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# Study the antibiotic spectrum change of antibiotic resistant aerobic bacterial spore in sterilized milk during storage

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**Abstract**
In the present study, four isolates of aerobic bacterial spores identified from raw milk samples such as *Bacillus licheniformis* A6, *Bacillus subtilis* A29, *Bacillus cereus* A35, *Bacillus tropicus* A50 were selected based on antibiotic resistant characteristics against various groups of antibiotics using antibiotic susceptibility test. These resistant *Bacillus* sp. were inoculated to sterilized milk and stored at room temperature (25±1 °C) for 21 days along with the control sample without test organism. During storage it was observed that there was a change in resistant characteristics among the isolates that signifies *Bacillus* sp. which were resistant at before storage became sensitive after storage and vice versa.

**Keywords:** Antibiotic resistance, Sterilized milk, Susceptibility, *Bacillus* sp.

## 1. Introduction

Milk is a highly nutritious substrate that can support the growth of a wide variety of bacteria. Aerobic spore-forming bacteria are important in the dairy industry for several reasons. The ubiquitous nature of these spore formers makes it basically impossible to prevent their presence in raw food and ingredients [1]. Aerobic spore-forming bacteria of the genus Bacillus are commonly present in raw milk. Their spores survive pasteurization and subsequently germinate, outgrow, and multiply. They have been responsible for the spoilage of pasteurized milk and milk product and UHT products [4]. It is necessary to use great care in the collection and handling of milk samples to prevent any extraneous contamination and to control the growth of organisms during transportation and storage of the milk.

Spores of *Bacillus* sp. appear regularly in stable environment and they usually represent a secondary contamination of milk during milking process. Besides predominant mesophilic species, e.g. *B. licheniformis, B. subtilis and B. pumilus*, dominant psychrotrophic isolates are represented by *B. cereus* [4]. Antibiotics are used not only in humans but also to treat individual animals with bacterial infections and prevent infections in herds or flocks. They are also used as growth promoting agents in animal husbandry.

Thermal processing of milk is an essential step for milk safety and has been adopted by the dairy industry. The heat treatment of milk reduces the microbial load and thus minimizes the risk of food poisoning and extends the shelf-life this improves the quality of this complex biological fluid but the heat resistant bacteria such as spore former carrying antibiotic resistant genes will survive and transfer these antibiotic resistant genes to other bacteria through horizontal gene transfer mechanism. The occurrence of antimicrobial resistance microorganisms and their genes in heat treated milk for human consumption has a remote potential to affect human health [7].

Antibiotic resistance genes which are present in partly inactivated, stressed cells get transferred to commensals and other pathogens, both in the foodstuff and after ingestion in the digestive system of humans. This may be achieved either by conjugation or by transformation and transduction. Dead cells may remain intact or be lysed because of cell membrane damage, releasing bacterial DNA including possible antimicrobial resistance genes into the environment. Dead cells cannot pass antimicrobial resistance genes to other bacteria by conjugation or transduction. As soon as DNA has been released antimicrobial resistance genes may be transferred by transformation [8].

## 2. Materials and Methods
### 2.1 Materials
a. Raw milk was procured from KVAFSU Dairy farm, Hebbal, Bengaluru.
b. *Bacillus* sp. isolates which were antibiotic resistant obtained from raw milk samples using antibiotic susceptibility test.
c. Muller Hinton Agar (MHA) for antibiotic susceptibility test
d. Autoclave for sterilization of raw milk

### 2.2 Methods
#### 2.2.1 Sample preparation
Raw milk samples which were obtained from the KVAFSU Dairy farm, Bengaluru were transferred to sterile glass bottles and subjected to sterilization at 121°C/ 15min. The sterilized milk samples were inoculated with characterized and selected antibiotic resistant *Bacillus* sp. obtained from raw milk samples of different sources using antibiotic susceptibility test (AST) at the rate of $10^3$ cells /ml and sealed. The control sample was kept without inoculation of *Bacillus* sp. The sealed glass bottles were kept at room temperature (25±1°C) for storage study till 21st day. The antibiotic resistant characteristics of *Bacillus* sp. were performed at the end of storage using disc diffusion test by Kirby- Baur [2].

#### 2.2.2 Antibiotic susceptibility test for *Bacillus* sp. in stored sterilized milk using disc diffusion method.
The *Bacillus* sp. were grown for 24 h at 37 º C in nutrient broth, 0.2 ml ($10^5$ cells/ml) of each isolate transferred to sterile labeled assay plate and then 10-15 ml of sterile molten Muller Hinton Agar (MHA) was poured into the respective plates. Antibiotic discs (HI Media, Mumbai) were placed on to the agar plates using the sterile forceps. Plates were incubated at 37°C for 24 h in the incubator. After the incubation the isolates were evaluated as resistant or susceptible based on the diameter of inhibitory zone around the discs [6].

### 2.2 Antibiotic discs used and Clinical laboratory and standards institute standards for antibiotics [3].
The standards for inhibitory zone of sensitivity, resistance and intermediate were shown in Table 1.

## 3. Results and Discussion
### 3.1 Effect of storage on antibiotic spectrum of *Bacillus* sp. in sterilized milk
*B. licheniformis* A6 showed sensitive against ampicillin, ceftriaxone, streptomycin, vancomycin and nalidixic acid with inhibitory zone values of 26, 28, 26, 28 and 28mm. Whereas against penicillin, erythromycin and azithromycin displayed resistance with 25mm and no inhibitory zone respectively and against bacitracin displayed intermediate with 9mm. During storage *B. licheniformis* A6 showed sensitive to ampicillin, ceftriaxone, streptomycin, vancomycin and nalidixic acid whereas against penicillin, erythromycin and azithromycin exhibited resistance and against bacitracin displayed intermediate. In this study after storage study the *B. licheniformis* A6 became sensitive against ampicillin and ceftriaxone but initially it was resistant whereas against azithromycin showed resistance which was sensitive initially and against other antibiotics no change was observed.

Similarly, *B. subtilis* A29 showed sensitive against streptomycin, vancomycin and nalidixic acid with inhibitory zone values 22, 23 and 27 respectively while against penicillin, ampicillin displayed resistance with 11mm each, erythromycin with no inhibitory zone azithromycin with 10mm and against ceftriaxone and bacitracin exhibited intermediate with 20 and 10mm respectively. *B. subtilis* A29 showed sensitive against streptomycin, vancomycin and nalidixic acid while against penicillin, ampicillin, erythromycin and azithromycin displayed resistance and against ceftriaxone and bacitracin displayed intermediate. After storage study the *B. subtilis* A29 became sensitive against vancomycin and azithromycin but initially they were resistant whereas against ceftriaxone and bacitracin showed intermediate which were sensitive and resistant respectively at initial period and against other antibiotics no change was observed.

In case of *B. cereus* A35 showed sensitive against streptomycin, vancomycin, azithromycin and nalidixic acid with inhibitory zone values of 16, 21, 32 and 25mm respectively while against penicillin and ampicillin displayed resistance with no zone and 8mm respectively while against ceftriaxone, erythromycin and bacitracin showed intermediate with 16, 14 and 11mm respectively. *B. cereus* A35 during storage showed sensitive against streptomycin, vancomycin, azithromycin and nalidixic acid while penicillin and ampicillin displayed resistance. After storage study the *B. cereus* A35 became sensitive against vancomycin but initially it was resistant whereas against erythromycin, bacitracin showed intermediate which were resistant initially whereas against ceftriaxone changes from sensitive to intermediate and against other antibiotics no change was observed.

*B. tropicus* A50 showed sensitive against streptomycin, vancomycin, erythromycin and azithromycin with inhibitory zone values of 26, 17, 31 and 28 respectively while against penicillin, ampicillin displayed resistance with no zone and 7mm respectively. Whereas against ceftriaxone, nalidixic acid and bacitracin exhibited intermediate with 15, 16 and 11mm respectively (Figure 1). In case of *B. tropicus* A50 during storage showed sensitive against streptomycin, vancomycin, erythromycin and azithromycin whereas against penicillin, ampicillin displayed resistance and against ceftriaxone, nalidixic acid and bacitracin displayed intermediate. After storage study the *B. tropicus* A50 became sensitive to erythromycin and vancomycin which were resistant initially whereas for ceftriaxone and nalidixic showed intermediate which were sensitive and resistant initially and bacitracin exhibited intermediate but initially it was resistant whereas against other antibiotics no change was observed. Statistically significant differences ($P$=.05) were observed within the *Bacillus* sp. against various antibiotics except bacitracin.

**Table 1:** CLSI Standards for antibiotic susceptibility

| Antibiotics name | Antibiotics group | Antibiotics Code | Disc Content | CLSI standards (mm) | | |
|---|---|---|---|---|---|---|
| | | | | S | I | R |
| Penicillin | ß-lactam | P | 10units | 29 | - | 28 |
| Ampicillin | | AMP | 10µg | >17 | 14-16 | ≤13 |
| Ceftriaxone | | CTR | 30µg | ≥21 | 14-20 | <13 |
| Streptomycin | Aminoglycoside | S | 25µg | ≥15 | 12-14 | ≤11 |
| Vancomycin | Glycopeptide | VA | 30µg | 17 | 15-16 | 14 |
| Erythromycin | Macrolides | E | 15µg | 23 | 14-22 | 13 |
| Azithromycin | | AZM | 15µg | ≥13 | - | <12 |
| Nalidixic acid | Quinolones | NA | 30µg | ≥19 | 14-18 | < 13 |
| Bacitracin | Polypeptide | B | 10units | 13 | 9-12 | 8 |

In this study it was observed that the antibiotic sensitivity and resistance characteristics of *Bacillus* sp. may changes during their growth in milk with respect to certain antibiotics and again it was observed that the change was not antibiotic specific. The change in sensitivity and resistance was not similar in all the *Bacillus* sp. the sensitivity or resistance varies with species and the change was not specific to antibiotics. The antibiotic resistant characteristics of all 4 *Bacillus* sp. were shown in Table 2.

In accordance to the present study, Munsch-Alatossava *et al.* [5] determined antibiotic resistance of mesophiles against four antimicrobial agents gentamicin (G), ceftazidim (C), levofloxacin (L), and trimethoprim-sulfamethoxazole (TS) with concentration of 4, 8, 2 and 4 (T), 76 (S) mg/l respectively in raw milk stored at 4 °C for 4 days by aseptically removing 500 µl of raw milk at each day and serially diluting the sample in a saline solution and spreading 50 µl on Mueller-Hinton agar added with antibiotics. Plates were incubated at 30°C for 2days. At day 0, the antibiotic resistance prevalence was highest for ceftazidime and trimethoprim-sulfamethoxazole for mesophiles with 13.8–40 and 10-30% respectively and lowest to levofloxacin (0-5%) and gentamycin (0-30%) in mesophiles. At day 2 antibiotic resistance showed highest of 50-100% to trimethoprim-sulfamethoxazole followed by ceftazidime (10-80%) compared to gentamycin and levofloxacin. At day 4 the antibiotic resistance of ceftazidime, trimethoprim-sulfamethoxazole, gentamycin and levofloxacin was reduced to 7, 35, 2 and 1% respectively. Considering the antibiotic resistance, milk at day 4 could have a lower risk though this milk was spoiled and inappropriate for consumption.

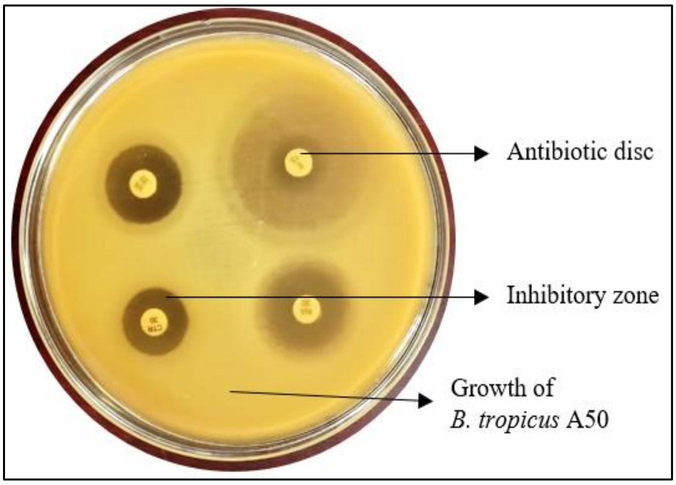


**Fig 1:** Antibiotic susceptibility *B. tropicus* A50 after storage in sterilized milk against VA, E, NA and CTR

**Table 2:** Antibiotic susceptibility of antibiotic resistant *Bacillus* sp. in sterilized after storage at room temperature (25±1 °C) using disc diffusion method

| ***Bacillus* sp.** | **Antibiotic group** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **ß-lactam** | | | **Aminoglycoside** | **Glycopeptide** | **Macrolides** | | **Quinolones** | **Polypeptide** |
| | **P** | **AMP** | **CTR** | **S** | **VA** | **E** | **AZM** | **NA** | **B** |
| | **Diameter of inhibitory zone (mm)** | | | | | | | | |
| *B. licheniformis* A6 | $25^a$ (R) | $26^a$ (S) | $28^a$ (S) | $26^a$ (S) | $28^a$ (S) | $00^c$ (R) | $00^d$ (R) | $28^a$ (S) | $9^a$ (I) |
| *B. subtilis* A29 | $11^b$ (R) | $11^b$ (R) | $20^b$ (I) | $22^b$ (S) | $23^b$ (S) | $00^c$ (R) | $10^c$ (R) | $27^{ab}$ (S) | $10^a$ (I) |
| *B. cereus* A35 | $00^c$ (R) | $8^c$ (R) | $16^c$ (I) | $16^c$ (S) | $21^b$ (S) | $14^b$ (I) | $32^a$ (S) | $25^b$ (S) | $11^a$ (I) |
| *B. tropicus* A50 | $00^c$ (R) | $7^c$ (R) | $15^c$ (I) | $26^a$ (S) | $17^c$ (S) | $31^a$ (S) | $28^b$ (S) | $16^c$ (I) | $11^a$ (I) |
| CD (*P*=.05) | 1.36 | 1.93 | 1.93 | 1.93 | 1.93 | 1.36 | 1.73 | 1.93 | 1.93 |

**Note:**

- The results were average of three trials.
- Same superscript show non-significance while different indicate statistically significant difference (*P*=.05)
- S- Sensitive; I- Intermediate; R- Resistant

## 4. Conclusion

The characterized and selected antibiotic resistant *Bacillus* sp. were inoculated to sterilized milk to study the change in antibiotic resistant characteristics during storage. Antibiotic spectrum of *Bacillus* sp. showed variation in their inhibitory zone and showed resistance to different group of antibiotics like β-lactam, aminoglycosides, quinolones, fluoroquinolones, tetracyclines and nitrobenzene when compared to before and after storage. Use of antibiotics in veterinary medicine should be moderate and careful. If the remaining spores have a resistant trait and are contaminated by bacteria after heating, these germs could cause health issues if milk and milk products are consumed.

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