Spectrum of Antibiotic Resistant Aerobic Bacterial Spore from Raw Milk

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Milk is a very nourishing medium that can help a variety of microorganisms grow. For a range of reasons, aerobic spore-forming bacteria are significant in the dairy sector. It is practically hard to avoid these spore formers' presence in raw food and products due to their ubiquity (Aouadhi *et al*. 2014).

Raw milk frequently contains aerobic Bacillus spore-forming bacteria. Their spores endure pasteurisation, germinate, develop, and then reproduce. They are to blame for the deterioration of UHT products, pasteurised milk, and milk products (McGuiggan *et al*., 2002). Milk samples must be handled carefully during collection to avoid any unintentional contamination and to limit the growth of microorganisms during transit and storage of the milk (Pervin *et al*. 2016).

In stable environments, Bacillus spp. spores frequently indicate a secondary contamination of milk during the milking process. Other than the most common mesophilic species, such as *Bacillus licheniformis, Bacillus subtilis, and Bacillus pumilus*, the dominant psychrotrophic isolates are those of the Bacillus cereus strains (Yacoub *et al*. 2017).

Make sure that raw milk contamination is kept to a minimum to prevent aerobic spore-forming bacteria from contaminating milk and dairy products (Scheldeman *et al*. 2005). Bacterial spores in dairy products with liquid bases rarely pose a health risk, but they might degrade products if pasteurisation or storage procedures are insufficient, resulting in product degradation and revenue losses. For dairy powders, thermophilic spore-forming bacilli can survive in the final product (Burgess *et al*. 2010).

Antibiotics are used not just to treat bacterial illnesses in humans but also to protect flocks or herds from infection. In animal husbandry, they are also employed as growth-promoting substances. The US Food and Drug Administration (FDA) first permitted the use of antibiotics to boost "feed efficiency," or the ability to grow animals quicker, in the early 1950s. This practise led to a shorter period to harvest animals with better economic benefits for farmers and lower costs for consumers (Shea, 2003). Antibiotic resistance is mostly caused by the reckless use of antibiotics in human, animal, and environmental medicine. (Canica *et al*. 2019).

Antibiotic susceptibility testing (AST) is frequently used in clinical settings to identify the antibiotic resistance patterns of bacterial isolates, to direct antibiotic treatment choices, and to forecast the success of therapeutic interventions. The most used techniques for AST testing include the disc diffusion method, the E-strip test, and genotypic techniques like PCR and DNA hybridization. (Syal *et al*. 2017).

Heat-resistant spore-forming microorganisms that carry antibiotic resistance genes cause non-sterility issues in UHT milk products. If they survive, they pass those genes to other bacteria through a process called horizontal gene transfer. There is a distant possibility that the presence of antibiotic-resistant microbes and their genes in heat-

treated milk for human consumption will harm people (Te Giffel *et al*. 2002).

Material & Methods

Sample collection

20 raw milk samples were collected from different sources like individual cow milk, Bulk tank milk, pooled milk from different areas of Bengaluru, Chikkaballapur, Doddaballapur, Kolar and were kept in ice box and transferred to microbiological lab for analysis.

Enumeration of aerobic spore forming bacteria

11ml of well mixed raw milk samples were transferred to 99ml sterile saline diluent and mix the sample thoroughly and heated to 80°C /10min and cool immediately to <10°C using ice cold water. From this make serial dilutions. Dilutions of 1:100 and 1:1000 were prepared. Transfer 1ml of each dilution to sterile petri plates. Transfer 10-15ml of sterile molten 2% Nutrient agar to petriplates and mix the contents slowly. Allow the medium to solidify and incubates the plates in inverted position at 37°C 24-48h. Select the plates having colonies between 30-300. Count the number of colonies present in each dilution, take the average and express the results as cfu/ml (Harrigan, 1998).

Isolation and maintenance of aerobic spore-forming bacterial isolates

Select the countable plate having colonies with erose, curled, flat, irregular morphology. Inoculate loopful of colony into nutrient broth. The isolates were transferred to nutrient agar slants and subcultured once in a month and maintained in refrigerator at 5°C.

Characterization of aerobic bacterial spore isolates

A total of 50 isolates were subjected to preliminary test to confirm the genus and further specific biochemical tests conducted helped to place them in species. Preliminary tests include Gram staining, Motility test, Catalase test, Oxidase test and specific tests includes Oxidative- Fermentative test, Starch hydrolysis, Casein hydrolysis, Voges-proskaur reaction, Nitrate reduction, Egg yolk reaction, Growth at 50°C (Harrigan, 1998).

Antibiotic susceptibility test for aerobic bacterial spore isolates

Characterized isolates of aerobic bacterial spore were subjected to antibiotic susceptibility test using E- strip method against different groups of antibiotics like ß-lactam, Aminoglycoside, Quinolones, Flouroquinolones, Tetracyclines Nitrobenzene. The isolates were grown at 37 °C for 24 h in nutrient broth, 0.2 ml (10^5 cells/ml) of each isolate transferred to sterile labelled assay plate and then pour 10-15 ml of sterile molten Muller Hinton Agar (MHA) into the respective plates. Allow to solidify and swab the isolate in nutrient broth on the solidified agar using sterile cotton swab. Antibiotic strips (HI Media, Mumbai) were placed on the agar plates using applicator. Group of antibiotics like ß-lactam- Ampicillin (0.016-256 µg/ml), Ceftriaxone (0.016-256 µg/ml), Cefotaxime (0.016-256 µg/ml), Aminoglycosides- Streptomycin (0.016-256 µg/ml), Gentamycin (0.016-256 µg/ml), Quinolones-Ciprofloxacin (0.016-256 µg/ml), Fluoroquinolones-Enrofloxacin (0.02-32 µg/ml), Tetracycline (0.016-256 µg/ml), Nitrobenzene- Chloramphenicol (0.016-256 µg/ml) were used. Plates were incubated at 37 °C/24 h. Read the MIC (Minimum Inhibitory Concentration) (CLSI, 2018) value where the edge of the inhibition ellipse intersects the side of strip. Further, the isolates were determined as resistant or susceptible based on the MIC (µg/ml) (Madhusudan, 2016).

Statistical analysis

The data was analyzed using R software [R. version 4.1.2] for statistical computing. Data on the respective variables were collected for three replications for each of these treatments. ANOVA tables were prepared to analyse the data and where the F value is significant, the critical difference was calculated (P=.05) and used to identify where significant differences existed and was indicated in the table use superscripts.

The formula for the critical difference (CD) is

$$CD = \frac{\sqrt{2}\times MSS(E)}{R}\ t\alpha \quad @0.05$$

Where, MSS (E) = Mean Sum of squares of the error

R = number of replications

tα = table t value of the α level of significance

Results

Enumeration of total aerobic bacterial spore from individual cow milk (ICM), bulk tank storage milk (BMST), pooled milk (PM) of different regions

The results showed that the average count of total aerobic bacterial spore in individual cow milk was ranged between 1.0 log cfu/ml to 2.0 log cfu/ml and average count of total aerobic bacterial spore in bulk tank storage milk was ranged between 1.0 log cfu/ml to 2.146 log cfu/ml and similarly the average count of total aerobic bacterial spore in pooled raw milk was ranged between 1.0 log cfu/ml to 2.0 log cfu/ml. The average count of aerobic bacterial count for raw milk samples of different sources were shown in Table 1.

Characterization of aerobic bacterial spore isolates obtained from raw milk samples

Total of 50 isolates were subjected to preliminary tests, results showed that among 50 isolates 40 isolates were aerobic spore forming bacteria. 80% of isolates were Gram positive, rod shape, motile, catalase positive, aerobic in nature. Preliminary identified bacterial isolates were grouped under the Genus Bacillus

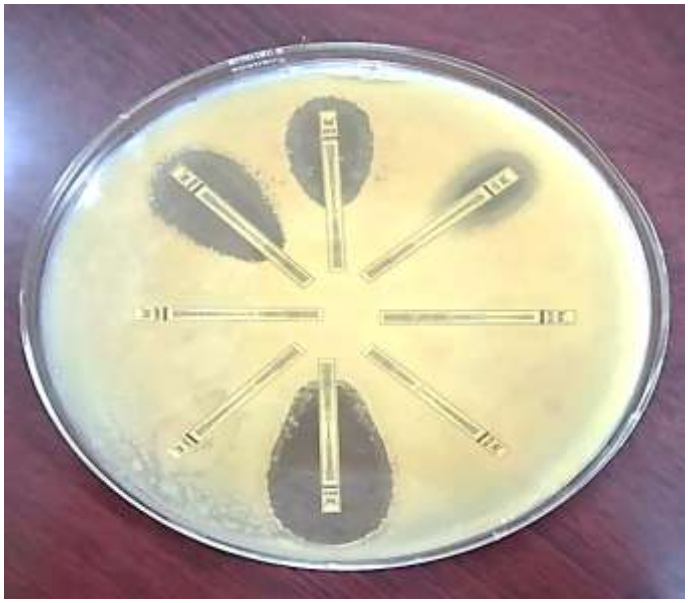
Preliminary identified isolates of Genus Bacillus were subjected to specific biochemical tests like OF test, Starch hydrolysis, Casein hydrolysis, Voges-proskaur reaction, Nitrate reduction, Egg yolk reaction, Growth at 50°C (Harrigan, 1998). Among 40 isolates of 15 isolates were identified as *Bacillus licheniformis*, 10 isolates as *Bacillus subtilis*, 10 isolates as *Bacillus cereus* and 5 isolates as *Bacillus tropicus*. All identified isolates had the ability to produce catalase enzyme, nitrate reductase, growth at 50°C and hydrolyze starch and casein. *Bacillus licheniformis* and *Bacillus subtilis* showed oxidase negative. Except *Bacillus subtilis* (O+F-) all the isolates showed positive to the Oxidative and fermentative test.

Out of 40 isolates 37.5% were *Bacillus licheniformis*, 25% were *Bacillus subtilis*, 25% were *Bacillus cereus* and 12.5% were *Bacillus tropicus*.

Antibiotic susceptibility properties of isolated Bacillus species from raw milk

The identified isolates of *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus, Bacillus tropicus* were subjected to antibiotic susceptibility test using E-strip method. The results showed that *Bacillus licheniformis* and *Bacillus subtilis* showed resistant to ß-lactam (ampicillin, Ceftriaxone, Cefatoxime) and tetracycline antibiotics, *Bacillus cereus* showed resistant to ß-lactam (Ceftriaxone) whereas *Bacillus tropicus* showed resistant to nitrobenzene (chloramphenicol). The MIC of each Bacillus isolate was shown in Table 2 and intersection of elliptical inhibitory zone was shown in figure 1.

Figure 1: Representing antibiotic susceptibility characteristics of Bacillus species against various group of antibiotics. The intersection of the elliptical zone at the sides of antibiotic strip indicates MIC of a Bacillus species.



Discussion

Spore-formers are ubiquitous and can be isolated from a wide variety of environments, including soil, sediments, dust, and natural waters. In the present study from the 20 raw milk samples aerobic spore formers were isolated and characterized. The total aerobic bacterial count from different milk samples of ICM, BMST and PM showed the range between 1 to 2.146 log cfu/ml. The total of 40 isolates were characterized as *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus* and *Bacillus tropicus.* In contrast to the present study, Khater and Abdella (2017), collected 10 raw milk samples from Cairo market and examined for aerobic bacterial spore count. Aerobic spore forming bacterial count was ranged between 3.505 to 4.431 log cfu/ml. Out 20 isolated aerobic spore formers from raw milk samples 18(90%) were *Bacillus anthracis* and 2(10%) were *Bacillus mycoides*.

Antibiotic susceptibility test of aerobic bacterial spore isolates of raw milk such as *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus* and *Bacillus tropicus* was performed using E strip methods using MHA agar. MIC of inhibition zone is notes and determined the isolates as susceptible and resistant to antibiotics of different groups by comparing with standars of CLSI. The results showed that *Bacillus licheniformis* and *Bacillus subtilis* were resistant to ß-lactam (ampicillin (AMP), Ceftriaxone (CTR), Cefatoxime (CTX)) and tetracycline (TET) antibiotics, *Bacillus cereus* showed resistant to ß-lactam (Ceftriaxone (CTR)) whereas *Bacillus tropicus* showed resistant to nitrobenzene (chloramphenicol (CHL)). Similarly, to the above study Jaber et al. 2021 performed antibiotic susceptibility test for aerobic bacterial spore isolates named as *Bacillus thuringiensis*, *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis* using disc assay method. The results showed that all the isolates were resistance to cadazolid (CDZ) and chloramphenicol (C) and medium sensitivity for tetracycline (TE), erythromycin (E) and vancomycin (VA), its show highest sensitivity to ciprofloxacin (CIP) and cephalexin (CN).

Conclusion

The aerobic bacterial spore isolates were characterized and subjected for antibiotic susceptibility test. Bacillus species namely *Bacillus licheniformis, Bacillus subtilis, Bacillus cereus, Bacillus tropicus* were identified. Antibiotic susceptibility property of the Bacillus species showed variation in their minimum inhibitory concentration (MIC) and showed resistance to different group of antibiotics like β-lactam, aminoglycosides, quinolones, fluoroquinolones, tetracyclines and nitrobenzene. 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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Table 1. Enumeration of total aerobic bacterial spore from raw milk obtained from from different resources

No. of samples	Source		Count log cfu/ml	Codes of isolates	Identified organism
1	Individual cow milk (ICM)	ICM, Kanchenahalli, Chikkaballapur	1.0^{d}	A1, A2, A3, A4, A5	*Bacillus licheniformis (15)*
2		ICM, Nallimaradhalli, Chikkaballapur	0^{e}	Nil	
3		ICM, Kondenahalli, Chikkaballapur	1.845^{ab}	A6, A7, A8, A9	
4		ICM, Medihalli, Kolar	1.903^{ab}	A10, A11, A12, A13	
5		ICM, Tarigenahalli, Kolar	2.0^{ab}	A14, A15, A16, A17	*Bacillus subtilis (10)*
6		ICM, Naganahalla, Kolar	1.602^{bc}	A18, A19, A20, A21	
7		ICM, Yelahanka	0^{e}	Nil	
8		ICM, KVAFSU dairy farm	0^{e}	Nil	
9	Bulk milk storage tank (BMST)	BMST1, BAMUL	2.146^{a}	A22, A23, A24, A25	
10		BMST2, BAMUL	1.94^{ab}	A26, A27, A28, A29	
11		BMST3, BAMUL	1.30^{cd}	A30, A31	
12		BMST4, BAMUL	1.0^{d}	A32	*Bacillus cereus (10)*
13		BMST5, BAMUL	2.0^{ab}	A33, A34, A35, A36	
14	Pooled milk (PM)	PM, SEDP	1.0^{d}	A37, A38, A39	
15		PM, KVAFSU Dairy farm	0^{e}	Nil	
16		PM, Yelahanka	1.903^{ab}	A40, A41, A42,	
17		PM, Devanahalli	2.0^{ab}	A43, A45, A46	
18		PM, MPCS, Doddaballpur	0^{e}	Nil	
19		PM, MPCS, Chikkaballapur	1.8^{abc}	A47, A48	
20		PM, MPCS, Kolar	2.0^{ab}	A49, A50	*Bacillus tropicus (5)*
CD (*P*=.05)			**0.28**	**50 isolates**	**40 isolates**

Note:

- The results were average of three trials.
- Same superscript show non-significance while different indicate statistically significant difference (*P*=.05)

Table 2: Antibiotic susceptibility properties of isolated Bacillus species from raw milk

Antibiotic class	Antibiotics Strips (µg/ml)	MIC (µg/ml) of Bacillus species			
		B. licheniformis	*B. subtilis*	*B.cereus*	*B.tropicus*
B-lactam	Ampicillin (AMP)	0.0^{e} R	0.0^{e} R	0.19^{de} S	3.0^{b} S
	Ceftriaxone (CTR)	0.0^{e} R	0.0^{e} R	0.0^{e} R	2.0^{c} S
	Cefatoxime (CTX)	0.0^{e} R	1.5^{d} S	1.0^{c} S	1.0^{d} S
Aminoglycosides	Streptomycin (STR)	2.0^{c} S	48.0^{b} S	4.0^{a} S	2.0^{c} S
	Gentamycin (GEN)	0.25^{de} S	0.94^{e} S	0.38^{de} S	0.25^{e} S
Quinolones	Ciprofloxacin (CPH)	0.50^{d} S	0.64^{e} S	0.047^{de} S	0.094^{e} S
Fluoroquinolones	Enrofloxacin (EFX)	0.19^{de} S	0.47^{ef} S	0.032^{de} S	0.032^{e} S
Tetracyclines	Tetracycline (TET)	64.0^{a} R	64.0^{a} R	0.50^{d} S	$0.50d^{e}$ S
Nitrobenzene	Chloramphenicol (CHL)	8.0^{b} S	3.0^{c} S	1.5^{b} S	24.0^{a} R
CD (*P*=.05)		**0**	**0**	**0.298**	**0.344**

Note:

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

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