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CURRENT TREND IN ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF *CLOSTRIDIUM TETANI* ISOLATED FROM SOIL SAMPLES IN KANO

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ABSTRACT
The need for a regular assessment of the antimicrobial sensitivity patterns among tetanus causing as well as infectious members of Clostridia found in soil-human settlement provides a justification for the present study. Accordingly, soil from various locations of Bayero University Campus, Kano was screened for the isolation of C. tetani by anaerobic culturing procedures. The organism was detected in 60% of the soil samples. The isolates were tested against commonly prescribed drugs including sparfloxacin, ciprofloxin, chloramphenicol, cloxacillin and metronidazole by disc diffusion technique. Flouroquinolones; sparfloxacin (30µg), ciprofloxacin (30µg), metronidazole (5µg), gentamycin (10µg) and tetracycline (10µg) showed greater in vitro inhibitory effect. The activity of erythromycin (5µg) and chloramphenicol (30µg) was moderate. However, all penicillin derivatives (augmentin (30µg), amoxicillin (25µg), cloxacillin (5µg) and penicillin V (30µg) as well as co-trimoxazole (25µg) were all inactive against the organism. The flouroquinolones, genta,mycin, chloramphenicol and tetracycline remain the drugs of choice against infections due to the organism, while co-trimoxazole and members of the penicillin group of antimicrobials appeared to loose their in vitro potency and effectiveness..

Keywords: Clostridium tetani, Soil isolate, Chemotherapy, Antimicrobial Susceptibility

INTRODUCTION

Clostridium tetani is a cosmopolitan, spore – forming anaerobic Gram positive bacillary bacterium that is widely distributed in the soil. It produces the second most potent microbial endotoxin known to human and it is the etiologic agent of tetanus (Kenneth, 2004). Tetanus is an acute and often fatal infectious disorder that is characterized by increase muscle tone and episodic muscular spasms. The disease usually results when a wound or laceration accidentally become contaminated with the spores of the organism, and under anaerobically favourable condition, the spore germinate and the vegetative cells produce toxin, which is released into the surrounding interstitial spaces. The possible consequence of the absorption of this toxin into the central nervous system (CNS) is the blockage of the release of inhibitory neurotransmitters, gamma amino benzoic acid (GABA) and glycine. This leads to dangerous over activity in the muscles called tetanus. Despite the World Health Organization's intention to eradicate tetanus by the year 1995, it still remains an important cause of death worldwide, particularly in developing world. There are estimated 800,000 to 100,000 deaths from tetanus each year (Daniel, 2003).

Tetanus can be diagnosed bacteriologically by isolating *C. tetani* from the infected wound. But this is not usually done, because the disease is so easily diagnosed clinically on the basis of its unique, recognizable and archetypal signs. However, the wound from which the specimen can be collected for bacteriological investigation might sometimes, not be obvious and the organism is non – invasive, therefore, only very few isolates of the bacterium from clinical cases have been studied. *C. tetani* is commonly found in soil samples in all parts of the world (Cook *et al.,* 2001).

Considering the epidemiological consequences of *C. tetani* (Kenneth, 2004; Prescott and John, 1999), it is imperative for researchers to continue to investigate the occurrence of the organism in human immediate environment on regular basis. It is therefore, the aim of this research to investigate the occurrence of *C. tetani* from soil samples of Bayero University, Kano old campus, as well as to re-examine its current antimicrobial susceptibility pattern. This was with a view to assessing the current suitability of the drug agents for use in its chemotherapy.

MATERIALS AND METHODS

Isolation of the Organism

The method applied in this research was a modification of one described by Ichiro and Shoki (1964). Soil specimens (2g) were collected from five different sites, namely, botanical garden, central mosque entrance where shoes are kept, fronts of theater 1 and 2, and male hostel). These were aseptically heated in a test tube at temperature of 60^{o}C for an hour. Sterile water was added to each, until the suspension was 10ml, making a dilution of 0.2g/ml. One milliliter (1ml) aliquot of each of the dilution was inoculated onto fresh blood agar medium by means of pour plate technique. After solidification, the plates were incubated in anaerobic jars containing sodium boronhydrate and sodium carbonate.

Establishment of Anaerobic Condition

To create anaerobic environment, 2g each of sodium boronhydrate ($NaBH_2$) and sodium trioxocarbonate (IV) ($NaCO_3$) (May and Baker Ltd, England) were put in small container and placed in the anaerobic jar and the cap of the jar was replaced immediately. This is a modification of the oxoid gas pack (Mukhtar *et al.,* 2008).

Sodium boronhydrate and sodium carbonate in the container react with water separately to form hydrogen gas (H_2) and carbondioxide (CO_2) respectively. The hydrogen gas neutralizes the oxygen in the jar with the subsequent formation of water. CO_2 on the other hand made the environment more suitable for the growth of the target organisms.

After incubation, cultures were examined for cultural and colonial characteristics. Colonies that appeared flat, translucent with irregular and rhizoid margins, which is the description of *Clostridium tetani* (Paul and Stanley, 1963) were carefully picked and aseptically subcultured on fresh blood agar medium and incubated anaerobically for 24 hours at 35^{o}C again. Cultures were examined for cultural and colonial characteristics of an axenic culture of the organism.

Microscopical and Biochemical Identification

Colonies from axenic cultures were used for staining and biochemical tests. Gram's and spore staining techniques were conducted on young (24 hour old) and old (7 day old) cultures. Catalase and coagulase producing ability of the isolates was examined. Motility was also observed by microscopic examination of the wet preparation of the isolate (Cheesbrough, 2002).

Antimicrobial Sensitivity Testing

The isolates, which were confirmed to be *C. tetani,* were subjected to antimicrobial sensitivity testing. Disc diffusion technique was adopted and fresh blood agar media were used. Commercially prepared antimicrobial discs (ABTEK, Biological Limited, UK) impregnated with disc potent amoxicillin (25µg), chloramphenicol (30 µg), tetracycline (10 µg), erythromycin (5 µg), augmentin (30 µg), co – trimoxazole (25 µg), metronidazole (5µg), penicillin V (30µg), gentamycin (10µg), cloxacillin (5µg), sparfloxacin (30 µg) and ciprofloxacin (30 µg) were used.

Preparation of Standard Culture

Loopful of colony from axenic culture was suspended in sterile saline to make a suspension with turbidity equals to that of 1% barium sulphate solution. This was enough to produce about 3.3 x 10^6 cfu/ml (0.5 Macfarland standards). Three loopfuls were used to inoculate the agar surface by streaking method. After 10 minutes prediffusion time, replicates of test drugs discs were aseptically fastened onto the culture surface using a pair of forceps. These were anaerobically incubated at 35^{o}C for 18 hours. Where no growth was observed, incubation continued until 24 hours. The plates were examined for zones of inhibition at the individual discs. Zones were accurately measured and recorded using millimeter rule to the nearest whole number. The Table of National Clinical Laboratory Standards was consulted for the interpretation of the susceptibility patterns of the organism to the various drugs (Cheesbrough (2002).

RESULTS

During the examination of the incubated culture that were inoculated with the soil specimen, a strong odour was perceived immediately after uncovering the jar, droplets of water was seen by the sides of the inverted cultures as well as along the wall of the jar. Colonies of varying shape and size were formed and clear zones of haemolysis were observed. The colonies which were suspected to be *C. tetani* were about 3 – 6mm in diameter. The colonies were detected in 24 (60%) of the samples with mean colony count of 6.29 x 10^2 cfu/ml.

The subcultured colony swamped all over the surface of the media, with odour perceived. Zones of hemolysis were also observed.

Microscopic examination of the Gram – stained smear obtained from 1 day old cultures revealed Gram – positive bacilli, but the smear obtained from the 7 day old culture showed Gram – negative bacilli, many of which have drumstick – like appearance. Spore staining of the 1 – day old culture produced the same result with that of Gram staining. But 7 – day old culture revealed pink coloured bacilli with light green colour at their terminals. Neither catalase nor coagulase was produced by the isolates, but motility was conspicuously observed.

Antimicrobial Sensitivity Results

Besides the cultural and colonial characteristics similar to that observed during the pre – confirmative stages, clear zones of inhibition were observed around some antimicrobial discs with others devoid of such zones (Table 1).

The antimicrobials that have activity against the isolate were erythromycin, tetracycline, gentamycin, chloramphenicol, metronidazole and ciprofloxacin, with sparfloxacin been the most active. Others including the penicillins were found to be inactive (Table 1).

Table 1: Mean diameter of the zones of inhibition (mm) produced by the antimicrobials against the isolates

Antimicrobials	Potency	Mean diameter of zone of inhibition (mm)	Remarks on activity
Erythromycin	5	10	+
Tetracycline	10	18	++
Augmentin	30	0	-
Amoxicillin	25	0	-
Chloramphenicol	30	14	+
Co-trimoxazole	25	0	-
Metronidazole	30	18	++
Gentamycin	10	16	++
Cloxacillin	5	0	-
Penicillin V	30	0	-
Sparfloxacin	30	32	+++
Ciprofloxacin	30	26	+++

Key:
- inactive, + moderately active, ++ very active, +++ highly active

DISCUSSION

Isolation of *C. tetani* is in fact, one of the more tedious practices in bacteriology. A strong odour perceived in this research was indicative of fermentation. *Clostridium* species do not oxidize carbohydrate or water and carbondioxide, because of their inability to utilize oxygen, or rather due to their inability to carry out complete biodegradation of inorganic substances. The odour usually gives a suggestion that *Clostridium* species was present.

C. tetani is an obligate anaerobe, and therefore its isolation requires an environment, which is totally devoid of even a trace of oxygen. The technique applied in this research to create an anaerobic condition was found to be effective, less expensive and reliable. This was because the drops of water observed in the jar and at the sides of the inverted cultures were evidence that absolute neutralization of oxygen in the surrounding jar was achieved. The use of sodium boronhydrate and sodium carbonate can be encouraged in trying to improvise for the costly and usually scarce gas packs in common laboratories that face economic crunch.

The irregular and rhizoid appearance of *C. tetani* observed in this research was due to vigorous and restrictive motility of the organism, which in trying to escape away from the colony, form such branching projections. But the organism swamped on the surface of fresh agar because of its motility was unrestricted (Paul and Stanley, 1963).

During the decline phase of growth (e.g. in old culture) many bacteria establish a mechanism which enhance resistance against the unbearable condition (exhaustion of nutrient due to overcrowding, low water activity, ageing and other inconveniences). *C. tetani* was known as a producer of terminal endospores (which give it a distinctive drumstick appearance) that confer resistance to such adverse conditions (Paul and Stanley, 1963). This research has empherically demonstrated this phenomenon although during the active growth of the organism (in young cultures) spores were not observed. But in 7 – day old cultures, spores were perspicuously seen through microscopic examination of both Gram and spore stained smears. The isolate was established to be toxigenic in laboratory mice in one parallel study by Mukhtar *et al.* (2008).

The sensitivity of *C. tetani* to metronidazole as observed in this research has been globally known for long and the drug is being used in the treatment of tetanus. The present work still proves it to be appreciably active. Tetracycline, erythromycin, chloramphenicol and gentamycin were also found to have tremendous activity against the isolate. These were described to be suitable as alternative drugs (Cook *et al.,* 2001). Sparfloxacin and ciprofloxacin (both of which are members of flouroquinolones) were found to have greater *in vitro* activities (32mm and 26mm zone diameters respectively). Members of florouquinolones family were described to operate pharmacological principle of interactive inhibition. The drugs affect DNA gyrase which is an enzyme that has a variety of activities including the introduction of negative superhelical twists into double stranded DNA (a reaction that creates tension in the double helix that favours unwinding of the double strands) and the catenation and decatenation of two deplex DNA circles interlocked like links in a chain (Prescott and John, 1999). This maintains the drugs high activity trend against *C. tetani.* On the other hand, the sensitivity to penicillins, which hitherto this investigation was the most well known and frequently used antibiotics as far as treatment of tetanus is concerned, was found to be poor currently.

Conclusion and Recommendation

There was exuberance of *C. tetani* on soil samples around human habitations as shown in this research, and the organism is active to a wide range of commonly used antibiotics. The most active chemotherapeutic agents are the sparfloxacin, ciprofloxacin, metronidazole, chloramphenicol, erythromycin, gentamycin and tetracycline. However, members of penicillin drugs appeared no more active on the bacterium.

The public should therefore be encouraged on personal hygiene and the health authorities should continue to pay attention to the use of anti-tetanus vaccine as well as the few active drugs in both prophylaxis and chemotherapy of the infection. However, it is imperative for the global health authorities to put members of the florouquinolones, such as sparfloxacin, ciprofloxacin under consideration, with more emphasis on their large scale production, so that they can be affordable for use in the treatment of tetanus. In view of the antimicrobial sensitivity status of *C. tetani,* it is also advisable for such authorities to continue to support the global examination of the different antimicrobials against various strains of the bacteria. In fact, this will overcome the problem of emergence of resistant strains of the pathogenic bacteria.

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