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Research Article

# Use of antimicrobial susceptibility pattern as an identification marker of Gram positive, aerobic spore forming bacilli, isolated during vaccine manufacturing process

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**Abstract:** Bacilli used in this study were *Bacillus megaterium, Bacillus cereus, Bacillus mycoides, Bacillus anthracis and Bacillus subtilis.* All of the bacilli tested were Gram-positive while hemolysis on blood agar was variable. All bacilli were motile and catalase positive except for *Bacillus anthracis*. The Lecithinase reaction on egg yolk agar was also variable whereas urease activity was absent in all the organisms including *Bacillus anthracis*. Gelatin hydrolysis and anaerobic growth were positive in all bacilli. Sodium Chloride (NaCl) at concentration of 6% was tolerated by *B. megaterium* and *B. subtilis*. Free or endospores were observed in all including *B. anthracis*. Acid was produced from glucose, salicin and xylose in oxidation/fermentation reactions, whereas utilization of mannitol, maltose and lactose varied. The organisms have opaque to creamy ground glass consistency colonies on agar surfaces compared to broth where homogenous growth and pellicle formation were the main features and varied according to organism. Under the microscope; short to long chains made up of Gram-positive rods were observed. Forty four (44) antibiotics from different groups were used in this study. Of these 9 were from cephalosporin group, 6 from quinolone group, 5 from penicillin group, 3 from ß-lactam

inhibitors combination group, 2 from tetracycline group, 4 from aminoglycoside group and 15 from diverse group. The extensive antibiogram of a number of *Bacillary* species envisage to explore selected antibiotics to which the *B. anthracis* is resistant and other isolated *Bacilli* are sensitive. During this study the co-trimoxazole (trimethoprim+sulphamethoxazole) at the concentration of 25 µg/disc allowed the growth of *B. anthracis* (i.e. resistant) but did not allow the growth of other four *Bacilli*.

**Keywords**: bacilli, Antimicrobial, Identification, Marker, vaccine

## INTRODUCTION

The type genus *Bacillus*, of the family *Bacillaceae* currently comprises more than 60 species of aerobic or facultatively anaerobic Gram-positive *Bacilli* that produce endospores[1]. Large number of *Bacillus* species are frequently encountered as contaminants in the bacteriology laboratory, and as contaminants in pathological specimens. Of these species the most commonly found are *Bacillus subtilis*, *Bacillus mesentericus*, *Bacillus mycoids* and *Bacillus megaterium*. They are occasionally found as primary agents in mild localized lesions, but one species *Bacillus anthracis* is an important pathogen of animals and man [2].

*Bacillus anthracis* is the etiological agent of Anthrax, a highly infectious disease of considerable economic importance. Domestic livestock, such as cattle, sheep, goat and horses are the most common victims of the disease[3]. The disease is somewhat unusual in that it spreads from a dead or dying host. *Bacilli* are shed in large number from the body orifices of infected animals during the terminal stages of illness and these readily sporulate in soil at temperature of 20-30 °C; cattle or sheep consequently feeding on contaminated ground are liable to become infected[4,5].

The *Bacillus anthracis* 34F2 Sterne strain has been used to produce vaccine against Anthrax for 60 years. Introduction of this live attenuated spore vaccine in the late 1930s resulted in a decrease in the incidence of the disease both in cattle and in humans[6]. Although reservations regarding the use of naturally occurring mutants or strains attenuated in the laboratory as vaccines have been stressed, the contribution that such vaccines have made in controlling economically important disease in the field should not be forgotten. In the case of Anthrax, the live attenuated spore vaccine derived from an avirulent non-capsulated variant of *Bacillus anthracis* has had a pronounced effect in limiting economic losses resulting from Anthrax[7]. Until 1993 of the 36 worldwide producers of animal anthrax vaccine, 31 use the 34F2 Sterne strain for seed stock[8].

The proposed study is hoped to provide impetus to the vaccine production against *Bacillus anthracis* through availability of a cheapest and reliable monitoring system for purity of culture during various stages of vaccine manufacturing process. Since purity of culture is of paramount importance in vaccine production therefore, monitoring the bacterial biomass for purity will ensure quality and usefulness of vaccine. The study results are anticipated to cut down the economic losses occurring due to non availability of proper monitoring system of purity of biomass during vaccine production process this study will also add knowledge of repeatedly tested antibiograms of vaccinal as well as of wild strain of *Bacillus anthracis*.

## MATERIALS AND METHODS

**Microorganisms used in the study:** All *Bacilli* strains were obtained from different Veterinary Research Institutes. These *Bacilli* were incubated over night on Brain Heart Infusion Agar (BHIA). Pure colonies of *Bacilli* were picked up from the agar surface and maintained in Brain Heart Infusion (BHI) for further use.

**Growth conditions:** Organisms used in the study were routinely grown on BHI and BHIA for primary propagation. All incubations were performed at 37 °C in the conviction air incubators over night (16-24 hours) or if stated otherwise.

**Antibiotic sensitivity testing:**

**Preparation of McFarland nephelometer standards:** A chemically induced precipitation reaction was used to approximate the turbidity of a bacterial suspension.

**Disk diffusion testing:** The disk susceptibility method as proposed by Bauer *et al*,[9] was adopted. For this purpose Muller Hinton Agar (MHA) was used.

**Macro dilution broth susceptibility test:** This method as proposed by the Ericsson and Sheris[10], Washington *et al.* [11] and Barry[12] was used to determine the Minimum Inhibitory Concentration (MIC) of the selected antibiotics.

## RESULTS AND DISCUSSION

**Cephalosporins:** Nine different antibiotics of cephalosporin group showed mixed patterns of sensitivity and resistance against various organisms in the study. The vaccinal strains of *Bacillus anthracis* and extraneous contaminants namely *Bacillus megaterium, Bacillus cereus, Bacillus mycoides and Bacillus subtilis* showed varied diameters but sensitivity to cephradine, cephalexin, cefoperazone and cefaclor.

Among these antibiotics cephradine, cephalexin and cefoperazone were moderately effective. The *Bacillus megaterium* was less sensitive to cefaclor compared to the wild and vaccinal strains of *Bacillus anthracis* and *Bacillus cereus, Bacillus mycoides* and *Bacillus subtilis* which were highly sensitive to cefaclor. Cefotaxime, ceftizoxime, cefixime and ceftazidime had no effect on any of the organisms under study. Ceftriaxone among cephalosporins however presented different profile, where vaccinal strains of *Bacillus anthracis* and *Bacillus megaterium* were resistant, but *Bacillus cereus* and *Bacillus mycoides* were moderately sensitive and *Bacillus subtilis* showed a typically poor sensitivity. The results indicate that among cephalosporins ceftriaxone can be used as an identification marker for the organisms used in the study; whereas *Bacillus anthracis* is resistant to it and *Bacillus cereus, Bacillus mycoides* and *Bacillus subtilis* can grow in the presence of this antibiotic excluding *Bacillus megaterium* which does not grow as do the *Bacillus anthracis.*

**Quinolones:** All quinolones including ofloxacin, ciprofloxacin, enoxacin and lomefloxacin actively inhibited all microorganisms under study. Reaction to pefloxacin was moderate (inhibition zone ranged from 20-28 mm). Nalidixic acid was poor in response where inhibitory zone ranged between 15-22 mm. The vaccinal *Bacillus anthracis* has a difference of 7 mm in the inhibition zone, which could be of significance where wild strain inhibition zone was 15 mm and it was comparable to other *Bacilli* in the

study except vaccinal strain of *Bacillus anthracis* whose inhibition zone was 22 mm. Further study in this direction may therefore prove useful to elucidate whether this difference can be used as an identification marker of vaccinal and wild strains of *Bacillus anthracis*. It is known that Nalidixic acid possesses the basic quinolone structure compared to other quinolones used in the study, which have altered structure named flouro-quinolones.

**Penicillins:** All penicillins whether modified or in combination effectively inhibited the vaccinal strains of *Bacillus anthracis*. The inhibition zone ranged from 21-33 mm however, *Bacillus megaterium* has a very low sensitivity against these antibiotics and was resistant to Penicillin G. The other three organisms in the study had a moderate to high sensitivity towards this group. This study therefore establishes that vaccinal and most probable extraneous contaminant *bacilli* are well inhibited by many members of Penicillin group except *Bacillus megaterium* which was resistant to Penicillin G only. Briefly, wild and vaccinal strains of *Bacillus anthracis* were almost equally sensitive to Penicillin G (P10), while *Bacillus megaterium* was resistant. This finding coincides with other studies. This study also establishes that *Bacillus megaterium* is fairly less sensitive to this group of antibiotics.

**Tetracyclines:** Tetracycline group of antibiotics including tetracycline and doxycycline were very much inhibitory to all *bacilli* including *Bacillus anthracis*. Tetracycline alone or with modification of long activity are one of the most widely used antibiotics in the veterinary medicine. The results in this study show that tetracycline are also effective against *Bacillus anthracis* and can therefore be confidently used during out breaks and clinical therapy.

**Aminoglycosides:** Members of aminoglycoside antibiotics used in this study were gentamycin, tobramycin, amikacin and kanamycin, all antibiotics showed good inhibition against all microorganisms studied. To vaccinal strains of *Bacillus anthracis,* tobramycin's inhibitory zones were 32 mm, Sensitivity of the other *Bacilli* (*B. megaterium, B. cereus, B. mycoides* and *B. subtilis*) against aminoglycosides ranged from 20-30 mm inhibitory zone.

**Other antibiotics:** These antibiotics included, clindamycin, erythromycin, chloramphenicol, vancomycin, novobiocin, polymyxin B, aztreonam, meropenam, trimethoprim, co-trimoxazole, nitrofurantoin, metronidazole, pipemedic acid, penicillin + sulbactam, tazobactam + piperacillin, fucidine, piperacillin, penicillin G and trimethoprim + sulphadiazine. *Bacillus subtilis* was resistant to clindamycin. Aztreonam inhibited all the *Bacilli*. Trimethoprim completely inhibited *B. anthracis* and *B. megaterium.* Co-trimoxazole (sulphamethoxazole + trimethoprim) inhibited (ie found resistant) only *B. anthracis*, whereas all other *Bacilli* used in the study were highly sensitive to this combination formula of trimethoprim and sulphamethoxazole.

*Bacillus subtilis* was found resistant to meropenam. Majority of the *Bacilli* were less sensitive to chloramphenicol, vancomycin, novobiocin and polymyxin. *Bacillus cereus* was almost resistant to chloramphenicol (zone 11 mm). *Bacillus anthracis* was less sensitive to chloramphenicol, vancomycin, novobiocin, polymyxin, meropenam and resistant to aztreonam, trimethoprim and co-trimoxazole. All *Bacilli* were found resistant to metronidazole while fairly sensitive to nitrofurantoin.

Study have shown that *B. cereus* is sensitive to streptomycin, neomycin, gentamicin, amikacin, oxytetracycline and sulphonamide[13]. This study not tested strptomycin where as aminoglycosides tested

including gentamicin, tobramycin, amikacin and kanamycin inhibited the growth of *B. cereus*. The organism was also found sensitive to tetracycline.

Weber *et al.*[14] conducted in vitro susceptibility of *Bacillus* species and indicated resistance of *B. cereus* against chloramphenicol which is partially in compromise with that study and similar to this study where sensitivity has been shown against gentamicin and ciprocloxacin.

**Determination of MIC:** The Minimum Inhibitory Concentration (MIC) of the selected antibiotic was also performed, during this study the selected antibiotic was Co-trimoxazole (Sulphamethoxazole + Trimethoprim) the results are given in **Table.**

| S. No. | Organisms | Concentration of Co-trimoxazole (µg per ml of media) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 | 12.5 | 25 | 50 | 100 | control |
| 1. | *B. anthracis* | + | + | + | + | + | + | + | + | - | + |
| 2. | *B. megaterium* | + | + | + | + | + | + | - | - | - | + |
| 3. | *B. cereus* | + | + | + | - | - | - | - | - | - | + |
| 4. | *B. mycoides* | + | + | + | + | - | - | - | - | - | + |
| 5. | *B. subtilis* | + | + | - | - | - | - | - | - | - | + |

In this method the antibiotic was serially diluted as 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.2 µg/ml, 6.4 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. All the four organisms were inoculated in the said dilutions of antibiotic concentrations.

*Bacillus anthracis* grew in all concentrations except in 100 µg/ml. The *Bacillus megaterium* showed no growth in concentrations of 50 and 100 µg/ml.

The *Bacillus cereus* showed growth at 0.4, 0.6 and 1.6 µg/ml concentrations. The *Bacillus mycoides* showed growth at 0.4, 0.8, 1.6 and 3.2 µg/ml concentrations whereas the *Bacillus subtilis* showed growth at 0.4 and 0.8 µg/ml concentrations.

There appears to be no study existing where comparative MICs have been studied with such a combination of *Bacilli* group of organisms which warrants further studies in this direction.

## CONCLUSION AND SUGGESTIONS

Immunoprophylaxis is a procedure of choice for the control of anthrax, in any given area. It involves use of live spore vaccine of quality. Less efficient vaccine manufacturing process usually suffer from problem of contamination from the surroundings. These contaminants usually belong to bacillus species.

The contamination can be controlled by observing strict parameter test of culture purity at each stage of vaccine production. This study aimed at differentiation of *Bacillus anthracis* vaccinal strain and other commonly related environmental bacilli through the biochemical tests, antibiotic susceptibility and determination of MICs. In the light of results obtained during this study the following recommendation and conclusions are set here forth to improve the understanding of identification procedures of the member organisms of *Bacilli* especially *Bacillus anthracis* for use in the procedures of applied microbiology such as that of laboratory identification and vaccine production.

1. Conventional bacteriological, biochemical and sugar fermentation tests are not sufficient to differentiate between vaccinal and wild *Bacillus anthracis,* however these tests differentiate species between the *Bacilli* to a limited extent. It is therefore recommended that advance techniques for differentiation and identification must be realized and used for the purpose.
2. None of the majority antibiotics differentiate vaccinal and wild strain of *Bacillus anthracis,* therefore other antibiotics may be tested to confirm and expand the observations made in this study.
3. This study showed an extensive antibiogram profile of *Bacillus anthracis* and other Bacillus species. There sensitivity and resistance should be taken into consideration while treating infections from a member of Bacillus species.

Differentiation of *Bacillus anthracis* and other important members of *Bacilli, which are common* extraneous contaminants, can be easily differentiated by Co-trimoxazole (MIC 50-100 μg/ml). Therefore the use of this antibiotic at a given concentration may be used in the laboratory procedures to differentiate *Bacillus anthracis* from other bacilli. This antibiotic at given concentration allows growth of *B. anthracis* while other common contaminating bacilli are inhibited and as such this procedure may be adopted in the processes of applied microbiology

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