INCIDENCE OF PATHOGENIC CLOSTRIDIAL SPECIES IN SELECTED AREAS OF KADUNA AND NIGER STATES

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In revered memory of my

Guardian

MR. P. Y. IBRAHIM

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ABSTRACT

30 soil samples from the market places, play grounds and motor parks were examined for the presence of clostridial species. 50 wounds from a rural clinic and an out patient department of an urban hospital were also examined for the same purpose. An additional 20 wounds were examined from the wards and sterilized surgical instruments. Thioglycolate was used as transport medium in all cases as this stimulates clostridial spore germination. Isolation of organisms were made by sub-culturing into 5% human blood agar and 5% egg yolk medium. Plates were incubated in vaccum jars at room temperature for 3 days, after which the isolated colonies were subcultured into cooked meat, medium.

Spore production by the various species was excellent in cooked meat when incubated at 34°C for 3 days except for C. perfringens. C. perfringens were made to sporulate after heat-shock treatment at 80°C for 5 minutes and then incubated overnight at 34°C.

Identification of the species was made by using their cultural and biochemical properties, namely; spore shape and position, their activity in cooked meat medium, production of

lecithinase and lipase in egg yolk agar, gelatinase production and their ability to coagulate, or digest, acidify or produce gas in crossley milk and ability to ferment glucose, lactose, maltose and sucrose.

Species isolated from the soil samples include C. perfringens. C.. septicum, C. novyi, C. histolyticum, C. sporogenes, C. tetani and C. botulinum. Similar species were isolated from both the rural and urban clinics except for C, botulinum. There were no isolates of Clostridia from the sterilised surgical instruments.

The most prevalent of the species from both the soil samples and clinical materials was C., perfringens. C. perfringens was found to occur in 55 (61.1%) of the soil samples; this was closely followed by C. histolyticum with *54* (60,0%) incidence of occurence in soil. C. septicum, C. novyi and C. sporogenes had 23(27.8%), 15(16.7%) and 13 (14.4%) incidence of occurence in soil. In the clinical materials, C. perfringens and C. histolyticum had 4O (33.3%) and 9 (7.5%) incidence respectively while that of C. septicum was 17 (l4.2%), C. novyi 14 (11.7%) and C. sporogenes 19 (15.8%).

The most invassive of the clostridial species, C. botulinum and C. tetani had 23 (25.6%) and 12 (13.3%) incidence of occurence in soil samples. In the clinical materials, the incidenoe of C. botulinum and C. tetani were nil and 1 (0.8%) respectively.

INTRODUCTION:

The word Clostridium, as defined by Bergey's manual of determinative bacteriology, is from two Greek words; closter, meaning a spindle, and dim, which means small. Clostridium, therefore means a small spindle. The organisms are rods, often swollen at sporulation, producing clostridial, clavate or novicular forms, Most species are motile by means of peritrichous flagella, except for Clostridium perfringens which is non-motile. These organisms are generally gram positive.

Many of the species are saccharolytic and fermentative producing various acids (generally butric and acetic), gas (carbondioxide, hydrogen and some time methane) and various amounts of neutral products such as alcohols and ecetone, other species such as Clostridium botulinum and C. sporogenes are proteolytic. Some species are active in the fixation of free nitrogen. They are strickly anaerobic, or moderately anaerobic or aerotolerant forms. A few species are obligately thermophillic. Exotoxins are sometimes produced. The Clostridia comprise of a very large and diverse group of essentially saprophytic organisms, which are widely distributed in nature (in soil, in dust, in clothing) as well as in the alimentary canal of man and most animals. The type specie is Clostridium butyricum (Broks et al, 1959).

The occurence and persistence of cetain pathogenic microorganisms in the soil has been largely overlooked as a problem in disease control. Few workers have been attracted to this study because of the complexity of the soil as a natural enviroment for microbial populations and the scarcity of methods required for assessing the behaviour of these organisms under varied soil conditions. Reports on sporadic outbreaks of human and animal diseases caused by soil borne pathogens, however, have assumed increasing significance as a public health problem. (Maclennan, 1962; Smith, 1949).

Although many pathogens are short lived in the soil, others, notably the spore forming Clostridia persist for long periods. These anaerobes are reported to be widely distributed in soils, decomposing plants and animal residues, and could cause disease under appropriate condition, (Waksman, 1945). Garcia and Mackay in 1969, reported that Clostridium septicum, which is known to cause gas gangrene, braxy in sheep and malignant oedema in other animals can even survive and multiply in dairy soil. In 1949, Smith and Gardner also isolated a high proportion of heat susceptible Clostridium perfringens from different soils indicating widespread occurence of vegetative Clostridia under various soil conditions.

This is in contrast to the general belief, that infections due to anaerobic bacteria are neither very rare, nor, on the whole

very grave; for these organisms are so common and so widespread and at the same time exacting, unreliable and relatively inefficient pathogens. Indeed, except Clostridium botulinum and with a great deal of reserve Clostridium tetani, it is only when these organisms invade the blood stream or the muscle that they become really dangerous. (Maclennan, 1962).

It is necessary however to distingush between the simple contamination and the actual invassion of muscle tissues by anaerobic bacteria. These organisms are so common and so widely distributed in nature that their presence in a wound is the rule rather than the exception; and very few wounds fail to involve the muscles.

True invassive myonecrosis and botulism are fortunately, relatively rare complications. They are diseases, as Ogilvie has happily remarked, associated with a particular opportunity rather than a particular organism (Maclennan, 1962 and Smith, 1977).

Fortunately, of the 300 species recognised in the genus Clostridium, 61 species have been studied, 13 of which are pathogenic for laboratory animals and produce one or more toxins, (see table I). An additional 16 species have been found in infected wounds or infections of animals or humans C fallax, C. subterminale, C sporogenes, C plagarum, C. sphenoides, C. indolis,

C. tertium, C. pseudotetanicum, C. ramosum, C. cochlearium, C. innocuum, C. perenne, C. cadaveris and C. putrificum, are species that are known not to produce detectable toxins. However, there is the possibility that some of them may have a synergistic action in a mixed bacterial infection, Furthermore only eight out of the 13 pathogenic species are established and well documented pathogens of man. These are Clostridium botulinum, C. tetani, C. perfringens, C. novyi, C. septicum, C. histolyticum, C. sordellii (C. bifermentans) and C. fallax. The role of C. difficile in the pathogenesis of man is not clear, but it is thought to play some role in antibiotic-associated pseudomembranous colitis (PMC), (Arbuthnott, 1978; Collee et al 1971 and Duncan et al 1975).

Table I: Pathogenic Clostridial Species.

Species	Diseases
Clostridium botulinum (8 tyes)	Botulism
C. tetani	Tetanus
C. perfringens (*C. welchii*)	Wound infections; Enterotoxaemia.
C. novyi (*C. oedematiens*) (3 types)	Wound infections
C. septicum	Wound infections
C. haemolyticum	-"-
C. sordellii	"
C. limosum	Wound infections
C. chauvoei	Wound infections
C. histolyticum	Wound infections
C. difficile	Wound infections
C. carnis	Wound infections
C. fallax	Wound infections

Reproduced from Duncan, C. L. (1975)

Infections caused by pathogenic clostridial species, in addition to the ones listed above, include, though uncommon, uterine infections usually inolving the gravid uterus.

Gas gangrenous infection, such as those originating in fibromata, have been described. The majority of cases resulting from attempts at criminal abortion, and less commonly during labour, spontaneous abortion or puerperium. (Maclennan, 1962). Gas gangrene of the eye and gas gangrene of the brain have been reported in the literature. These are acute and destructive infections, almost invariably due to Clostridium perfringens alone.

In general, the Clostridia have minimal invassive capacity, in other words they are opportunistic pathogens that are able to produc disease only when the tissues of the primary lesion have been altered to suit their limited and perculiar needs (Arbuthnott, 1978; Smith et al 1974).

The aim of this work was to investigate the occurence of pathogenic clostridial species in areas that are heavily congested with human activities, and to investigate their incidence in wounds and sterilized surgical instruments as sources of human infection. The findings, it is hoped will further highlight the public health importance of current preventive measures against the most commonly occuring clostridial organisms pathogenic for man.

LITERATURE REVIEW:

Tetanus:

Tetanus resulting from an infection with *Clostridium tetani* has long been recognized as a disease of wounds of humans and animals. Even minor wounds are exposed to tetanus and nowadays minor wounds are more commonly associated with tetanus than major wounds because of the omission of protective measures in instances of minor cuts or abrasions that do not require medical attention.

The desease is a simple toxinosis in which the organism multiplies in the oxygen-free enviroment and produce tetanospasmin. Smith et al in 1974, showed that growth and toxin production by tetanus bacilli follows immediately after infection by *C. tetani* spores. Tetanospasmin is the toxin entirely responsible for the effects of the disease. Incubation period of tetanus varies from few days to several weeks.

In 1966, Lettl and his co-workers, as cited by Maclennan (1962), postulated that de novo synthesis of toxin in the bacterial cell takes place during the autolysis process. Thus the importance of tetanus toxin derives first from the fact that it is capable of producing all the symptoms of clinical tetanus and second from the fact that tetanus is today a world-wide problem: several hundred thousand people all over the world are affected each year by this toxigenic infection.

In the last two decades the toxin has been isolated in a homogenous state, and its physical and chemical properties have been defined in various laboratories in different parts of the world. The production of the toxin in high yields has been found to be dependent on several factors, namely, (i) the presence in the culture medium of particular toxigenic factors, (ii) the intrinsic toxigenic capacity of Clostridium tetani strains, and (iii) the mode of cultivation. (Arbuthnott, 1973; Duncan, 1975; and Evans, 1947).

Although the toxigenic factors are still to be identified, semisynthetic and synthetic media enabling the production of tetanus toxin to varying degrees have been formulated. Nowadays the most widely used medium is the one described by Latham et al (1962). More recently it has been found that Clostidium tetani can grow in both liquid and solid forms of a defined and commercially available tissue culture medium 109 if it is supplemented with cystein and ascorbic acid. (Watt et al 1979; Willis et al 1970).

It is also interesting that toxigenic strains of C. tetani lose their toxigenicity to varying extents after repeated subcultures in liquid medium.

It has been established that the decrease in the capacity of the organism to produce the toxin is dependent to some degree on the medium used. Toxigenicity was better preserved when the

organisms were cultured on semisolid complex medium with glucose than when they were cultured in liquid complex medium or semisynthetic medium. According to Nielsen (1968) the decreasing ability of toxigenic strains of C. tetani to produce the toxin is likely to be related either to a biochemical mutation or to a modification of the cell wall receptor mechanism. The former assumption is supported by the observation that toxin synthesis is influenced by supplementing the culture medium with certain amino acids. Subsequently, Mellanby (1968) has reported that an excess of glutamate in the medium accelerates the growth of the bacteria and reduces the time necessary for autolysis to start as compared with the time taken in conventional Mueller medium. Since less toxin is synthesized in the presence of excess glutamate, it appears likely that most of the toxin is being produced during the stationary growth phase. In the presence of an excess of glutamate the change induced in the bacteria, resulting in a shortened stationary phase, could then account for the apparently decreased toxigenic capacity of the C. tetani strain (Bizzini, 1979; Diamond et al 1971).

Tetanus bacilli are pleomorphic filamentous rods, motile by means of long peritrichous flagella. The spores are early in development. The fully developed spore is characteristically terminal and spherical, two to four times the diameter of the

bacillus (drumstick). It is a strick anaerobe, with temperature range between 14°C - 43°C, and optimum temperature for growth is 37°C. It can grow on ordinary nutrient agar but grow best on cooked-meat medium or freshly poured blood agar plates (Shoemaker et al 1976).

Colonies on solid medium show fine branching projection, or fine spreading growth may extend over the entire surface of medium which may not be readily seen on examination. It shows haemolysis on horse blood agar in the region of initial growth and may not extend to the region of spreading growth (Hoeniger and Tauschel 1974).

It liquifies nutrient gelatin slowly. Litmus milk medium may show no coagulation or there may be delayed clotting. Slight blackening (digest) of cooked-meat medium occurs due to breakdown of amino acids, but no carbonhydrates are fermented by typical strains of C. tetani. (Watt and Brown 1975; Willis and Williams 1970).

Ten antigenic types are recognized by agglutination test involving flagellar H antigens. Type 6 consists of non-flagellate strains. All types produce the same neurotoxin and toxigenic and non-toxigenic strains may belong to the same group. (Smith and MacIver 1974).

The symptoms of tetanus are primarily spasmodic muscular contraction which ultimately may or may not be lethal depending on

the wound, the site of infection and the treatment. The natural disease does not confer immunity because the lethal dose is insufficient to induce it. The disease is preventable by active immunisation with toxoid. This has proved effective in protecting populations against tetanus. Antitoxin is effective prophylactically only if the toxin has not become fixed to its specific receptor in the nervous tissue. (Ipsen, 1961; Price et al 1977).

(i) Nature of tetanospasmin:- It was the second bacterial toxin to be discovered (diphtheria being first), (Arbuthnott, 1978). It is a heat labile protein that may be inactivated by heating for 20 minutes at 60°C. The isoelectic point is between 5.0 to 5.2. The toxin is of a single antigenic type that can exist in a biologically active state in the form of a monomer with molecular weight of 67,000 or a dimer with a molecular weight of 150,000 which is non-toxic (Arbuthnott, 1978; Bizzini, 1979; Smith, 1977).

Tetanus toxin is a holoprotein with most reports of the amino acids composition being in fairly good agreement. The toxin has been found to contain six free - SH groups and two disulphide bridges. The presence and nature of amino-terminal groups are controversial but most investigators identified either leucine or glycine as the single amino-terminal residue.

Two structural models have been proposed for tetanus toxin. Bizzini and his co-workers (1979) investigated toxin derivatives modified in various ways by using the techniques of sodium dodecyl sulphate - gel electrophoresis, gel filteration and ultracentrifugation. From their results they proposed that tetanus toxin might consist of two identical subunits with molecular weights of 75,000, each subunit being formed from two non-identical chains with molecular weights of 50,000 and 25,000, respectively, held together by disulphide links. The second structural model was first described by Craven and Dawson in 1973 and has been confirmed and extended in more recent reports. According to this model, the intracellular toxin would consists of a single polypeptide chain with a molecular weight of 150,000 and the extracellular toxin would consists of two non-identical polypeptide chains with molecular weights of 100,000 and 50,000 respectively. Subsequently, Matsuda and Yoneda in 1975 postulated that extracellular toxin is nicked intracellular toxin. These authors nicked intracellular toxin by mild trypsinization and showed that the two constituents subchains could be separated by gel filteration. They also succeeded in reconstituting the toxin molecule from the separated constituent subchains.

Each of the constituent subchain of tetanus toxin has been found to exhibit residual toxicity. This toxicity is probably due to contamination of each subchain preparation by a trace of the other, allowing some reconstitution of the toxin, (Maclennan, 1962).

In cell cultures, the prototoxin has been found to be converted into biologically active toxin by nicking of the polypeptide by a bacterial protease.

(ii) Site of action:- Tetanospasmin is a neurotoxin that does not act on any isolated cell or fluids of the body. The substance responsible for fixation of tetanospasmin in nerve tissue is ganglioside. The ability of a specific ganglioside to bind toxin is dependent on the number of sialic acid (N - Acetylneuramic acid) residues and their position in the ganglioside. Ganglioside with two sialic acid residues attached to each other have the greatest binding capacity. Gangliosides GDib and GTI were active, being capable of binding up to 20 times their weight of tetanospasmin. Ganglioside which contain only one sialic acid residue in the middle of the oligosaccharide chain has poor toxin-fixing ability, (Arbuthnott, 1973; Bizzini, 1979).

How the tetanospasmin reaches the central nervous system (CNS) from the local site of infection is still not clear. It is suggested that it reaches the CNS via blood or neurally, by way of

tissue space of the peripheral nerves. In local or ascending tetanus, the toxin appear to be absorbed by motor nerve endings and moves from the tissue along major regional nerve trunks to its site of action in the central nervous system. In general or descending tetanus, large amounts of toxins that are not totally absorbed by the local motor nerve endings may be taken into lymphatic and circulatory systems with subsequent absorption by all of the motor nerves. The sequence of spasmatic response of the various muscle groups may represent the degree of sensitivity of the various neural centers to tetanospasmin, (Bizzini, 1979; Smith, 1973).

The degree of susceptibility of animal species to tetanospasmin varies. Cold-blooded animals are quite resistant, birds are relatively resistant and mammals are very sensitive. The resistance of some animal species does not appear to be due to absence of the specific-binding ganglioside. Frog brain has a low toxin-binding capacity, while man has a high toxin-binding capacity. The difference in toxin binding capacity is thought to be that, in frog the complex of brain ganglioside with other constituents may differ from that of mammalian brain, (Arbuthnott, 1978).

Principal route of entry of tetanospasmin is the abraded skin. A few cases of tetanus in humans have developed from inhalation of dust of powdered toxin. It is considered harmless when administered in moderate amounts by rectum, vagina, conjectiva or trachea. Large doses are toxic by the oral route, (Duncan, 1975).

(iii) Mode of Action: There is no simple in vitro assay for tetanospasmin. Its biological activity can be demonstrated only in the whole animal or in nerve muscle preparations. This makes the task of investigating its mode of action at molecular level mere difficult. At the physiological level, tetanospasmin, as earlier mentioned acts mainly on the CNS. It produces uncoordinated muscular contractions of skeletal muscles by blocking inhibitory neurons which control the motor activities of the CNS. This results into a spastic paralysis in contrast to botulism which produces flaccid paralysis. It has however been shown that, intramuscular injection of tetanus bacilli into rabbits produced in botulism, (Arbothnott 1978). There is also evidence that the toxin can act peripherally at the level of neuromuscular transmission by interfering with the release of acetylcholin in a manner similar to botulinum toxin, (Bizzini, 1979)

Diamond and Mellanby in 1971 demonstrated that in goldfish the peripheral action of tetanospasmin produces flaccid, rather than spastic paralysis, and that the toxin appear to block neuromuscular transmission yet did not affect the muscle response to direct electrical stimulation. There was also no effect on either inhibition or excitation of the central nervous system, indicating only a peripheral effect of the toxin. Recent studies (19 by Bigalke and his co-workers demonstrated that acetylcholine synthesis was inhibited by tetanus toxin. However, the activity of choline acetyltransferase was not affected by the toxin. The inhibitory action of tetanus toxin on choline uptake was assumed to be due to an indirect "stabilizing" effect of the toxin on the intracellular pool of acetylcholine, preventing its release from the cell. Paralysis as a result of tetanus toxin action has also been reported to occur when the toxin was prevented from accumulating in the spinal cord. This phenomenon might also account for the flaccid paralysis caused in animals by the injection of low-nitrated toxin derivatives, bearing in mind that these derivaties have lost their capacity to bind to gangliosides. From comparison of actions of tetanus toxin and type A botulinum neurotoxin, using the above cholinergic systems,

Bigalke and his co-workers concluded that the two toxins differed in their effects only on a quantitative basis, (Smith, 1973).

Thus tetanospasmin may induce two pharmacological responses.

(i) Spastic paralysis via action on the CNS.

(ii) Flaccid paralysis of muscles by a direct peripheral action.

Whether the interference with the neuromuscular transmission occur presynaptically as in botulism or postsynaptically is not known. But it is known that in man botulinum toxin only acts peripherally rather than on the CNS to produce flaccid paralysis, (Arbothnott, 1978).

(iv) Other tetani toxins:- Clostridium tetani also produces a haemolysin known as tetanolysin. Tetanolysin is reversibly inactivated by oxygen. It is heat-labile and it is serologically related to the oxygen-labile haemolysins produced by other bacteria including Clostridium perfringens; C. novyi, Streptococci and Pneumococci, (Arbothnott, 1973). It is responsible for the haemorrhagic lesion of tetani infection. In addition C. tetani also produces a nonspasmogenic neurotoxin that affects the peripheral neuromuscular junction. The nonspasmogenic neurotoxin has been reportedly neutralized by tetanus antitoxin and it is not fixed by ganglioside. Its molecular weight is between 35,000 to 40,000. The role of this neurotoxin in tetanus is unknown, (Smith, 1973).

Botulism:-

The C. botulinum group of organisms produce neuroparalytic toxins that are considered to be the most toxic substances known to man. One microgram of purified C. botulinum type A toxin contains about 250,000 minimal lethal dose for the mouse.

Eight immunologically distinct neurotoxins (A,B,C_1,C_2,D,E,F and G) are produced by C. botulinum group of organisms. These toxins produce similar pharmacological effects, and they form the basis of grouping C. botulinum group of organisms into types A to G. The proteolytic strains of types A,B and F and a few of type E have been implicated in cases of botulism in man, whereas the nonproteolytic strains, types C,D and E have been responsible for most instances of botulism in domestic and wild animal populations. Type G was isolated from soil and has not been implicated in cases of natural botulism. (Duncan, 1975; Sugiyama, 1980).

Botulism is caused by ingestion of toxin preformed during growth of the organism in the food. Cases of wound associated botulism is thought to be similar to those in tetanus, in which the organism multiplies in the wound and produces the lethal toxin, (Solomon et al 1971; Sugiyama, 1980).

In some cases of botulism, the incubation period is prolonged. The symptoms may reappear after therapeutic treatment and recovery of the patient. A toxicoinfectious theory has been advanced for the prolonged incubation period and recurrence of botulism. It implies that botulism is as a result of growth and toxin formation in the intestinal tract. Evidence against this theory is based on the fact that C. botulinum lacks the ability to compete against other organisms for a niche in the intestinal tract. (Arbuthnott, 1973).

The symptoms of botulism in man are associated with its paralytic action. The incubation period depends on the dose but it may be only a few hours to several days. Nausea and vomiting are common in cases with a short incubation period. Other symptoms of the disease in man include difficulty in swallowing and breathing, double vision and flaccid paralysis of the extremities. In later stages of the disease respiration becomes difficult, (Smith, 1977).

Administration of type specific antiserum neutralizes the toxin that has not been fixed to its receptor in the nervous tissue.

This is of therapeutic value when administered in the early stages of the disease. Toxoids are also of value in protective immunization, however as in tetanus, natural immunity is thought not to occur because the lethal dose is insufficient to give rise to immunity, (Duncan, 1975; Sugiyama, 1980).

(i) Culture - toxin relationships:- The property that separates Clostridium botulinum from other clostridial organisms is the production of a neurotoxic protein which causes flaccid paralysis by acting at peripheral cholinergic junctions. The antigenically different toxins are identified as toxin types. The same neurotoxin is consistently produced by a particular culture, but toxins of different antigenic specificities are produced by different groups of culture strains,(Boroff and Fleck 1966; Solomon et al 1971).

The established toxin types are A,B,C1, C2, D, E, F and the recently identified type G, (Sugiyama, 1980). Generally a culture type produces one type of toxin. C. botulinum is considered to contain four culture groups with respect to their proteolytic activity. Group I cultures are ovolytic (proteolytic) strains which produces toxin type A,B or F; group II cultures are non-proteolytic strains which produce toxin type B,E or F; group III

cultures are weakly proteolytic or non-proteolytic strains which produce toxin type C1, C2 or D and group IV contains the proteolytic but non-saccharolytic strains that produce type G toxin. Proteolytic strains that produce type E toxin have been isolated recently by Nakane in 1977.

Cultures of one group are closely related among themselves but differ in important ways from cultures of another group, so that the cultural characteristics are not always evident when a strain is identified only by its toxin type. Except for the antigenicities of the toxins, a type B culture of group I is very similar to a type A strain but quite different from a type B culture of group II. Intragroup homogeneity and intergroup heterogeneity are shown by the antigens of vegetative cells and of spores by homologies of DNA and RNA and by susceptibility to bacteriophages, (Lee and 1970).

All human botulism cases are caused by cultures of group I and II. Cultures of these groups differ in their properties which influence the potential for causing food poisoning. Spores of group I cultures have high heat resistance relative to spores of group II cultures and group II cultures can grow at a lower temperature (about 4° versus 10°C) than cultures of group I.

Group III cultures are important causes of botulism in several animal species, (Smith, 1977).

As a rule, the toxin in culture fluids of group I strains is at or near its maximum possible toxicity, whereas the toxin of group II cultures must be activated with trypsin before it becomes fully toxic. This difference is due to the enzymes produced by the cultures and is reflected in the molecular form of the purified toxin, (Bonventre and kempe 1959; Das Gupta 1971; Eklund and Poskoy 1972).

(ii) Pathogenic forms of botulism:- The toxin that causes botulism can be acquired by one of three ways. The classically known botulism food posoning is due primarily to toxin that is formed in and digested with a food. In infant botulism, toxin is produced in vivo during intraintestinal growth of the organism. Wound botulism is also caused by toxin formed in vivo, but in this case the toxin is formed in a wound, (Sugiyama, 1980).

Epidermiological reports suggest that outbreaks of food poisoning botulism involving most cases occured in 1977 and caused illness in 58 persons and another in 1978 which involved 34 cases in United States. Home prepared foods are more important than commercially processed foods, and vegetables

and fruits are more common vehicles of botulism than are meat or fish. Most of the cases were due to C. botulinum types A and B, a few of type E and one outbreak due to type F. Definite proof of clinical infant botulism was obtained in 1976 when C. botulinum and/or toxin was demonstrated in the faeces of infants suffering from constipation and neuromuscular weakness. Only type A and B cases have been reported. Among specimens from 211 sudden death cases, the bowel contents of 9 infants contained C. botulinum, and two of these also had the homologous type of toxin. These findings contrast with the occurence of C. botulinum in only one faecal specimen from 160 agar-matched healthy (control) infants. Infant botulism occur only in children between 3 and 26 weeks old. Type A cases are slightly more frequent than type B cases. Since no case can be attributed to a food that contained toxin, the presence of C. botulinum and its toxin in faeces of acutely ill infants and convalescents indicates that the illness is a toxico-infection in which the organism colonizes and produces toxin in the intestinal tract. Botulism is therefore a food poisoning in the sence that the organism is most likely acquired, spores probably being the infective form, (sugiyama, 1980).

Wound botulism is reported to be the rarest type. It is as a result of C. botulinum infecting a wound by itself or with other microorganisms and producing toxin, which reaches other parts of the body via the blood stream. A local lesion distinguished this botulism from food posoning, but the neurological effects are comparable. Only types A and B cases have been reported in United States, (sugiyama, 1980).

(iii) Nature of botulinum toxin:- Botulinum toxin is also an intracellular toxin that is released from cell after autolysis or mechanical rapture. It is the most toxic substance known to man; one microgram of purified type A, C. botulinum toxin contains 250,000 minimal lethal dose for mouse, (Arbuthnott, 1978).

The toxins of non-proteolytic strains types C,D,E and G and a few of types B and F strains seem to be synthesized as protoxins and must therefore be activated by an enzyme, trypsin, before they can exhibit their full toxigenic potential, (Sugiyama, 1980). Trypsin cleaves the toxin molecule into at least two polypeptides. It acts by reducing the disulphide bond linking them. This activation of prototoxins of non-proteolytic strains of C. botulinum in the intestinal tract, is thought to occur by proteases before its absorption into the blood.

The toxins of proteolytic strains are activated by an endogenous trypsin - like enzyme present in the culture.

Characterisation of the molecular properties of botulinum toxins is complicated by the fact that purified toxins are complexes of neurotoxin (M.Wt 150,000) and other components like haemagglutinin. However, type A toxin has been crystallized as a single protein of 900,000 M.Wt. It is found to be a complex consisting of a 120,000 to 150,000 neurotoxins and a 500,000 haemagglutinin. The Molecular weight of the neurotoxin of types B, E and F and probably of types C and D are thought to be in the range of 150,000 to 167,000. Type E toxin has a 350,000 neurotoxin and nontoxic components,(Duncan, 1975).

The size of the toxic unit, the role of haemagglutin and other non-toxic components in pathogenesis of the various types of neurotoxins is not clear. One suggestion is that haemagglutinin protects the neurotoxin from the digestive enzymes before it enters the blood stream. This suggestion is based on the fact that the neurotoxin by itself is labile to digestive enzymes and to low PH of the stomach. It is also found that several thousand times more neurotoxin than the toxin complex is required to cause botulism in animals by feeding, (Arbuthnott, 1978).

(iv) Site and mode of action:- Site of action of the toxin is the peripheral nervous system. And unlike the tetani toxin, the central nervous system is not affected. This may be due to lack of receptive sites for the toxin in the CNS. The toxin binds specifically at the neuromuscular junction and inhibits the release of acetylcholine (Ach) at the motor end plates. It acts presynaptically to block the release of acetylcholine. The mechanism by which this is done is not know. But it is known that neither the synthesis nor degradation of acetylcholine is affected by the toxin. It is suggested that botulinum toxin in a way antagonises serotonin - mediated transport of calcium ions across the cell membranes. This in turn affects acetylcholine release, since the amount of acetylcholine released per nerve impulse is a function of the extracellular calcium ion concentration, (Simpson and Morimoto 1979).

Observations made of presynaptic site of action of C. botulinum toxin in the early studies with intact animals have been confirmed by in vitro experiments with nerve-muscle junctions (NMJ), such as the isolated phrenic nerve diaphram isolated from rats and mice.

When the toxin is added to a bath fluid, the muscles progressively lose their responsiveness to indirect stimulation but their activity is restored when acetylcholine is added or when it is electrically stimulated, (Sugiyama, 1980).

These observations could be explained in several ways, but impossible explainations can be eliminated. Paralysis could result if toxin blocks transmission through the terminal nerve fibrils, where there is no myelin sheath, but this is unlikely since action potentials can be recorded from this part of the nerve, (Arbuthnott, 1978).

Nerve-muscle junction becomes paralyzed from a lack of transmitter if acetylcholine synthesis is inhibited, but observations show that botulinum toxin does not affect the availability of choline and acetyl-coenzyme A or their synthesis into acetylcholine. The toxin does not inhibit acetylcholine synthesis by choline acetyltransferase preparations, synaptosomes (responsible for the synthesis of acetylcholine), or brain slices, and the diaphrams of poisoned and control animals have similar amounts of transmitter. Direct stimulations of paralyzed nerve-muscle junctions release as much acetylcholine as indirect stimulations of controls, (Sugiyama, 1980).

Unless acetylcholine reacts with postsymaptic receptors and is rapidly inactivated by acetylcholinesterase, the receptors do not respond to the transmitter that is released next. Moreover, a poisoned nerve-muscle junction still responds to exogenous acetylcholine. The elimination of these possibilities leaves as the most likely explaination an action at the nerve ending itself, which prevents release of transmitter, (Simpson and Morimoto 1969).

In vitro experiments with central nervous system have shown that the effects produced by intracerebral injection of impure toxin samples are difficult to attribute to the neurotoxin itself. The responses that follow intravenous challenges with solutions of type A toxic crystals are more acceptable as being caused by toxin. Most workers believe that a blood-brain barrier prevents botulinum toxin from entering the brain tissue itself, (Sugigama, 1980)

Death from botulinum intoxication is a result of flaccid paralysis of the autonomic nervous system. This in turn interferes with breathing and terminal asphyxia (Duncan, 1975).

Two different chemicals that have been used in the treatment of botulism are guanidine hydrochloride and germine monoacetate. Guanidine hydrochloride is thought to act presynaptically to

enhance the release of acetylcholine from nerve terminals, while germine monoacetate acts postsynaptically to increase the muscle acetylcholine intake. A combination of the two chemicals in animals produces a greater treatment effect than either drug used alone, (Puggiayi and Chorington 1978).

(v) Similarities of tetanus toxin:- Botulinum and tetanus (tetanospasmin) toxins are neurotoxic proteins produced by closely related Clostridia. Tetanus toxin was originally determined to be a 4S molecule, but all recently determined values are closer to 7S. Its molecular weight, as determined in different laboratories, ranges from 127,000 to 174,000, with most values between 140,000 and 160,000. These values are within the range of molecular weights which have been determined for the botulinum neurotoxins, (Arbuthnott, 1973).

Tetanus toxin has not been reported to form complexes comparable to those of botulinum toxin, but different purification methods may be the possible reason for this. The specific lethalities of toxins which produce different paralytic effects may affect vital functions to varying degrees but cannot necessarily be compared meaningfully. Despite this reservation, it is interesting that the mouse killing potencies of the two toxins are so similar.

The values for tetanus toxin are often in the range from $1x10^8$ to 3×10^8 minimal lethal dose per mg. of N (or 1.6×10^7 to 4.8×10^7 MLD per mg. of protein if a 16%N content is assumed). Since one LD_{50} can be expected to have less toxin than one MLD, the specific lethality of tetanus toxin for mice is within the lower range of the specific lethality of botulinum toxins, (Bizzini, 1979).

Tetanus toxin is like the toxin of proteolytic botulinum cultures in that it is synthesized as a single - chain protein and is nicked into a dichain molecule by an enzyme(s) of the culture after it becomes extracellular. The effect of trypsin on intracellular tetanus toxin confirms that this toxin is synthesized as unnicked molecules, which are converted by trypsin into dichain molecules that are indistinguishable from the naturally formed dichains recovered from the culture fluids.

Tetanus results primarily from effects on the spinal cord, whereas botulism, is due to interference at cholinergic junctions. Since tetanus toxins is normally acquired from a wound infection, its route to the spinal cord has been studied intensively, and it is now accepted that the toxin reaches the

spinal cord by centripetal neural transport, particularly in motor nerves. The movement of radiolabelled toxin has been used to trace this migration. Intramuscularly injected botilinum toxin also enters the spinal cord by a similar pathway. The two toxins cause muscular paralysis by inhibiting transmitter release. The paralytic effects differ because the two toxins act at different sites with different kinds of synapses, (Arbuthnott, 1973; Bizzini, 1979).

Clostridial Wound Infections other than tetanus:

Apart from the rare cases of anaerobic streptococcal infection, the microorganism of prime importance in gas gangrene are obligate anaerobic, spore-bearing bacilli; that is, they are clostridial species. The Clostridia comprise a very large and diverse group of essentially saprophytic organisms, which are widely distributed in nature: in soil, in dust, in water and in the alimentary tract of most animals. Their large numbers and wide distribution thus render them one of the commonest types of bacteria to be found in wound; indeed in most parts of the world any large wound is likely to contain Clostridia. Fortunately only very few species are generally

accepted as pathogenic and still fewer as capable of causing gas gangrene in man, (Maclennan, 1962). This last group are six in number, Clostridium perfringens, C. novyi C. septicum, C. bifermentans (C. sordellii), C. histolyticum, and C. fallax. And all owe their pathogenicity to the pssession of soluble exotoxins which have the power to destroy blood and tissue cells, (Arbuthnott, 1978).

Mention may also be made of Clostridia difficile. Stock (1974) have it that this histotoxic anaerobe was first isolated by Hall and O'Toole from the meconium and faeces of newborn children. It proved to be pathogenic for experimental animals but until world War II had never been found in any pathological lesion of man or animals.

Clostridial species, generally are so common and so widely distributed in nature that their presence in any wound is the rule rather than exception; and very few wounds fail to involve muscle. True invassive myonecrosis, is, fortunately, a relatively rare complication. It is a disease, as Ogilvie has happily remarked, quoted Maclennan (1969), associated with a particular opportunity rather than a particular organism. Thus, Clostridial wound infections are not bacteriological entities, they are clinical

conceptions, and accordingly must be defined in clinical terms. On this basis, clostridial wound infections may be divided into three classes, (Duncan, 1978; Maclennan, 1962). These are:-

(a) Simple contaminations.

(b) Anaerobic cellulitis.

(c) Clostridial myonecrosis (gas gangrene).

Although the majority of these infections are sufficiently common and distinct to be called separate "diseases" few are so defined from the pathological point of view; many fade into or overlap one another, and between any pair it is rarely possible to draw a rigid dividing line. For example in simple contaminations of Clostridia, one or more species may be present but they do not invade the underlying tissues. There is a little pain and as a rule systemic form of the infection is not involved, and resolutely the wound refuses to heal. Any type of wound may be affected in this way, even small superficial abrasions. Clinically such lesions are rarely recognised as clostridial infection at all; but it will be found that the elimination of the anaerobic bacilli from the wound flora almost invariably results in prompt healing, (Arbuthnott, 1978).

However, simple contaminations of wound by Clostridial species may progress to anaerobic cellulitis. This is a heavy clostridial infection of the necrotic tissue which has already been killed not by bacterial action but by ischemia or by direct trauma. Infact healthy muscle layers are not involved. Toxaemia or shock is also not involved. But when proteolytic Clostridia are involved in anaerobic cellulitis, the wound may have a brownish, foul discharge as a result of proteolysis, of the necrotic tissue, (Duncan, 1975).

Clostridial myonecrosis or true gas gangrene entails acute invasion of true healthy living muscles. In the terminal stages, severe prostration and apathy and irreversible shock may occur. Death follows immediately. The reason for this is unkown, but it appears to be related to intoxication in which the toxin reaches and affects critical points in the body. The incubation period of gass gangrene is usually short, almost always less than three days and in a majority of cases less than 24 hours. An incubation period of one hour is on record, (Maclennan, 1962). It is also worth noting that clostridial spores may remain dormant in scar tissue for considerable length of time and then give rise to gas gangrene years after the original injury as a result of quite minor degrees of trauma which need not involve an open lesion, (Duncan, 1975).

Table II: Clinical Differenciation of Clostridial Varieties of anaerobic myonecrosis.

Creterion	Clostridial Spcies					
	C perfingens	C novyi	C septicum	C histolyticum	C bifermentans	C f'allax
Incubation	18-24hrs	2-6days	1-3days	2-3days	4days	2-3days
Onset	Sudden	Sudden	Sudden	?	Sudden	Sudden
First symptom	Pain	Weight	Pain	?	Pain	?
Swelling Oedema	++	+++	++	+	+++	Nil
Gas	++	-or1	+++	Nil	1	Nil
Smell	+	-	+	Foul	Foul	Fishy
Wound Exudate (i) Amount	++	++++	+++	+++	+++	?
(ii) Nature	Haemo-rrhagic	Yellow	Very haemo-rrhagic	Brownish	Haemorrhagic	?
Taxaemia	++	++++	+++	++	+++	+++
Rapidity of course	++	+++	++++	1	+	+

Reproduced from Machennan, J. D. (1962).

(i) Cultural Characteristics of Wound-associated Clostridial species:-

(a) Clostridium perfringens:- Equally well known as C. welchii and Bacillus aerogenes capsulatus, this organism was first described by Welch and Nattal in 1892 as cited by Machennan (1962), and it is at once the commonest, the most important, and the best known of the gas gangrene Clostridia. In a smear made from an acute case, C. perfringens appears as a short, plump, Gram positive rod with the rounded ends and a well marked capsule.

C. perfringens is unique among the pathogenic Clostridia in being non-motile, and it is usual in that those strains associated with gas gangrene very rarely form spores in the tissues or even in the common laboratory media. It is, however, an easy organism to cultivate. On blood agar it forms typical round, flat, creamy colonies, about 3mm in diameter, surrounded by a well marked zone of beta-haemolysis and frequently by further concentric rings of less complete haemolysis.
This target appearance on blood agar with the colony forming the bull's eye is due to presence of several distinct haemotoxins.
If the organism is grown on nutrient agar to which has been added either sterile human serum or a sterile suspension of egg yolk (lecithovitelin (LV) agar plates), the colonies will be

seen to be surrounded after a few hours of incubation by a zone of opacity, owing to the splitting of the lecithovitellin in the medium. This, the so called "Nagler Reaction" or"LV reaction" has been much used in the rapid identification of C. perfringens, particularly in a mixed culture, (Maclennan, 1962).

The bacillus liquefies galatin, and ferments most of the common carbonhydrates with the evolution of acid and large amount of gas. In litmus milk the breakdown of lactose produces an acid clot which is rapidly disintergrated by the gas evolved. This stormy fermentation of milk is fairly characteristic of C. perfringens and has been advocated as a test of diagnostic importance. It is worth noting that C. septicum and E. coli give the same general reaction, therefore as a single diagnostic creterion, stormy fermentation can be regarded as of very little value. (see table VI). (Blair, [illegible] and Truant, 1970).

(b) C. novyi:- It is also known as C. oedematiens and is one of the earliest pathogenic anaerobes to be isolated, but since it proved so difficult to grow and still more to obtain in pure culture, its significance in gas gangrene was long thought to be slight.

Today it is rightly recognized as a most important cause of gas gangrene in man, and a number of severe infections in domestic animals, (Collee, 1979).

In smears from wound exudate, C. novyi is seen as a large Gram positive rod, often endowed with a swollen subterminal spore. It is motile and non-capsulated. In artificial culture it is always slow and often difficult to grow and therefore can easily be missed in a culture. Initially the strictest anaerobic conditions are required, together with presence in the medium of blood or serum. On solid medium it has a tendency to spread in a continous film or even to produce motile colonies; but if discrete colonies are obtained, they are flat, delicate, irregular, rough and "maplike". On blood agar, they are usually surrounded by a narrow zone of partial haemolysis, (Bard and McClung 1948; Collee, 1979).

C. novyi gives a weak Nagler reaction owing to the production of lecithinases distinct from that of C. perfringens. It liquefies gelatin, digest casein and blackens cooked meat medium. Of the commonest carbonhydrates, this organism ferments only glucose and maltose.

(c) C. Septicum:- This was the first pathogenic anaerobe to be identified by Pasteur and Joubert in 1877, as quoted by Machennan (1962). In smears from wound it is usually seen as a slender, motile, Gram positive rod, often with a large subterminal spore. Occassionaly it shows pleomorphism in both wound smears and artificial culture.

C. septicum is relatively easy to grow, but difficult to isolate because of its tendency to form continous films of growth on solid meida rather than discrete colonies. But individual colonies, when they occur, are flat, very delicate and filamentous, and surrounded by a well marked zone of haemolysis. Gelatin is slowly liquified, and all the common sugars are fermented with the importance exception of sucrose. (see table VI) stormy fermentation occurs in litmus milk, but to a lesser extent than in the case of C. perfringens, (Garcia and Mckay 1969; Willis and Williams 1972).

C. septicum is of greater importance in animal than in human infections. It resembles C. chauvoei very closely to the extent that it is difficult to separate them into different species. Moussa, in his studies suggested that C. septicum and C. chauvoei might well be considered as types A and B of a single specie, presumably C. septicum,(Moussa, 1958).

(d) C. histolyticum:- This organism has never been considered a commonly found organism. Maclennan (1962) quoted that 'it was not until 1916 when it was first described by Weinberg and Seguin and since then has only been periodically identified!. In wound smears it resembles, C. perfringens closely, except for the absence of a well marked capsule and its sluggish motility which is often difficult to demonstrate.

It grows best under anaerobic conditions, but scanty growth can be obtained aerobically and this property has been used as an aid in its isolation. On blood agar the colonies are small, rough, and irregularly round and surrounded by a narrow zone of partial or (more rarely) complete haemolysis. In artificial culture rough variants occur. Unlike the Clostridia so far described, C. histolyticum has a powerful digestive action on native proteins and, in addition to gelatin, rapidly liquefies coagulated serum, egg white, and meat particles. Litmus milk is readily peptonized. C. histolyticum produces no action on any of the common carbonhydrates. Although the organism does not elaborate a lecithinase, it may give rise to slight turbidity in lecithovitellin solutions, presumably due to the release of bound fat by its proteolytic action, (Jennison, 1947, Maclennan, 1958).

(e) C. bifermentans (C. sordellii):- The exact status of this organism is uncertain. Maclennan (1962) cited that an organism known as C. bifermentans was isolated from putrefying butcher's meat by Tissier and Martelly in 1902. It was nonpathogenic but was frequently found thereafter, both in wounds and in normal intestinal tract. He continued saying that, 'in 1922, Sordelli, isolated a new, highly toxigenic Clostridium from a case of gas gangrene, and from there afterwards similar strains were reported. The organism was ultimately named C. sordellii, but Cark, Hall and Stewart produced evidence strongly suggesting that C. bifermentans and C. sordellii were simply innocent and pathogenic strains of the same organism'. They are biochemically related except that C. sordellii produces urease whereas C. bifermentans does not, (Brooks and Epps 1959; Maclennan, 1958).

In wound smears C. bifermentans resembles C. perfringens very closely; i.e. it is a large Gram positive bacillus with rounded ends. Motility is difficult to establish and spores are rarely formed in the animal body, but readily appears in culture; they are central and slightly distend the bacterial cell.

It is a difficult organism to grow, requiring stickly anaerobic conditions and the presence of blood or serum. On blood agar it forms coarse, irregularly round colonies. The colonies are surrounded by a well marked zone of beta-haemolysis, although non-haemolytic variants are said to exist. It gives a well marked Nagler reaction which can be neutralized by C. perfringens antitoxin. It is less proteolytic than C. perfringens, but as its name indicates, liquefies gelatin, coagulates serum, egg albumin and slowly digests meat. Of the common sugars it ferments only glucoose and maltose, (see Bergey's manuel 7th ed.).

(f) C. fallax:- Maclennan cited that this was first isolated by Weinberg and Seguin in 1915. Since then it has rarely been found. In wound smears this organism resembles C. perfrigens very closely (hence its name), even in the possession of a capsule. It is however motile and in cultures forms spores readily.

C. fallax is a difficult organism to isolate and maintain in culture and requires the most strickly anaerobic conditions and large quantities, of blood or serum. On blood agar it elaborates small, round, smooth colonies surrounded by a zone of weak haemolysis.

It does not give the Nagler reaction, a point of some importance in rapid differenciation from C. perfringens. Unlike the other clostridial species already described, it is without action on gelatin. It ferments glucose, lactose and maltose only. (Macleennan 1958).

(ii) Wound - associated C. perfringens toxins:-

Clostridium perfringens produces 12 types of soluble antigens that may be involved in pathogenesis. Four of these toxins alpha., beta., epsilon and iota - are used to group the species into five toxigenic types A-E. In additon to the 12 types of toxins, several other biological active substances, have been described. The role of these substances in pathogenesis is unknown Gamma - and eta - toxins have also been reported in the literature but their existence is said to be questionable. (Duncan, 1975).

Type A strains of C. perfringens are most frequently encountered in wound infections. Types B,C,D and E are associated with gastroenteritisof man or animals. The toxins produced by type A strairs that are involved in pathogenesis of wound infections are alpha-, kappa-, theta-, lambda, and nu-toxins, (Arbuthnott, 1973; Duncan, 1975).

Smith and Arbuthnott in 1974 investigated the properties of C. perfringens type A toxins, purified by electrophoresis. Electrophoresis of supernatant indicated five major extracellular components which were thought to be toxins and enzymes. They were found to be acidic proteins with isolectric point between 4.5 to 6.5. The components, were identified to be collagenase, hyaluronidase, alpha-toxin, neuraminidase and theta-toxins. The most lethal of these is the alpha-toxin. Further electrophoresis of the alpha-toxin revealed two forms (X_A and X_B) with isoelectric point of 5.50 and 5.25 respectively. X_A was found to be the main component and constitutes about 80-90%. Alpha-toxin possessed hot-cold haemolytic, phspholipase C and lethal activities. It haemolyses lecithin and sphinogomyelin. And it was shown to be cardiotoxic, (Smith et al 1974).

Wilsdon, Glenny and Oakley and their collaborators in 1954 as cited by Oakley (1954) clearly demonstrated the multiplicity of the toxins produced by C. perfringens. Today at least twelve such agents are recognised. (see table III).

Table III: Toxins of C. Perfringens.

Toxin type	Biochemical activities			Biochemical nature
	Haemolytic	Necrotizing	Lethal	
Alpha	+	+	+	Lecithinase
Beta	-	+	+	?
Gamma	-	-	+	?
Delta	+	-	+	?
Epsilon	-	+	+	?
Eta	-	-	+	?
Theta	+	+	+	?
Iota	-	+	+	?
Kappa	-	+	+	Collagenase
Lambda	-	+?	=	Proteinase
Mu	-	-	?	Hyaluronidase
Nu	-	-	?	DNAse

Reproduced from Oakley and Warrack (1953) and Oakley (1954).

The most important and most, established of these toxins are discussed below:

(a) Alpha-toxin is a calcium-dependent phospholipase C toxin, and it is also known as lecithinase. It is a zinc metalloenzyme of about 53,000 daltons. It acts by hydrolysing membrane-associated lecithin and blood phospholipid complex, to yield phosphoryl choline and diglyceride. In an infection it destroys the erythrocytes and leukocytes. Calcium ion is necessary for phospholipase C activity by producing a positive charge on the lecithin. The activity of the toxin can therefore be inhibited by phosphate or other substances like citrate or pyruvate that can bind the calcium ion, (Duncan, 1975).

The haemolytic and hydrolytic activities of phospholipase C can be functionally separated by heating at a temperature of 56°C in the presence of calcium ions. Under these conditions, the haemolytic activity is inhibited while the hydrolytic activity is retained. And it was suggested by Sabban et al as cited by Duncan (1975) that the haemolytic activity of phospholipase C is necessary to expose the phospholipids of the membrane to the hydrolytic activity. Alpha-toxin is also dermonecrotizing and lethal and it is certainly the most important constituent of type A toxic filterates. It was tentatively identified by MacFarlane et al in 1941. It has a close antigenic relationship between the alpha-toxin of

C. perfringens and the essentially similar lecithinase produced by Clostridium bifermentans.

(b) Kappa-toxin is a collagenase and it is also active on gelatin. It has a molecular weight of 80,000. It hydrolyses the non-polar regions of the collagen molecule. Experiments with guinea pigs injected with collagenase subcutaneously or intramuscularly suggested that the toxin attacks healthy connective tissues in the skin, where collagen fibres constitute the main component. This may result into extensive destruction of the muscle, (Arbuthnott, 1978; Duncan 1975).

(c) Theta-toxin is a thermolabile haemolysin. It is also oxygen-labile that can be reactivated by reducing agents e.g. cystein. In 1973, Hauschild et al purified C. perfringens type C theta-toxin by ammonium sulphate precipitation, gel filtration and ion-exchange chromatography. It yielded a single protein band with L-lysine as the terminal amino acid. It is haemolytic and histotoxic, with a M. wt: of 74. 000 (Hauschild, 1973). Smith in 1975, identified and purified multiple forms of theta-toxin of C. perfringens type A.

He found that it contained four components with different PH. Gel-electrophoresis gave a reaction of identity.

In any case the ability of the toxin to be adsorbed on erythrocytes depends on the toxin being in the reduced state. Theta-toxin, tetanolysin, pneumolysin and streptolysin are all oxygen-labile, and each toxin can be neutralized by antisera of the other three. It is therefore possible that they possess a common prosthetic group(s). In contrast to alpha-toxin, which is specie specific, theta-toxin lyses the erythrocytes of all species except the mouse, (Duncan, 1975).

(d) Lambda - toxin is a non-specific proteinase. It can attack azocoll and gelatin but not natural collagen. The toxin is thought to function in degrading dead tissue at the site of infection. Some of the toxin may be absorbed and distributed systemically, thereby contributing to generalized toxaemia, (Duncan, 1975).

(e) The nu-toxin is a deoxyribonuclease. It is a non-lethal antigen, nonhaemolytic and has no necrotizing activity. It is suggested that it destroys leukocytes and deoxyribonucleic acids.

(f) Miu-toxin:- Brilliant work by Chain and Duthie as quoted by Maclennan (1962) demonstrated that the so called "spreading factors" that numerous other investigators had been able to extract from certain mammalian tissue, pyococci, and from gas gangrene bacilli, were infact enzymes, whose property was to hydrolyze the cementing mucopolysaccharide of the tissue to which Meyer and Palmer has given the name "hyaluronic acid". Most type A strains of C. perfringens produce a hyaluronidase, sometime in large amounts. Oakley and Warrack have suggested the name "miu-toxin" for this enzyme, (Oakley and Warrack 1953),

(g) Other agents:- The above metioned toxins are well defined in the literature, in othr words their biological effect and neutralization by a suitable antisera are well documented. There are other reactions which are pathologically important but not yet related to any of the toxic factors so far listed.

Reed et al in 1943 have stated that certain strains of C. perfringens can produce a fibronolysin. Little is known about its activity, but certainly it is an enzyme. But it has been claimed that C. perfringens antiserum is without anti-fibronolytic power.

Fredette and Frappier (1946) also made mention of a "bursting power" which is found in young and otherwise non-toxic culture filterates of C. perfringens. It is said to produce a severe but temporal oedema on intramuscular injection into experimental animals. Its exact nature and status, remain to be determined and as Smith (1975) has suggested, it may best be regarded for the time being as the non-commital "aggressin"

(iii) Other Clostridial toxins associated with Wound infections:-

Clostridium novyi (C. oedematiens) is an important agent of wound infections in man. They are divided into three toxigenic types A,B and C based on their ability to produce eight recognized toxins. Clostridium haemolyticum is considered to by type D, Clostridium novyi because of similar cultural characteristics and its sharing of beta-, eta-, and theta-toxins with C. novyi type B. C. haemolyticum is however associated with diseases of cattle variously known as "red water disease" or "bacillary haemoglobinuria", (Duncan, 1975; Smith et al 1957).

Table IV: Toxigenic types of Clostridium novyi.

Type	Alpha	Beta	Gamma	Delta	Epsilon	Zeta	Eta	Theta
A	+	-	+	+	+	?	-	-
B	+	+	-	-	-	+	+	-
C	-	-	+?	-	-	-	-	-
D	-	+++	-	-	-	-	+	+

Reproduced From Machennan J. D. (1962).

C. novyi produces alpha-toxin which is necrotizing and lethal and it is the main toxin involved in wound infections. It also produces gamma- and beta-toxins and two distinct haemolysins, two distinct lipases and a tropomyosinase which may also play a role in the infection of wounds.

Boyd, Thompson and Walker, in 1972 investigated the prevention of experimental C. novyi and C. perfringens gas gangrene by active immunisation. Sheep were immunized with a mixed vaccine containing the toxoids of C. perfringens, C. septicum and C. novyi. The sheep were challenged at different intervals by a high-velocity of either C. novyi or C. perfringens.

Immunization was found almost completely effective in preventing gas gangrene due to C. novyi or C. perfringens even after one year of vaccination. Survival rate was about 90%.
In unvaccinated controls survival rate was about 3%.

C. septicum is another specie of Clostridium associated with wound infections. C. septicum and C. chauvoei produce four type of toxins, and are thought to be two types of the same specie. They produce alpha-toxin which is lethal, necrotising, haemolytic and leukocidic; beta-toxin which is an oxygen-labile haemolysin, (Moussa, 1958). The alpha-toxins produced by the two species are antigenically different while the other toxins are antigenically related. The histological appearance of tissues infected with C. septicum and C. chauvoei is similar to that of C. perfrigens infection. The alpha-toxin of C. septicum is also antigenically related to that of C. histolyticum except for the fact that, that of C. histolytiam is only lethal and necrotizing but not haemolytic.

The alpha-toxin of C. septicum is principally responsible for the effects produced during infection. It causes haemorrhagic necrosis in the local lesion and exerts toxic effects on the cardiovascular system by some unknown mechanism.

Other recognized clostridial species that have been associated with wound infections include C. sordellii, C. histolyticum, C. limosum, C. difficile, C. cam'is and C. fallax, (Duncan, 1975).

C. sordellii has been isolated from gangranous war wounds and causative agent of post-operative gas gangrene and spontaneous peritonitis. Arseculeratine et al in 1969 investigated the toxins of C. sordellii. They showed that it produces two types of toxins and the two together produce oedema, haemolysis, fibrolysin and callagenase effects in rabbits.

C. Sordellii and C. bifermentans are closely related and it is thought that C. bifermentans represents a non-pathogenic strain of C. sordellii. Both produce phospholipase C that are antigenically related, though not as lethal as that produced by C. perfringens. It is therefore puzzling that C. bifermentans are non-pathogenic. In addition to this lethal component, all strains of C. sordellii and C. bifermentans whether pathogenic or apathogenic, elaborate a substance causing a well marked Nagler reaction on lecithovitelin plates. This factor which is known to be a lecithinase, has been studied in some details by

Miles and Miles (1947) who reported that it is antigenically related to the theta-toxin of C. perfringens, but differs from it in the PH optima at which it is active. It has little or no haemolytic action andwshows no toxicity to laberatory apinals.

C. histolyticum is known to produce five types of soluble antigens that are biologically active and may function in an infection. This specie is common in soil but has not been isolated from wound infection. Below is the table of toxins elaborated by C. histolyticum, (Duncan, 1975).

Table V: Toxins of Clostridium histolyticum.

Toxin	Biological acitivity			Biochemical Nature
	Lethal	Nectrotizing	Haemolytic	
Alpha	+	+	-	?
Beta	+	+	-	Collagenase
Gamma	?	?	-	Protease
Delta	?	?	-	Elastase
Epsilon	?	?	+	?

Reproduced from Maclennan (1962).

C. limosum, C. difficile, C. cargis and C. fallax are commonly found in associations with other clostridial species in wound infections. Little, if at all is known about their role in wound infections. But they are tought to play some synergistic effect in wound, (Duncan, 1975; Maclennan, 1962). And as earlier stated, it would not be possible or even helpful to describe the biological characteristics of the numerous other Clostridia which may be found in gangranous wounds. Although they are not generally regarded as pathogenic, there can be little doubt that these organisms play an important ancillary role in the infective process, most likely because of the powerful proteolytic and saccharolytic enzymes which many of them produce.

Clostridial Enterotoxaemia:

Major cause of clostridial enterotoxaemia in man and animals is due to C. perfringens. All the toxigenic types of C. perfringens have been incriminated in enterotoxaemia of man or various animal species. Clostridium perfringens is a normal flora of the intestine. Enterotoxaemia is as a result of ingestion of large

number of viable cells, as in the case of human food-poisoning. The reason for the increased multiplication of the organism in the intestine is not clear, but it is known that toxins are produced and when this reaches a sufficient level, it induces symptoms of enterotoxaemia (Hauschild et al 1971). It is thought that the viable cells are ingested, passed to the intestine, sporulate and lyse to release the enterotoxin, (Labbe et al 1976).

Hauschild in 1970, investigated this enteropathogenic factor. He obtained it from extracts of sporulated cells of C. perfringens type A. He observed that it produced diarrhoea and fluid accumulation in ligated loops of rabbits and lambs. It also caused erythema in guinea pigs and rabbits injected intradermally with the extract. The activities of this enteropathogenic factor is not neutralized by immune sera against extracts of non-sporulating cell or C. perfringens type A antitoxin. The experiment also failed to incriminate any of the known toxins of C. perfringens and the corresponding antitoxins have no effect.

In another exercise, Hauschild et al in 1970 investigated the response of ligated intestinal loops of lambs to this enteropathogenic factor of C. perfringens type A. It caused accumulation of fluid in the loops.

They observed that the factor is antigenic, heat-labile and of large molecular weight. Immune sera against the enteropathogenic factor inhibits its effect.

To confirm this findings, Nillo et al (1971), injected parentarally formalized cell extracts of C. perfringens type A into sheep. This induced antibody formation against the erythemal and enteropathogenic factors. Protective immunity was however not evident when these lambs were challenged by the intraduodenal and ligated intestinal loop methods. This may explain the reccurence of clostridial enteritis in man. They confirmed that the enteropathogenic factor is distinct from any other toxins produced by C. perfringens type A. This they did by separetely challenging adult monkeys (Mecaca fascicularis) of both sexes with enterotoxin plus saline; enterotoxin plus normal serum; and enterotoxin plus antiserum. Results showed that the enterotoxin is responsible for emesis and diarrhoea in monkeys. And the antiserum is active against the enterotoxin. In man emesis does not occur.

It is not known whether the toxin is preformed in food prior to ingestion as in the case with botulinum toxin. But in monkeys, as shown above, preformed toxin can induce diarrhoea the major symptom of this type of food-poisoning and vomiting. Conceivably it could be the case in human beings. The argument against this idea of preformed toxin in foods is that the toxin is not synthesized during vegetative growth but during sporulation. And the organism sporulates poorly or not at all in most foods such as meat and poultry dishes that are usually associated with this type of food poisoning. The toxin is known to be associated with bacterial spore coat as a structural protein. Uemura (1978) showed that it is released during sporulation and heat treatment (75°- 95°C) enhances production of the enterotoxin.

Purification in attempt to characterise the enterotoxin of C. perfringens, Hauschild (1973) showed that it contained 18 amino acids including aspartic, serine, leucine and glutamic acids as the predominats. It gave a sedimentation coefficient of 2.85, with molecular weight using various techniques of between 33,000 to 40,000.

The major toxins include the enterotoxin, beta-toxin, and epsilon-toxin. In addition to these, the various haemolysins, collagenase, and deoxyribonuclease are thought to contribute to the pathogenesis of C. perfringens enterotoxaemia in which invassion of the mucosa occurs. Whether iota-toxin is involved is unknown. But if it is, it together with epsilon-toxin must first be activated by proteolytic enzymes before they can exhibit their full toxigenic potential, since they are synthesized as the prototoxin, (Duncan, 1975).

C. perfringens type D, the cause of enterotoxaemia in sheep is known to be notorious for epsilon-toxin synthesis, the toxin causes an increased permeability to the intestine, allowing rapid absorption of the toxin from the lumen. The toxin has a high affinity for nervous tissue and the kidneys. At these sites, it causes oedema and necrosis which results in convulsive seizure and death. The specific binding sites and mechanisms of action of toxin are unkown. Buxton (1978), showed that C.perfringens type D epsilon-toxin stimulates adenyl cyclase to produce increased amounts of cyclic adenosine - 3' 5' - monophosphate (cAMP), which inturn increases fluid production in the intestine

in a manner similar to that of E. coli and vibrio enterotoxaemia. In another experiment, Worthington et al (1979) injected C. perfringens type D epsilon-toxin into sheep. It was observed that the toxin caused large increase in catecholamin and cyclic 3' 5' - Amino-phosphate levels and a moderate increase in cyclic - 3', 5' - Quanosine monophosphate levels. Haemaconcentration also occured. In another development, Buxton (1978) used immunoperoxide technique to investigate by light and electron microscopy the sites of binding of C. perfringens type D epsilon - toxin in mice. He injected the toxin into mice intravenously and found that thetoxin bound to the luminalsurface of the endothelial lining of certain blood vessels, cells lining the loop of Helen, convoluted tubules of the kidney and hepatic sinusoids.

Type C strains of C. perfringens are known to produce beta-toxin. Beta-toxin is thoutht to be responsible for intestinal inflammation and extensive mucosal degradation in necrotic enteritis. From theliterature, much work is still expected on food-poisoning due to type C C. perfringens. Unfortunately, food-poisoning due to it is rare. But it is thought that, it

produces enterotoxin which was originally associated with type A strains of C. perfringens. As to whether other strains of C. perfringens produces entero- toxin is yet to be seen.

In 1979, Skjelkvalo et al showed that lecithinase positive strains of C. perfringens type A produce enterotoxin and a higher proportion of them are non-haemolytic. On the other hand haemolytic strains produce higher levels of enterotoxin. Lecithinase negative strains were regarded as variants (C. plagarum) of Clostridium perfringens. They showed that they do not produce enterotoxin and that they can be serotyped with C. perfringens antisera. Nakamura et al in 1976 compared Lecithinase negative variants of C. perfringens. They have similar biochemical properties including sugar fermention, production of greenish colonies on blood agar and chromatographic analysis of glucose end-product metabolism. Distinction between the two are that C. plaganum is lecithinase negative and non-haemolytic on blood agar, and does not produce enterotoxin.

The enterotoxin has been purified and characterized as a heat-labile protein with a Molecular weitht of 35,000. The toxin has been shown to produce diarrhoea in human volunters,

In 1974, Niilo investigated the response of ligated intestinal loops of seven week - old chickens to the enterotoxin of C. perfringens. He found that injection of 20 - 30 microgram of the enterotoxin into ligated loops caused fluid accumulation, In another investigation, Skjelkvale and Uemura (1977) observed the effect of oral admission of C. perfringens enterotoxin in in human voluteers. They showed that it provoked diarrhoea and abdominal pain identical with C. perfringens food-posoning. Five of the nine volunteers showed antienterotoxin in serum and all showed enterotoxin in faeces. They also concluded that detection of enterotoxin in faeces is reliable for epidermiological studies of C. perfringens food - poisoning outbreak.

In conclusion to this aspect of clostridial enterotoxaemia, other species of current interest will now be discussed. C. difficile is known to play a role in pseudo-membranous colitis (PMC). Bartlett (1979) investigated this role of toxigenic strains of C. difficile. He observed that in cases of antimicrobial agent-associated PMC (i) toxigenic strains can most of the time be isolated and isolation rate from health individuals is in the

Joseph Silva Jr., in 1979 made a quantitative bacteriological stool examination in antibiotic-challenged hamsters. Fourteen hamsters were challenged with clindamycin. He observed that a numerical reduction occured in Clostridial species in clindamycin-treated animals. These species include C. butyricum, C. septicum, C. sphenoides, C. bifermentans, and C. subterminale. Quantitative increase occured in C. difficile, C. sordellii, C. sticlandii and C. perfringens counts. He infered that these increase could be related to the ability of C. difficile and C. perfringens to produce diarrhoea and colitis. He also observed that the mechanism of antibiotic-induced colitis include (a) Increase in antibiotic-resistant organisms (b) decrease in antibiotic-sensitive clostridial species and (c) supression of normal flora.

Wilkins(1979), in another investigation, showed that clindamycin (a lincomycin derivative) is one of the best antibiotics for the treatment of anaerobic infections, but unfortunately a few people develop a disease of the colon after taking the antibiotic. And sterile filtrate of the colon can reproduce the disease in healthy hamsters. He identified the factor to be similar to C. difficile toxins.

(ii) Isolation of Species:-

By means of a sterile wire loop a loopful from each of the thioglycolate bottle was separately inoculated into freshly poured 5% egg yolk medium and 5% human blood agar plates, both to which one gram per litre of neomycin and 0.25% of cystein hydrochloride had been added. All the plates were incubated in tightly sealed jars and air was evacuated from them by means of a vaccum pump. The jars were left at room temperature for three days.

The various colonies on blood agar plates were examined macroscopically for colonial morphology and haemolysis. Smears of the colonies were made on clean microscopic slides, fixed, gram stained and then observed macroscopically for lecithinase and lipase production.

(iii) Spore Production and Inoculum:-

In cases where discrete colonies or pure cultures were obtained on blood agar plates, the various colonies were picked and inoculated aseptically into cooked meat medium contained in bijou bottles in duplicates. The bottles were then incubated at 34°C for three days. Where isolation of discrete colonies failed on

blood agar plates due to spreading growth of some species, a subculture of such plate was made into freshly poured blood agar plates and incubation was repeated, but for a shorter duration (about 48 hours) to obtain pure cultures or discrete colonies. The cooked meat medium was observed for blackening (proteolysis) or reddening (saccharolytic activity).

Loopfuls of sets of the cooked meat cultures were aseptically smeared on clean microscopic slides, fixed, gram stained and observed under the microscope for the bacilli, spores, their position and shape. Where spores were not observed, the other set of cooked meat cultures were heat - shocked by placing them in a steam bath at 80°C for five minutes and then re-incubated at 34°C overnight. Smears were then made aseptically onto clean microscopic slides, fixed, gram stained and then observed under the microscope.

The unheated sets of cooked meat cultures were used as inoculum for the subsequent biochemical tests.

(iv) Biochemical tests:-

(a) Sugars:- 1% each of glucose, lactose, maltose and sucrose were prepared and sterilized, with phenol red as the

indicator in cotton-plugged test tubes. The sugars were aseptically inoculated on the day of preparation, using the unheated set of cooked meat cultures. The tubes were immediately layered with sterile paraffin oil up to about 5mm depth above the sugar level. The tubes were incubated at 37°C for three days.

(b) Gelatin Liquefaction:-

Nutrient gelatin agar in screw-capped tubes were seeded with the various clostridial species from the cooked meat medium and incubated at 37°C for 14 days. Gelatin liquefaction was then observed after refrigeration of the tubes for about 20 minutes.

(c) Crossley Milk:-

Crossley milk with cremo-cresol purple as the indicator in screw-capped tubes were inoculated using the cooked meat cultures as the inoculum. The tubes were incubated at 37°C for three days. Gas production, acid production, clot or digestion were then observed.

(d) Lecithinase (LV) and Lipase Production:-

5% egg yolk medium in which 0.25% of cystein hydrochloride and one gram per litre of neomycin have been incorporated was inoculated using the unheated set of cooked meat cultures as the inoculum.

The plates were incubated in the vaccum jars, described above, and were left for three days at room temperature. Presence of whitish precipitation within, under and around the colonies were read as positive lecithinase, while presence of irrediscent zone at the edges of the colonies were read as positive lipase.

(e) Biochemical properties:-

Below is a table of biochemical properties used in the identification of the various clostridial species. It was adapted from the Manual of Clinical Microbiology by J.E. Blair and Clinical Microbiology: 2nd Edition by Lennette et al.

Table VI: Cultural & Biochemical Characteristics of Some Clostridial Species.

Specie	Cooked Meat Medium			Egg Yolk Agar		Biochemicals					
	Digestion	spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose
C. perfringens	-	OT	Thick	+	-	ACG	+	+	+	+	+
C. septicum	+	OS	Normal	-	-	A	+	+	-	+	+
C. novyi	+	OS	Large	+	V	V	+	+	-	+	-
C. histolyticum	+	OS	Normal	-	-	D	+	-	-	-	-
C. sporogenes	+	OS	Thin	-	+	D	+	+	-	+	-
C. tetani	-	RT	Normal	-	-	C	+	-	-	-	-
C. botulinum											
(a) Proteolytic	+	OS	Normal	-	+	D	+	+	-	+	-
(b) nonproteclytic	-	OS	Normal	Slight	+	A	+	+	-	V	-

Notes on table VI

+ = Positive Reaction; - = Negative Reaction; V = Variable

C = Clot; D = Digestion; O = Oval; R = Round; S = Subterminal;

T = Terminal; G = Gas; LV = Lecithinase, A = Acid.

RESULTS:

(i) Soil Samples: Tables XIII to XV show the various pathogenic clostridial species in 30 soil samples each from the market places, play grounds and motor parks. Seven species were isolated, these are clostridium perfringens, C. septicum, C. novyi, C. histolyticum, C. sporogenes, C. tetani and C. botulinum. A total of 195 isolates were obtained from the three areas that were investigated of which 56 isolates were from the market places, 74 from the play grounds and 65 from the motor parks. The most commonly encountered species were C. perfringens and C. histolyticum with 55 and 54 isolates respectively. This is 28.2% and 27.7% of the total number of isolates or 61.1% and 60.0% of the incidence of occurence in soil respectively.

The least encountered species were C. tetani, C. sporogenes and C. novyi with a total isolates of 12, 13 and 15 respectively. This is 6.1%, 6.6% and 7.7% of the total number of isolates or 13.3%, 14.4% and 16.7% of the incidence of occurence respectively. A total of 23 isolates of each of C. septicum and C. botulinum were obtained from the 90 soil samples. This is 12% of the total number of isolates or 25.6% incidence in soil.

Out of the 23 isolates of C. botulinum, 5 were proteolytic types of which 3 were from the market places, 2 from the motor parks and none from the play grounds.

In 51 of the soil samples, two types of species were encountered, in 24 samples, three types of species and in 2 samples four types of specie , while in 13 samples one type of specie was isolated. There was no soil sample without clostridial specie .

(ii) Clinical materials:

Tables XVI to XVIII show the various pathogenic clostridial species isolated from the wounds and surgical instruments. Six types of species were isolated from 85 out of the 120 wound swabs that were examined. These are C. perfringens, C. septicum, C. novyi, C. histolycum, C. sporogenes and C. tetani. There were no clostridial species isolated from the sterilized surgical instruments.

Out of the 50 wound swabs examined from the rural clinic, 40 have clostridial species isolated from them. This represents 80% presence of clostridia in wounds from the rural area Wounds from the urban outpatient department, showed 31 out of the

50 examined with clostridia, while the wards gave 14 out of the 20 that were examined. This is 62% and 70% incidence of clostridia in urban OPD and wards respectively.

C. perfringens was the most prevalent species isolated from the wound swabs with a total of 40 isolates. This is 40% of the total incidence or 33.3% incidence of occurence in wounds. Out of the 100 isolates, 19 were C. sporogenes, 17 C. septicum and 14 C. novyi, this is 19%, 17% and 14% of the total incidence or 15.8%, 14.2% and 11.7% of incidence of occurence in wounds respectively. C. histolyticum and C. novyi were the least encountered species. 9 isolates of C. histolyticum were obtained, while only one of C. tetani was isolated and this was from the wards.

C. botulinum, C. sordelli (C. bifermentans) and C. fallax were not isolated from the wound swabs.

The characteristic colonial morphologies of the various clostridia as observed in 5% human blood agar plates are as follows:

C. perfringens: Colonies are small, circular, and greenish, forming double zone of haemolysis. The inner zone is of complete haemolysis while the outer zone is of partial haemolysis.

C. septicum: It forms haemolytic, transparent and continous film, with numerous rhizords on blood agar. The transparency of the colonies is such that can easily be missed on the plate.

C. novyi: Colonies of C. novyi are delicate (very thin), filamentous with partial haemolysis. They can also easily be missed on the plates because of the delicacy.

C. histolyticum: The colonies are small, rough and irregularly round, surrounded by a narrow zone of partial haemolysis.

C. sporogenes: It forms fairly large "medusa head" colonies. The colonies are round, haemolytic and yellowish in colour; at times it forms continous film on blood agar.

C. tetani: It forms spreading colonies as a thin film. In most cases, it is accompanied by cell elongation and nuclear division. And where discrete colonies occur, they are small, grey, fimbriated and haemolytic.

C. botulinum: The colonies are large, transparent, fimbriated and haemolytic.

Table VII: Occurence of Pathogenic Clostridial Species in 30 soil specimens each from three sites:

Species	Market Place	Play Ground	Motor Park	Total number of each specie
C. perfringens	18	23	14	55
C. septicum	3	13	7	23
C. novyi	1	5	9	15
C. histolyticum	14	22	18	54
C. sporogenes	1	5	7	13
C. tetani	6	3	3	12
C. botulinum	13	3	7	23
C. sordellii	0	0	0	0
C. bifermentans				

Table VIII: Incidence of Pathogenic Clostridial Species From 90 Soil Samples:

Species	Total Incidence	% of Total Number	% Incidence
C. perfringens	55	28.2	61.1
C. septicum	23	12.0	25.6
C. novyi	15	7.7	16.7
C. histolyticum	54	27.7	60.0
C. sporogenes	13	6.6	14.4
C. tetani	12	6.1	13.3
C. botulinum	23	12.0	25.6
C. sordellii (C. bifermentans)	0	0	0

Table X: Occurence of Pathogenic Clostridial Species in Wounds and Surgical Instruments:

Species	Rural Clinic	Urban O.P.D.	Wards	Surgical Instruments
C. perfringens	17	14	9	0
C. septicum	9	5	2	0
C novyi	9	4	1	0
C. histolyticum	3	4	2	0
C. sporogenes	10	7	2	0
C. tetani	0	0	1	0
C. botulinum	0	0	0	0
C. sordellii (C. bifermentans)	0	0	0	0

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	L V	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
9a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
10a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
11a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
12a	-	CS	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	CS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
13a	+	CS	Thick	-	-	D	+	-	-	-	-	C. histolyticum
14	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
15a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
16a	+	CS	Normal	-	-	D	+	+	-	+	-	C. botulinum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
17a	+	CE	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
18	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
19	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
20	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
21a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
22	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
23	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
24a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
25a	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. [illegible]yi
c	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
26a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	+	OS	Normal	-	+	D	+	+	-	+	-	C. botulinum
27a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
c	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
28a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
29a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
30a	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum

LV = Lecithinase O = Oval spore R = Round spore T = Terminal spore
S = Subterminal spore; A = Acid production; C = Clot G = Gas production
D = Digestion + = Positive reaction - = Negative reaction.

Table XIV: Pathogenic Clostridial Species Isolated from Play Ground:

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
31a	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
b	*	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
c	-	CS	Large	+	+	AC	+	+	-	+	-	C. novyi
32	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
33a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
34a	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
c	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
35a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
36a	-	OS	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
c	-	OS	Normal	+	+	AC	+	+	-	+	-	C. novyi
37a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
c	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals							Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose		
51a	-	OT	Thick	+	-	ACG	+	+	+	+	+		C. perfringens
b	-	OS	Normal	-	+	A	+	+	-	-	-		C. botulinum
c	-	RT	Normal	-	-	C	+	-	-	-	-		C. perfringens
52a	-	OT	Thick	+	-	ACG	-	+	+	+	+		C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-		C. histolyticum
53a	-	OS	Normal	-	-	D	+	-	-	-	-		C. histolyticum
b	-	OS	Normal	-	-	A	A	+	+	-	+		C. septicum
c	+	OS	Normal	-	-	D	+	-	-	-	-		C. histolyticum
54a	-	OT	Thick	+	-	ACG	+	+	+	+	+		C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+		C. septicum
c	+	OS	Thin	-	+	D	+	+	-	+	-		C. sporogenes
55a	-	OT	Thick	+	-	ACG	+	+	+	+	+		C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+		C. septicum
c	+	OS	Thin	-	+	D	+	+	-	+	-		C. sporogenes
56a	0	OS	Normal	-	-	D	+	-	-	-	-		C. histolyticum
b	-	OS	Large	+	+	AC	+	+	-	+	-		C. novyi

Speimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digstion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
57a	-	CT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
58a	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
b	-	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
c	-	OS	Normal	-	-	A	+	+	-	+	-	C. septicum
59a	+	CT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
60a	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
b	-	OS	Normal	-	-	A	+	+	-	+	-	C. septicum

Notes On table XIV.

LV = Lecithinase

O = Oval Spore
R = Round spore

T = Terminal spore

S = Subterminal spore

A = Acid production

C = Clot

G = Gas production

D = Digestion

\+ = Positive Reaction

\- = Negative Reaction.

Table XV: Pathogenic Clostridial Species Isolated from Motor Park.

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
61a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
c	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
62a	+	OS	Normal	-	-	D	+		-	-	-	C. histolyticum
b	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
c	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
63a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
64a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
c	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
65a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. histolyticum
66	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
67a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	+	OS	Normal	-	+	D	+	+	-	+	-	C. botulinum

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
68a	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
b	-	CT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
69a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
70a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
c	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
71a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
c	-	CT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
72a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
73a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
c	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Mattose	Lactose	
74a	-	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
75	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
76a	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
77a	-	OS	Normal	-	+	A	+	+	-	-	-	C. botulinum
b	-	OS	Large	+	+	AC	+	+	-	-	-	C. novyi
78a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
79a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
80a	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
81a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	-	C	+	-	-	-	-	C. tetani
82a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OS	Normal	-	-	C	+	-	-	-	-	C. tetani
c	+	OS	Thin	-	-	D	+	+	-	+	-	C. sporogenes

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemical						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Malttose	Lactose	
83a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
84a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
85	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
86a	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
87a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
88a	+	OS	Normal	-	+	D	+	+	-	+	-	C. botulinum
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
c	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
89	+	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
90a	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
b	-	RT	Normal	-	-	C	+	-	-	-	-	C. tetani
c	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum

NOTES ON TABLE XV

LV = Lecithinase
O = Oval spore
R = Round spore
T = Terminal spore
S = Subterminal spore
A = Acid production
C = Clot
G = Gas production
D = Digestion
\+ = Positive Reaction
\- = Negative Reaction

Table XVI: Clostridial Species Isolated From Rural Health Clinic.

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
1	-	OS	Large	+	+	AC	+	+	-	+	+	C. novyi
2.	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
3a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	-	D	+	+	-	+	-	C. sporogenes
4	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
5	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
6												
7	-	OS	Large	+	-	AC	+	+	-	+	-	C. novyi
8	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
9	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
10	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
11a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
12	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
13												
14	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
15	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
16a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
17												
18	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
19	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
20a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
21	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
22	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
23												
24	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
25a	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
b	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
26	-	OS	Normal	-	- A	A	+	+	-	+	+	C. septicum
27												C. perfringens
28	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
29	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
30	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
31	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
32												
33	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
34	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
35a	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
b	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
36	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
37												
38	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
39	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
40												
41	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
42												
43a	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
44	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
45a	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
46	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
47												
48												
49	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
50	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi

Notes on table XVI

LV = Lecithinase;
O = Oval spore;
R = Round spore;
T = Terminal spore;
S = Subterminal spore;
A = Acid production
C = Clot
G = Gas production

C = Digestion
\+ = Postitive Reaction
\- = Negative Reaction

Blank specimen numbers indicate no Clostridium specie isolated from the specimen.

Table XVII: Pathogenic Clostridial Species Isolated From Urban O.P.D.

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Malttose	Lactose	
51	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
52												
53												
54	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
55a	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
56	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
57												
58	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
59												
60	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
61												
62	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
63	+	OT	Thick	-	+	ACG	+	+	+	+	+	C. perfringens
64	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
65												
66	-	OS	Normal	-	-	A	+	+	+	+	+	C. septicum

Sepecimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
67	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
68a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringes
b	-	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
69	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
70	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
71a	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
72												
73	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
74												
75												
76	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
77	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
78	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
79												
80	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
81												
82	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens

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Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
83												
84	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
85	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
86	-		Normal	-	-	A	+	+	-	+	+	C. septicum
87												
88												
89	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
90	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
91	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
92												
93	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
94												
95	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
96a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
97	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
98												
99												
100												

Notes on table XVII

LV = Lecithinase

O = Oval spore

R = Round spore

T = Terminal spore

S = Subterminal spore

A = Acid

C = Clot

G = Gas production

D = Digestion

\+ = Positive Reaction.

\- = Negative Reaction.

Blank specimen numbers indicate no Clostridium specie isolate from the specimen

Table XVIII: Pathogenic Clostridial Species Isolated From the Surgical Wards.

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
101	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
102	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
103	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
104												
105a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	+	OS	Thin	-	+	D	+	+	-	+	-	C. sporogenes
106												
107												
108	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
109	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
110												
111	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum
112												
113	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
114a	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
b	-	OS	Normal	-	-	A	+	+	-	+	+	C. septicum

Specimen Number	Cooked meat medium			Egg yolk agar		Biochemicals						Specie
	Digestion	Spore	Bacilli	LV	Lipase	Milk	Gelatin	Glucose	Sucrose	Maltose	Lactose	
115	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
116a	-	OS	Large	+	+	AC	+	+	-	+	-	C. novyi
b	+	OS	Normal	-	-	D	+	-	-	-	-	C. histolyticum
117	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens
118	-	RT	Thin	-	-	C	+	-	-	-	-	C. tetani
119												
120	-	OT	Thick	+	-	ACG	+	+	+	+	+	C. perfringens

Sterilized Surgical Instruments: There was no clostridial specie isolate from the twenty swabs taken from the sterilized surgical instruments.

Notes on table XVIII

LV = Licithinase
O = Oval spore
R = Round spore
T = Terminal spore
S = Subterminal spore
A = Acid production
C = Clot
G = Gas production
D = Digestion
\+ = Positive Reaction
\- = Negative Reaction.

Blank specimen numbers indicate no Clostridium specie isolated from the specimen.

DISCUSSION

Past works on the incidence of pathogenic Clostridia in soil from Europe, Belgium, Italy, Tunisia, Japan and Manchuria show that Clostridium perfringens has the highest incidence of occurence in soil, and infact 100% incidence was reported from some of these countires. This is closely followed by Clostridium histolyticum and then C. novyi and C. septicum, (Maclennan, 1962). This is fairly in agreement with this study in which C. perfringens, C. histolyticum, C. septicum and C. novyi have 61.1% 60.0%, 27.8% and 16.7% incidence of occurence in soil respectively.

C. sordellii (C. bifermentans) and C. fallax are known to occur both in soil and in wounds. None were isolated in this work this could probably be due to the technique of anaerobiosis employed and the incubation of the jars at room temperature, or probably they do not occur in our type of soil.

The significance of this work is clearly seen in the tables of occurence of pathogenic Clostridial species in both soil and in wounds, (tables XIII to XVIII). They show that the relative incidence of the pathogenic Clostridia in wounds and in soil were more or less the same, in view of the fact that the wound and soil specimens were obtained from different parts of the country, and the high incidence of histotoxic Clostridia in soil may account for the over 70% incidence of these species in the 120 wound swabs examined. Since C. tetani and C. botulinum have low incidence of occurence in soil, this may also explain the rare cases of tetanus and botulism in our society, though it is reported that some of these cases especially botulism escape undiagnosed.

C. histolyticum has a very high percentage occurence in soil with a low percentage occurence in wounds. The reason for this is unknow, but this finding agreess with the reports of most experiments carried out in various parts of the world, (Smith and Gardner 1949). May be it is related to its survival in wounds.

The view that in most parts of the world, the presence of Clostridial species in a wound is the rule rather than the exception should therefore be upheld, because out of the 120 wounds examined, only 35 wounds were Clostridia free. In most of the wounds examined, only one specie was isolated and in 15 wounds, two different species were isolated. This is in contrast to the soil specimens in which two, three or even four species of Clostridia were isolated from a single sample. In effect 70.8% of wounds examined have clostridial species isolated from them. These species which include C. perfringens, C. septicum, C. novyi, C. tetani, C. histolyticum and C. sporogenes are generally accepted as histotoxic Clostridial species. But happily enough, except may be for C. tetani they are inefficient pathogens with low invassive capacity, however their elimination in a wound results in prompt healing, (Duncan, 1975).

C. perfringens, C. tetani and probably C. sporogenes were the simplest species to be identified, at least presumptively, due to their colonial morphology on human blood agar. C. perfringens produces a double zone of haemolysis surrounding each isolated colony on blood agar. The inner zone is completely haemolyzed and the outer zone shows partial haemolysis, giving the colony a target appearance. There is also the production of greenish colouration in the medium. Growth of C. tetani is accompanied by a gradual spreading as a thin film across the surface of the blood agar plates. This swamming phenomenum is of diagnostic importance and can be used to locate C. tetani colony from a mixed culture, (Hoeniger and Tauschell 1974).

Onset of swamming is accompanied by cell elongation and nuclear division without cell division. The nuclei are evenly and neatly seen throughout the cytoplasm which is not seperated by a cross wall. "Medusa head" colonial morphology with raised yellowish colonies permit easy identification of C. sporogenes on blood agar plates.

C. novyi types, A and B are important in human infections and they produce the same lethal (alpha) toxin and are usually not differenciated.

However, the C. novyi species isolated from this experiment appear to belong to group A. They differ from type B C. novyi by their lipase production and their ability to acidify and clot Crossley milk. Inability to isolate types B, C and D, C. novyi may be related to their being more fastidious than C. novyi type A.

The major problem encountered was the differenciation of C. botulinum from C. sporogenes. This is due to the fact that the two species have similar biochemical characterisitcs. And as a rule confirmed identification of C. botulinum is made by animal pathogenicity test or by toxin/antitoxin neutralization test or by immunofluorescent technique using a monospecific antisera to the vegetative cell wall. This is because of the heterogenuity in the species of C. botulinum. Identification and speration of these two species were therefore made on the basis of their colonial morphology on blood agar after they have been confirmed by biochemical tests to belong to either of these two species. This may sound unmicrobiological, but for obvious reasons, most especially when efforts to obtain antisera failed, this is fair enough in as much as the two species were confirmed Clostridial species and moreover C. sporogenes is the only specie of Clostridia that elaborates "medusa head" type of morphology on blood agar

C. botulinum species isolated were mostly of the nonproteolytic type,, they do not digest cooked meat medium and crossley milk. Only 5 out of the 23 isolates of C. botulinum were of the proteolytic type of which 3 were from the market place, none from the play ground and 2 from the motor park. The distinguishing cultural characteristics between the proteolytic and nonproteolytic C. botulinum are that the proteolytic group can digest cooked meat medium, and Crossley milk and are maltose positive.

Though the vegetative forms of C. perfringens and C. septicum are known to occur in soil, Clostridial species occur mostly in their spore forms in soil. Thioglycolate is known to stimulate their germination. Most species of Clostridia produce spores in coooked meat medium when incubated some few degrees below their optimum growth temperature. Species, especially C. perfringens that fail to produce spores under this condition, are easily made to sporulate after heatshock treatment at 80°C for 5 minutes, and then incubated overnight at 34°C. Infact over 90% of the Clostridium perfringens were made to sporulate in this way, otherwise, other Clostridial species sporulate very well in cooked meat medium when incubated some few degrees below their optimum growth temperature.

Neomycin which is a broad spectrum antibiotic was incoporated into the solid media to eliminate contamination from external sources. One gram per litre concentration is nontoxic to Clostridial species and it is accepted to promote asepsis, while cystein hydrochloride, which is a reducing agent, reduces the oxidation - reduction potential of the media

Difficulties encountered in obtaining pure cultures of Clostridial species need mention at this junction. Commonly encountered Clostridial species, except for C. perfringens are very motile especially C. tetani, C. septicum C. novyi and C. sporogenes. Where a mixture of two or more of these species occur care was taken in subculturing them. But since C. tetani and C. sporogenes can be presumptively identified due their perculiar colonial morphology, they were easily located in a mixed culture on blood agar. The greatest problem arise when C. septicum and C novyi occur together. They both occur as very thin film on blood agar. It is interesting to note that these two species were separated by shortening the incubation period to 48 hours and in some cases overnight. Since C. novyi is slow growing may be due to its strick anaerobic requirement, a

relatively pure culture of C. septicum can be obtained after the shortened incubation period during which time the C. novyi colonies were just beginning to appear.

It is worth noting that cotton - plugged tubes were used for the sugar tests. This allows a reasonable anaerobic condition to be created during autoclaving, since most of the air would have been driven off the tube at the end of sterilization. The sugars were inoculated while warm and immediately layered with sterile paraffin oil.

The idea of incubating Clostridial species in a vaccum originated after reading through Sheena's work on the Carbondioxide requirement of anaerobic bacteria in 1980. Sheena came out with the brilliant findings that C. perfingens grows well in the presence or absence of carbondioxide and even in vaccum; improvement of growth of C. bifermentans, C. chauvoei and C. histolyticum occurs in the absence of carbondioxide and growth of C. sporogenes and C. septicum is unaffected in the absence or presence of carbondioxide. It is therefore not surprising that most of the known pathogenic species of Clostridia including the most anaerobic demanding C. tetani species were isolated from vaccum anaerobic jars.

This is in agreement with the findings of Tabaqchali et al in 1979 that different anaerobic organisms require different conditions for survival and laboratory growth and that most strains from clinical specimens belong to the "moderately anaerobic" group. These are capable of growth at oxygen concentration of 2 - 8% and can survive atmospheric air exposure for 60 to 90 minutes, and can still be recovered after 72 hours from pus specimen.

As much as the idea of vaccum anaerobic jars may not be perfect for the laboratory survival and growth of anaerobes, it is reasonably reliable for the routine work on anaerobes in our laboratories considering our present economy. In this circumstance one can then accept the isolation rate in this experiment as reasonably good both in comparison with the findings in other parts of the world, as well as a reliable index of the actual situation in the areas surveyed. It is also significant to note the low incidence of some of the most pathogenic species of these organisms in places like play grounds, motor parks and market places (C. tetani and C. botulinum).

The low incidence of these organisms in these areas further

suggests that with adequate public health preventive measures, inform of vaccination, the occurence of infection with these organisms can further be minimized. It is noteworthy that in an otherwise rural hospitals investigated, none of the pathogenic species was found in all the surgical instruments examined. This observation augurs well for the aseptic conditions of the normal sources of infections in rural areas.

However, the finding of some species of pathogenic Clostridia in hospital environment, further illustrates the need for cleaner hospital environment in our society if only to minimise the problems and increasing incidence of nosocomial infections.

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