14	-	amp, tet, cot, smx
N 10	-	amp, cot
W 22	-	amp, tet cot, smx
L 11	+	amp, tet, cot, smx
N 9	-	amp, cot, smx
W 32	+	amp, cot, smx
L 13	-	amp, tet, cot, smx
N 4	-	amp, cot smx
W 29	+	amp, cot, smx
W 26	+	amp, cot, smx
N 6	-	amp, tet, smx
W 30	-	amp, cot, smx
K 1	+	amp, tet, cot, smx
L 12	-	amp, cot, smx
W 25	+	amp, tet, cot, smx

L 20	-	amp, cot kan, smx
W 40(1)	-	amp, cot, smx
W 40(2)	-	amp, tet, cot, smx
N 18	-	amp, cot, smx
W 39	-	amp, cot, smx

Key

+ = enterotoxin production; - = no enterotoxin production

amp - ampicillin,   cot - cotrimoxazole; smx - sulphamethoxazole;

tet - tetracycline; chl - chloramphenicol; kan - Kanamycin;

gen - Gentamycin   str - streptomycin; NT- not tested.

APPENDIX 4: A comparison of enterotoxin production, sucrose fermentation starch hydroysis and molecular weights of plasmids found in various *Bacillus cereus* strains.

Laboratory isolate no.	Enterotoxin production	Sucrose ferment.	Starch hydroysis	Mol. weights of plasmid(s) in MDa.
NCTC 11143	-	+	-	3.9, 3.7
NCTC 11145	+	-	+	none
BC 1	+	+	+	35.8, 3.7
BC 3	+	-	+	3.7
BC 4	+	-	+	60, 4.8, 3.7
BC 7	+	-	+	35.8, 4.8, 4.5, 3.7
BC 12	+	+	+	35.8. 3.7
BC 13	+	+	+	none
BC 20	+	-	+	3.7
BC 24	-	+	+	60
BC 26	+	+	+	35.8
BC 27	+	+	+	3.4
BC 28	+	+	+	60
BC 29	-	+	+	35.8, 4.8. 3.7
BC 30	-	+	+	none
BC 31	-	+	+	35.8
BC 32	-	+	-	3.9
BC 33	+	+	+	1.5, 0.1
BC 35	+	+	+	3.7
BC 36	+	+	+	35.8, 4.8, 3.9
BC 37	+	+	+	none
BC 38	+	-	+	3.9
BC 39	+	+	+	35.8, 3.7
BC 40	-	+	-	none
BC 42	+	+	+	35.8
BC 43	+	+	+	none
BC 44	+	+	+	3.7

BC 45	+	+	+	3.7
BC 46	-	+	-	60
N 15.	-	-	-	60. 4.8. 3.9
N 19 (1)	-	-	-	60. 3.7. 2.9
N 19(2)	-	-	-	3.7
N 19 (3)	-	+	-	60
L 15	-	+	+	60, 3.7
W 38	-	-	-	none
N 7	-	-	-	3.9, 3.7
W 36	+	-	-	60. 58, 4.6, 3.7, 2.6
K 3	-	+	-	4.8. 3.7
L 14	-	+	-	0.1
N 10	-	+	-	60
W 22	-	+	+	3.7, 2.5
L 11	+	+	+	60
N 9	-	-	-	60
L 17	-	+	-	none
L 13	-	+	+	60, 35.8
N 4	-	-	-	none
W 29	+	+	+	60
W 26	+	+	+	60
N 6	-	-	-	none
W 30	-	+	+	none
K 1	+	+	+	60
L 12	-	+	-	none
W 25	+	+	+	60, 3.7
L 20	-	+	+	none
W 40(1)	-	-	-	none
W 40(2)	-	+	+	60, 3.7
N 18	-	-	-	60. 4.8, 3.7
W 32	+	+	+	none
W 39	-	+	-	60
N 8	-	-	-	none

APPENDIX 5: A comparison of enterotoxin production, chromosomal DNA dot blot reaction to 741 bp bceT probe and bceT gene detection by PCR in various B. cereus strains.

Isolate No.	Enterotoxin production[a]	BceT gene detection by PCR	Dot blot reaction to 741 bp DNA probe
1. DSM 4384	+	+	+
2. NCTC 11145	+	-	-
3. NCTC 11143	-	-	-
4. BC 1	+	-	-
5. BC 3	+	-	-
6. BC 4	+	+	+
7. BC 7	+	-	-
8. BC 12	+	-	-
9. BC 13	+	-	-
10. BC 20	+	-	-
11. BC 24	-	-	-
12. BC 26	+	-	-
13. BC 27	+	-	-
14. BC 28	+	+	+
15. BC 29	-	-	-
16 BC 30	-	-	-
17. BC 31	-	+	+
18. BC 32	-	-	-
19. BC 33	+	+	+
20. BC 35	+	-	-
21. BC 36	+	NT	NT
22. BC 37	+	+	+
23. BC 38	+	-	-
24. BC 39	+	-	-

25. BC 40	-	NT	NT
26. BC 42	+	-	-
27. BC 43	+	-	-
28. BC 44	+	+	+
29. BC 45	+	-	-
30. BC 46	-	-	-
31. N 15	-	+	+
32. N 19 (1)	-	-	-
33. N 19 (2)	-	NT	NT
34. N 19 (3)	-	NT	NT
35. L 15	-	+	+
36. L17	-	NT	NT
37. K3	-	NT	NT
38. W 38	-	+	+
39. N 7	-	+	+
40. W 36	+	-	-
41. N 8	-	+	+
42. L 14	-	+	+
43. N 10	-	+	+
44. W 22	-	+	+
45. L 11	+	+	+
46. N 9	-	+	+
47. W 32	+	+	+
48. L 13	-	-	-
49. N 4	-	+	+
50. W 29	+	-	-
51. W 26	+	-	-
52. N 6	-	+	+
53. W 30	-	-	-
54. K1	+	+	+
55. L 12	-	-	-

56. W 25	+	+	+
57. L 20	-	-	-
58. W 40(1)	-	+	+
59. W 40(2)	-	-	-
60. N 18	-	+	+
61. W 39	-	-	-
62. 8915(Munich)	NT	+	+

a - tested by BCET- RPLA Oxoid test kit.

NT - Not tested.