

CLOSTRIDIA IN LABORATORY MEDICINEDeepali P. Danave¹, S. N. Kothadia², N. K. Shaikh³**HOW TO CITE THIS ARTICLE:**

Deepali P. Danave, S. N. Kothadia, N. K. Shaikh. "Clostridia in Laboratory Medicine". Journal of Evolution of Medical and Dental Sciences 2015; Vol. 4, Issue 43, May 28; Page: 7430-7435, DOI: 10.14260/jemds/2015/1078

ABSTRACT: OBJECTIVES: The significance of anaerobic bacteria as human pathogens is well documented. We undertook this preliminary study to detect anaerobic clostridial infections from clinical samples. **MATERIAL AND METHODS:** 58 clinical samples from various sites were obtained depending upon diverse manifestations during five consecutive years. They were subjected to direct smears using Gram stain and cultured anaerobically using Robertson's cooked meat broth. Culture smears were read after incubation from 48 hours to 5 days. **RESULTS:** Out of the 58 samples only 18 samples (25.86%) grew Gram positive spore bearing bacilli. Most of the samples showed polymicrobial flora. There was a preponderance of Gram positive bacilli with subterminal and central spores (55.55%). **CONCLUSION:** Although diagnostic anaerobiasis is tedious and time consuming all clinical microbiology laboratories should start the work in view of the significance of anaerobic infections.

KEYWORDS: Anaerobes, Clostridia, Gram stain, Robertson's cooked meat broth.

INTRODUCTION: In 2015 it has been more than 100 years for the death of Louis Pasteur who was the discoverer of anaerobes. He classified bacteria as aerobes and anaerobes.

When World War I broke out the access to natural rubber was cut off and there was an urgent need to synthesize it. England needed butyl alcohol, 30,000 tons of acetone and this was commissioned to Dr. Weizman by Winston Churchill. Clostridium acetobutyricum was used and several anaerobes were shown to produce glycerol much needed for production of explosives. Without this knowledge of anaerobes the English would have lost the war.

Anaerobic bacteria are those micro-organisms which do not use oxygen for growth and metabolism but obtain their energy from fermentation reactions. They will not grow in the presence of oxygen but will grow at low or negative oxidation reduction potential (Eh). Anaerobic bacteria are closely associated with human body as commensals. Gram positive non-spore forming anaerobic bacilli are present as commensals on all possible sites of human body viz. skin, upper respiratory tract, mouth, intestine, female genital tract.^[1] Clostridia are normal flora of the colon.

The significance of anaerobic bacteria as human pathogens is well known. The common clinical conditions with anaerobic infections include deep seated abscesses, infection of closed spaces, wound infections, gangrene, infection in the vicinity of mucosal surfaces, septicemia. The clinical clues for anaerobic bacterial infections are presence of foul odour, gas in tissues, black exudate, and patient's not responding to aminoglycoside therapy. It may be stressed that clostridial infections are still very common in our country.

Conventional methods for culture of anaerobes are time consuming and it takes ten days for full working up of a clinical specimen and to send out the report. Hence we undertook this preliminary study to detect anaerobic clostridial infections from clinical samples based on high degree of suspicion.

ORIGINAL ARTICLE

MATERIALS AND METHODS: The study was conducted in our institute during the years July 2009-Sept. 2014. Samples were obtained from patients presenting to our hospital. Possible cases (infants to elderly) were chosen depending on diverse manifestations as gangrene, diabetic ulcer, deep seated abscess, incised, debrided wound etc. based on one or more clinical clues suggestive of anaerobic infection. Single sample was obtained from each patient amounting to total 58 samples from various sites. Unstained direct smears for examination were obtained from the clinicians.

These were observed using Gram stain. Samples were simultaneously inoculated in Robertson's cooked meat broth and sent to the laboratory. The samples were cultured anaerobically in this medium. It contains unsaturated fatty acids which take up oxygen, the reaction being catalysed by haematin in the meat and also a reduced oxidation reduction (OR) potential. The samples were incubated at 37°C from 48 hours to 5 days before discarding them. After 48 hours of incubation smears were done daily using Gram stain. Presumptive identification of clostridial species was done depending upon morphology seen in stained smears and then reported. Co-relation between direct smears and culture smears was also done.

RESULTS: A total of 58 samples were cultured anaerobically out of which 18 samples (25.86%) were positive while 40 samples (68.96%) were negative for Clostridia – Table 1. Maximum number of samples i.e. 38 samples (65.51%) were obtained from the Dept. of Surgery - Table 2. There was a preponderance of samples obtained from gangrene (20.68%), excised muscle/ tissue (13.79%), traumatic/ incised wounds (18.96%) followed by diabetic ulcers (6.89%) and deep seated abscesses (6.89%) - Table 3. Presumptive identification of clostridial species on the basis of location of spores is shown in Table 4. Gram positive bacilli with sub- terminal and central spores were maximum (55.55%).

Year	Total	Positive	Negative
2009	9	6	3
2010	18	2	16
2011	7	3	4
2012	9	1	8
2013	6	1	5
2014	9	5	4
Total	58	18	40

Table 1: Total samples with year-wise distribution

Dept. Year	Surgery		OBGY	Medicine		Pediatrics		Orthopedics		Total
	M	F	F	M	F	M	F	M	F	
2009	3	2	-	-	1	3	-	-	-	9
2010	6	5	6	-	-	1	-	-	-	18
2011	3	-	-	-	-	1	1	2	-	7
2012	5	4	-	-	-	-	-	-	-	9
2013	4	1	-	1	-	-	-	-	-	6
2014	3	2	-	-	1	2	-	1	-	9
Total	38		6	3		8		3		58

Table 2: Department and sex-wise distribution of samples

ORIGINAL ARTICLE

Year Site	2009	2010	2011	2012	2013	2014	Total
Gangrene	3	4	1	1	2	1	12
Diabetic ulcer	1			3			4
Abscess/pus		1	1	2			4
Incised wound/ intraoperative sample		4	1				5
Traumatic wound/ Debrided wound	1	2	2	1			6
Scrotal gangrene (Fournier's)		1	1				2
Cellulitis		2		1		1	4
Excised muscle tissue		1	1		3	3	8
Ear swab		1				1	2
Urine	1						1
Blood	1						1
Tracheal aspirate						1	1
Rectal swab	1						1
Unknown site	1	2		1	1	2	7
Total	9	18	7	9	6	9	58

Table 3: Site -wise distribution of samples

Species	Gram positive bacilli - Position of spores						Total
	Cl. perfringes/ Cl.septicum/ Cl.novyi		Cl.difficile/ Cl.tertium	Cl.tetani/ Cl.tetanomo- rphum	Sub- terminal and terminal	Central+ Sub- terminal+ terminal	
	Central	Sub- terminal	Oval and terminal	Spherical & terminal			
Position of spores Year							
2009		5				1	6
2010	2						2
2011		1			2		3
2012			1				1
2013			1				1
2014	1	1	1	1	1		5
Total	3	7	3	1	3	1	18

Table 4: Presumptive identification of positive samples.^[2]

DISCUSSION: Clostridia are widely distributed in nature in oxygen free habitats. Clostridia in addition to its main soil habitat, were found in dust, sewage, rivers, lakes, sea water, milk, vegetables, fresh meat, fish, insects and the intestinal tract.^[2]

ORIGINAL ARTICLE

The genus clostridium comprises nearly 100 species, sub- divided into a majority of non-pathogenic species, 25 to 30 minor pathogens and around 13 classical major pathogens. The notable major pathogens are classified on the basis of lesions and clinical signs into neurotoxic clostridia (*C. botulinum* group I, II and III *C. tetani*), histotoxic clostridia (*C. chauvoei*, *C. novyi*, *C. bifermentans*, *C. septicum*, *C. hameolytium*) and/or enterotoxigenic clostridia (*C. perfringens*, *C. ramosum*, *C. difficile*, *C. septicum*, *C. sordellii*). Clostridial infections like intra-abdominal infections, pulmonary infections, pelvic infections, brain abscesses, skin and soft tissue infections, oral and dental infections, bacteremia and endocarditis are caused by various species such as *C. perfringens*, *C. novyi*, *C. septicum*, *C. bifermentans* and two species *C. botulinum* and *C. tetani* that release neurotoxins are responsible for botulism and tetanus respectively. In addition recent innovations in food processing and packaging technology have created an increasing interest in the magnitude of botulinal spore contamination of various foods.

So the main objective of our study was to determine the prevalence of clostridia in various clinical samples.

Anaerobic bacteria are slow growing organisms usually occurring along with other aerobes or anaerobes. Identification of anaerobes is time consuming and technically demanding because of increasing awareness of role of anaerobes in human infections. There are three levels at which laboratories can operate with regard to identification:

Level I: Presumptive identification from primary cultures in conjunction with morphology, Gram reaction, aerotolerance, distinguishing characters.

Level II: Using simple tests to further group the anaerobes and speciate certain ones.

Level III: Identify isolates using PRAS biochemicals, miniaturized biochemical systems, rapid enzyme detection panels, gas liquid chromatography, toxin assays etc.

Accurate identification of anaerobes to species/subspecies level may not be available in all laboratories. However initial reports based on direct methods of detection such as Gram staining maybe very useful to the clinician in initiating appropriate therapy.

Diagnosis of anaerobic infections is still dependant to a large extent on conventional cultural methods which are time consuming and rather expensive. So the aim of our study was presumptive identification of isolates which would provide useful information to the clinician and not accurate identification to species/ subspecies level. Growth of clostridia is relatively slow on solid media. But clostridia grow in RCM broth rendering the broth turbid.^[3] Hence we chose this medium.

Anaerobic infections are usually endogenous and are caused by tissue invasion by bacteria normally resident on the respective body surfaces. Anaerobic infections generally follow some precipitating factor such as trauma, tissue necrosis, impaired circulation, haematoma formation or the presence of foreign bodies.^[3] Dental flora contributes to anaerobic infections of the lung. Any nick or even a needle puncture can spill enough anaerobes into the peritoneal cavity to produce disease. All injuries and surgery on the colon must be treated with anti- anaerobic antibiotics otherwise disastrous infections can be expected during the post- operative period.^[4] Anaerobic colonization of small bowel occurs in cases of tropical sprue,^[5] diabetes mellitus and perhaps AIDS. Colonic cancer and leukemia have also been associated with clostridial septicemias. *Clostridium difficile* is the etiological agent for almost all cases of pseudomembranous colitis and 15-25 % of antibiotic associated diarrhea.^[6,7,8]

ORIGINAL ARTICLE

Majority of the samples which we received were from surgical wards (Table 2). The samples were obtained predominantly from gangrenous tissue, diabetic ulcers, incised/ traumatic wounds, excised tissue, cellulitis (Table 3). This indicates that some underlying host risk factor was already present predisposing them to anaerobic infections. Pronounced cellulitis is a common feature of anaerobic wound infections. This was also seen in majority of the patients.

The anaerobic infections can often be of polymicrobial nature. Anaerobic infections may in fact be a concerted pathogenesis of aerobes and anaerobes. Alternatively anaerobes may co-exist with other aerobes. In gas gangrene generally several species of clostridia are found in association with anaerobic streptococci and facultative anaerobes such as *E. coli*, proteus and staphylococci. *C. perfringens* is the most frequently encountered (Approx 60 percent) with *C. novyi* and *C. septicum* next (20- 40 per cent). Others like *C. histolyticum*, *C. tertium*, *C. sporogens* may also be encountered.

This maybe a reflection of the distribution of the species in different soils.^[9]

In our study polymicrobial flora of gram positive cocci/ nonsporing gram positive bacilli/ gram negative bacilli was also evident in most of the smears along with gram positive spore bearing bacilli. Of the positive samples, ten (55.55%) had bacilli which showed central/ subterminal spores indicative of *C. perfringens*/ *C. septicum*/ *C. novyi*. Three samples (16.66%) had bacilli with oval & terminal spores indicative of *C. difficile*/ *C. tertium*. One (5.55%) sample showed bacilli indicative of *C. tetani*/ *C. tetanomorphum*. This was isolated from ear swab where otogenic tetanus was suspected. Four (22.22%) samples had gram positive bacilli with various arrangement of spores indicating that more than one species of clostridia were co- existing. Thus our isolates were indicative of diverse species.

In a study conducted by S Sathish et al^[10] it was shown that there is a large diversity of toxigenic clostridia species responsible for gas gangrene, wound infections and neurological disorders within the natural soil, aquatic and meat environment. Furthermore some species were found higher during storm events suggesting that they were also mobilized from widespread animal rearing and slaughter activities.

Secondly, an upsurge of anaerobic infections has also resulted due to the development of resistance to the commonly used anti-anaerobic drugs. The susceptibility patterns of anaerobic bacteria are undergoing changes and decreased in- vitro susceptibility to various antimicrobials has been reported in recent years.^[11] Resistance to metronidazole, β lactam drugs has also been on the rise. Hence attempts to isolate and identify anaerobes from clinical infections would prove beneficial.

CONCLUSIONS: Although anaerobic bacteriological work is tedious and time consuming, all clinical microbiology laboratories should start the work in view of the increasing significance of these bacteria in human infections. There is great scope for research as very limited data is available in our country with our patients. Development and standardization of simplified methods requires focus.

REFERENCES:

1. Gorbach SL, Menda KB, Thadepalli H and Keith L. Anaerobic microflora of the cervix in healthy women. *Am J Obstet and Gynaecol.* 1973; 117: 1053-1056.
2. Haagsma J. Pathogenic anaerobic bacteria and the environment. *Rev Sci Tech* 1991; 10: 749-64.
3. Anathanarayan & Paniker's. *Anaerobic Bacteria I: Clostridium*. In Arti Kapil, editor. *Textbook of Microbiology* 9th edn. Universities press (India) Pvt.Ltd.2013; p253.

ORIGINAL ARTICLE

4. Thadepalli H and Mandal AK. Precepts and practices of antimicrobial therapy in abdominal surgery. CRC press, Boca Raton, Florida. 1991.
5. Thadepalli H. On the etiology of tropical sprue. *Tropical Gastroenterology*. 1981; 2: 4.
6. Bartlett JG et al. Antibiotic anaerobe pseudomembranous colitis due to toxic producing clostridia. *N Engl J Med* 1978; 298: 531-534.
7. Bartlett JG. Clinical practice: Antibiotic associated diarrhea. *New Engl J Med* 2002; 346:334-9.
8. Vaishnavi C. Antibiotic associated diarrhea and colitis. In: Vaishnavi C, editor. *Gastrointestinal tract infections*. New Delhi: Paragon International Publisher; 2008. p 152-68.
9. Kriek NPJ, Ondendaal MW. *Clostridium chauvoei* infections. *Infectious diseases of livestock with special reference to Southern Africa*. In: Coetzer JA, Thomson GW, Tustin RC, editors. Standard ed. Oxford: Oxford University Press; 1994. p5
10. Sathish S, Swaminathan K. Genetic diversity among toxigenic clostridia isolated from soil, water, meat and associated polluted sites in South India. *Indian J Med Microbiol* 2009; 27(4): 311-20.
11. Morya F, Lozniewskia A, Blandb S, Sedallianb A, Grollierc G, Girard- Pipaud F, et al. Survey of anaerobic susceptibility patterns: A French multicentre study. *Int J Antimicrob Agents* 1988; 10: 229-36.

AUTHORS:

1. Deepali P. Danave
2. S. N. Kothadia
3. N. K. Shaikh

PARTICULARS OF CONTRIBUTORS:

1. Assistant Professor, Department of Microbiology, Dr. V. M. Government Medical College.
2. Professor & HOD, Department of Microbiology, Dr. V. M. Government Medical College.

FINANCIAL OR OTHER

COMPETING INTERESTS: None

3. Associate Professor, Department of Microbiology, Dr. V. M. Government Medical College.

NAME ADDRESS EMAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Deepali P. Danave,
Department of Microbiology,
Dr. V. M. Government Medical College.
Opp. District Court, Solapur-413003.
E-mail: milliondollarbaby@rediffmail.com

Date of Submission: 08/05/2015.
Date of Peer Review: 09/05/2015.
Date of Acceptance: 22/05/2015.
Date of Publishing: 26/05/2015.