Microbial based assay for specific detection of β -lactam group of antibiotics in milk

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Revised: 12 November 2011 / Accepted: 9 December 2011 / Published online: 24 December 2011
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Abstract The spore forming *Bacillus cereus* (66) was screened for the induction of β -lactamase in presence of an inducer using iodometric assay. A significant induction in marker enzyme was observed in *B. cereus* 66 at maximum residual limit (MRL) of penicillin, ampicillin, cloxacillin, amoxicillin, cefalexin, and cephazolin belonging to β -lactam group of antibiotics. A microbial based assay, where enzyme induction was optimized at pH 7.0, temperature 30°C, and whey powder (0.25%) after 4 h of incubation. The spore based assay was tested with milk samples spiked with 6 different β -lactam antibiotics. The results were 100 and 83.33% in correlation with microbial receptor and inhibition based assay, respectively. Overall, spore based assay can be a useful and cost effective tool for the specific detection of β -lactam group of antibiotics in milk.

Keywords β -lactam · Induction · β -lactamase · *Bacillus* · Spore · Assay

Introduction

Among the commonly used antibiotics (i.e. β -lactam, tetracycline, aminoglycoside, sulfonamide and macrolides), β -lactam is mainly used for the treatment of dairy cattle. The indiscriminate use of unapproved drugs, lack of medication records and failure to observe withdrawal period in lactating animals; causes substantial excretion of these residues into milk. These residues in milk could be allergic, carcinogenic and develop microbial drug resistance, including serious health consequences in consumers (Katz and Brady 2000; Kabir et al. 2004). In addition, the presence of antibiotic residues in milk supply can have adverse effects during processing like starter failure (Mayra-Makinen 1995), lowered efficiency of dye reduction and hence the product fail to meet the international standards.

Different types of assays based on inhibition of microbial growth (Pikkemaat et al. 2008), receptor binding, colorimetry, spectrophotometry, chromatography, differential scanning calorimetry (Yildiz and Unluturk 2009) and immunoassays (Navrátilová 2008) are used for the detection of antibiotic residues in milk (Pikkemaat 2009; Kirbiš 2007). These methods either are qualitative, quantitative or semi-quantitative and have one or more limitations in terms of precision, accuracy, sensitivity, cost and infrastructure requirement (Zvirdauskiene and Salomskiene 2007). Therefore, the present investigation was undertaken to develop a microbial assay for the specific detection of β -lactam antibiotics in milk based on induction of β -lactamase (Davis et al. 1974; Imsande et al. 1972; Pollock 1950; Pollock 1961; Fonze et al. 2002) in spore forming bacilli (Chen et al. 2003; Kotiranta et al. 2000; Drobniewski 1993). The optimized spore based assay was evaluated by analyzing spiked milk samples with β -lactam group of antibiotics and results were compared with AOAC

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approved microbial receptor assay (Charm Science Inc., USA) and microbial inhibition based test kit that was developed in our laboratory (Patent Reg No. 1479/DEL/2006).

Materials and methods

Screening for β -lactamase

B. cereus 66 procured from NCDC, NDRI, Karnal was activated overnight in nutrient broth at 37°C and were streaked on nutrient agar followed by incubation at 37°C for 12 h. Colony from respective plates was picked up for β -lactamase enzyme by iodometric method (Livermore and Brown 2001).

Induction of β -lactamase

The inoculum (250 μ L) of *B. cereus* was spread on agar plate containing 0.25% whey powder (WP) and penicillin solution (10 μ g. mL⁻¹), while control was free from penicillin. After incubation at 37°C for 5 h, the grown culture was scrapped from the agar surface for further experiment.

Quantification of β -lactamase

The scrapped culture was added into the ampoules containing 0.5 mL of phosphate buffer to get a visible turbidity. Five sets containing test experiments and control were run simultaneously. The ampoules were kept at 30°C for 30 min followed by addition of 20 μ L starch solution and 35 μ L iodine reagent (Catlin 1975) in each ampoule and observed for color change upto 30 min. A positive test was indicated by change in initial black color to colorless.

Control Control 1: Induced culture + phosphate buffer (0.5 mL); Control 2: uninduced culture + phosphate buffer (0.5 mL); Control 3: Penicillin G (3 mg) and phosphate buffer (0.5 mL)

Treatments Treatment 1: Induced culture + phosphate buffer (0.5 mL) + 3 mg penicillin G; Treatment 2: un-induced culture + phosphate buffer (0.5 mL) + 3 mg penicillin G

Optimization of microbial assay based on induction of β -lactamase

The assay was developed following iodometric test procedure (Ahmad and Yadava 1979) and was further modified in two stages, so that it may find an industrial application.

Vegetative cells/ spore suspension

Nutrient broth was inoculated with *B. cereus* 66 and incubated at 37°C for 36 h. Broth culture was centrifuged (eppendorf North America, Inc. USA, 2801R) at 8,994 g for 5 min at 15°C and washed twice to eliminate all the cell bound enzymes. The cell pellet obtained was suspended in equal volume of sporulation medium (100 mL) and incubated at 37°C for 15 h. Then it was centrifuged and spore suspension was washed twice using normal saline. Final cell/ or spore suspension was prepared in normal saline, OD was adjusted to 1.0 at 660 nm using microbiological plate reader (Perkin Elmer, model 2030 VictorX3) and total plate/ spore count were enumerated (Downes and Ito 2001).

Enzyme induction and its detection based on iodometric assay

A agar containing 0.25% WP (0.5 mL) and color indicator i.e. bromo cresol purple (0.006%) was used as a test medium. It was dispensed in sterilized eppendorf tube and inoculated with 0.2 mL of fresh bacterial/ or spore suspension before induction. Antibiotic free milk (10 μ L) collected from NDRI cattle yard/ or spiked milk at $\leq$ MRL was pre-heat treated at 100°C for 3 min in dry incubator and inoculated in three sets of ampoules as control, $<$ MRL and MRL in accordance with council regulation 2377/90/EC (EC 1990) and afterwards council regulation 37/2010/EU (EC 2010). The ampoules were incubated at 37°C for 4 h for induction of enzyme followed by addition of 0.1 mL of penicillin G solution (30 mg mL⁻¹ in phosphate buffer) in each ampoule. The ampoules were further incubated at 30°C for 1 h for enzymatic action followed by addition of 20 μ L of starch and varying concentration of iodine reagent. The time for color change

Table 1 Microbial based bioassay for specific detection of β -lactam group of antibiotics in milk based on induction of β -lactamase in *B. cereus* 66^a

Antibiotics (β -Lactam)	Antibiotics (μ g/ mL) in spiked milk		Iodine consumption in control/ spiked milk sample (μ L) for 30 min		
	<MRL	MRL	Control	<MRL	MRL
Penicillin	0.00004	0.004	55 $\pm$ 0.67	55 $\pm$ 0.55	85 $\pm$ 1.64
Ampicillin	0.00004	0.004	70 $\pm$ 0.72	75 $\pm$ 0.75	150 $\pm$ 4.28
Amoxicillin	0.00004	0.004	50 $\pm$ 0.23	70 $\pm$ 0.67	80 $\pm$ 0.81
Cloxacillin	0.00030	0.030	65 $\pm$ 1.21	60 $\pm$ 0.38	100 $\pm$ 2.54
Cefalexin	0.00100	0.100	65 $\pm$ 1.93	60 $\pm$ 0.32	95 $\pm$ 2.03
Cephazolin	0.00050	0.050	50 $\pm$ 0.50	50 $\pm$ 0.76	65 $\pm$ 1.02

^a Optical density (1.0 $\pm$ 0.05) with Log vegetative/ spore count 7.85 / 3.69; *Each observation is a mean of three replicate experiments ($n=3$); $\pm$ Indicates Standard Deviation

Table 2 Comparative evaluation of bioassay for induction of β -lactamase during vegetative growth/ or spore germination*

Culture	Sporulation (%)	Concentration of Penicillin ($\mu\text{g}/\text{mL}$) for induction added through milk		Iodine consumption in control/ spiked milk sample (μL) at 30 min			Net iodine consumption (μL)
		<MRL	MRL	Control	<MRL	MRL	
Spore suspension ^a	69	0.00004	0.004	85 $\pm$ 1.12	85 $\pm$ 0.72	150 $\pm$ 6.42	65 $\pm$ 6.56
Vegetative cell suspension ^b	47	0.00004	0.004	55 $\pm$ 0.95	55 $\pm$ 0.62	85 $\pm$ 1.53	30 $\pm$ 1.36

*Each observation is a mean of three replicate experiments ($n=3$); $\pm$ Indicates Standard Deviation ^a Spore suspension with optical density (1.0 ± 0.03) with Log vegetative/ spore count 7.60/ 5.23 ; ^b Vegetative cell pellet with optical density (1.0 ± 0.03) with Log vegetative/ spore count 7.85/ 3.69

was noted down. The optimal conditions for enzyme induction were also determined under different conditions of temperature, pH, incubation time and growth factors like WP and casein acid hydrolysate etc.

Screening of *Bacillus* spp. for minimum inhibitory concentration (MIC)

β -Lactamase producing *B.cereus* was screened for MIC against sixteen antibiotics belonging to β -lactam, tetracycline, aminoglycosides, macrolides, sulphonamides, and miscellaneous groups based on zone of inhibition (IDF 1991; Aureli et al. 1996). Ten mL agar medium containing 0.25% WP was poured in petridish pre-inoculated with vegetative cell pellet at the rate of 8 mL 100 mL⁻¹. Sterile paper discs soaked in antibiotic were placed on the medium followed by incubation at 37°C for 4 h. The MICs were determined based on the diameter of zone of inhibition.

Comparison of microbial assay, AOAC approved microbial receptor assay and microbial inhibition test kit

The microbial assay using *B. cereus* spore was evaluated for detecting β -lactam antibiotics group in milk and the results were compared with microbial receptor based Charm 6602 analyzer (Charm science inc. USA) and microbial inhibition test kit (Patent Reg No. 1479/DEL/2006).

Statistical analysis

Data was analysed statistically and expresses as the mean ($\pm$ SD) of three replicates according to Snedecor and Cochran (1980)

Result & discussion

B. cereus 66 was screened for β -lactamase production using iodometric method (Livermore and Brown 2001) with slight modifications. The test ampoules containing culture and penicillin G, showed color change within 15–25 min as a result of induction of β -lactamase indicating the presence of

inducer i.e. penicillin-G. Whereas, uninduced culture did not show any color change in the test ampoules, which means the basal enzyme produced was not sufficient to reduce the starch iodine mixture under experimental condition. The induction of β -lactamase in *B. cereus* 66 could be established based on modified iodometric test for application in development of microbial assay for detection of β -lactam group of antibiotics in milk.

Microbial assay comprised of three steps (i.e. induction of β -lactamase during growth of vegetative cell or spore germination followed by penicillin treatment and detection of induced enzyme) optimized using strains of *B. cereus* 66. Experiment carried out with six β -lactam antibiotics in spiked milk used for induction at MRL doses and it was observed that these strains consumed small quantity of iodine varying from 50 to 70 μL and 65–150 μL in control and treated samples, respectively with a net increase of iodine consumption in the range of 15–75 μL . It indicates that β -lactamase enzyme induction take place in treated samples (Table 1). The maximum enzyme induction was observed in *B. cereus* 66 in milk spiked with ampicillin (150 μL) followed by cloxacillin (100 μL) while, cephalosin showed

Table 3 Optimization of microbial based bioassay for enhanced β -lactamase enzyme using spores of *B. cereus* 66^a

Parameter	Iodine consumption ^b (μL) within 30 min after enzyme induction by penicillin at MRL		
Temperature (°C)	40 (65 $\pm$ 1.21)	37 (70 $\pm$ 23)	30 (70 $\pm$ 1.14)
pH	6.5 (65 $\pm$ 0.56)	7.0 (70 $\pm$ 57)	7.5 (25 $\pm$ 1.51)
Whey Powder (gm/L)	0.5 (25 $\pm$ 1.51)	2.5 (65 $\pm$ 0.32)	5.0 (25 $\pm$ 0.21)
Casein Hydrolysate (gm/L)	2 (ND)	4 (65 $\pm$ 1.16)	6 (25 $\pm$ 1.16)

^a Each observation is a mean of three replicate experiments ($n=3$);

^b Figures in parenthesis indicates the iodine consumption (μL); $\pm$ Indicates Standard Deviation

ND Not Detected

Fig. 1 Disc assay result on comparative inhibitory effect of antibiotic on *B.cereus* 66; A = Minimum Inhibitory concentration and B = European Union/ Codex Alimentarius Commission Maximum Residual Limit (EU/Codex MRL) ($n=3$)

minimal induction (65 μ L). The enzyme induction was also studied at lower limits of MRL against antibiotic residues; however, the response was more or less similar to their respective control. The kind of induction may be attributed to differential basal production of enzyme and its association with cell surface/ or extra cellular nature. Pollock (1965) reported that the ratio of induced and basal enzyme production was 100 times in *B. licheniformis* in contrast to 800 times reported in *B. cereus* 66 (Shah and Day 1963). It was interesting to see that enzyme induction was enhanced significantly during spore

germination. When cell pellet of *B. cereus* 66 with vegetative log count of 7.85 (spore log count 3.69) was used, the iodine consumption in control as well as in spiked milk at MRL levels were 55 and 85 μ L with net iodine consumption of 30 μ L (Table 2). The respective values with spore suspension (spore log count 5.23) were 85 and 150 μ L, respectively and the net iodine consumption enhanced significantly to 65 μ L, as a result of higher enzyme induction during spore germination in *B. cereus* 66. Fenselau et al. (2007) demonstrated higher *denovo* synthesis of β -lactamase type I in the outgrowth phase of

Table 4 Evaluation and validation of microbial bioassay for detection of β -lactam group of antibiotics in milk^d

β -lactam group of antibiotic spiked milk	MRL (ppb)	Antibiotics detection by microbial bioassay and reference method ^d					
		Microbial based bioassay ^c		Charm 6602 System		MDR test kit	
		Control	MRL	Control ^a	MRL ^b	control	MRL
Penicillin	4	–ve	+ve (5 min)	890 (–ve)	195 (+ve)	–ve	+ve
Ampicillin	4	–ve	+ve (9 min)	795 (–ve)	424 (+ve)	–ve	+ve
Amoxicillin	4	–ve	+ve (6 min)	1283 (–ve)	417 (+ve)	–ve	+ve
Cloxacillin	30	–ve	+ve (4 min)	827 (–ve)	349 (+ve)	–ve	+ve
Cefalexin	100	–ve	+ve (4 min)	866 (–ve)	352 (+ve)	–ve	–ve ^c
Cephazolin	50	–ve	+ve (7 min)	792 (–ve)	219 (+ve)	–ve	+ve

^a Zero control standard 959 (any figure between 654 and 959/ or above is considered as negative); ^b Control point at MRL dose 653 (any figure ≤ 653 is considered as positive); ^c Detection limit 150–200 ppb; ^d Each observation is a mean of three replicate experiments ($n=3$); ^e Figures in Parenthesis indicates time taken for enzyme induction in presence of antibiotics; MDR Microbial Drug Residues

spores of penicillin resistant *Bacillus* spp., when compared with enzyme produced during vegetative growth. Wong et al. (2011) reported that a crystal structure of a class A β -lactamase PenP from *Bacillus licheniformis* 749/C is conjugated with fluorescein residues 166 for rational design of β -Lactamase based biosensor to detect wide spectrum of β -Lactam antibiotic group.

Our results revealed that optimal β -lactamase production takes place under sub-optimal growth temperature (30°C) minimum, where iodine consumption was 70 μ L while at 37°C was lowest i.e. 55 μ L. The corresponding value with vegetative cell pellet was 30 μ L at 37°C. The pH 7.0 appeared to be optimal for enzyme production (70 μ L) at MRL dose of penicillin (Table 3). It was also observed that 0.25% WP was optimal for enzyme induction with a negative correlation was observed with casein hydrolysate supplementation. Induction of enzyme could not be established at 2 h of incubation, however, at 4 h it was significantly higher and may be attributed to *B. cereus* having generation time of 35 min (Imsande 1970) resulting in continued induction effect upto seven doublings in cell mass (Pollock 1956). The drastic reduction after 6 h of incubation indicates instability of the enzyme and is well supported by Kogut et al. 1956.

B. cereus 66 showed an inhibition zone of 7–9 mm at ≥ 100 mg kg⁻¹ for β -lactam group of antibiotics, which is a much higher dose when compared to respective MRL varying from 4 to 100 μ g kg⁻¹. The growth of *B. cereus* 66 was inhibited by ampicillin at 1,000 mg kg⁻¹, which is much higher as compared to MRL i.e. 4 μ g kg⁻¹. Antibiotics from non- β -lactam group that includes tetracycline, oxytetracycline, gentamycin, streptomycin, sulphamethazine, erythromycin, bacitracin, lincomycin, chloramphenicol, and trimethoprim were also investigated and showed inhibition at very high concentrations ranging from 2.5 to 1,000 mg kg⁻¹ (Fig. 1). An inhibition zone of 9 mm was observed against trimethoprim at 10 mg kg⁻¹. However, rest of antibiotics showed inhibition zone at ≥ 100 mg kg⁻¹. Based on sensitivity pattern of *B. cereus* 66, it appears that the microbial based bioassay could be safe in terms of interference of antibiotics other than β -lactams.

The time required for color change of starch iodide complex was recorded. Any color change observed within 10 min was considered as positive for β -lactam group. Milk sample spiked with 6 β -lactam antibiotics i.e. at MRL doses varying from 4 to 100 μ g kg⁻¹ were used for checking the efficacy of microbial assay. These antibiotics could be detected within a time limit of 4–9 min on the basis of iodine color change. No color change was observed even after 45 min of incubation in control. The results were compared with microbial receptor charm 6602 Assay, where all the six spiked milk samples were positive with test reading as count per minute in the range of 195–424 against, control of 653 showing 100% correlation. The test results were also compared with microbial inhibition

based assay kit (Patent filed Reg No. 1479/DEL/2006) and 5 out of 6 antibiotics spiked milk could be detected with the exception of cefalexin that could not be detected on account of less sensitivity of assay at MRL (Table 4).

Conclusion

An innovative approach for the detection of β -lactam group of antibiotics in milk was attempted based on the induction of β -lactamase produced by spore forming bacteria. Induction in β -lactamase was established in *B. cereus* 66 during vegetative growth/ or spore germination. The spore based assay was optimized and critical growth factors leading to enhanced induction of β -lactamase in indicator strain were identified. Optimized spore based assay was evaluated for its performance by analyzing spiked milk with β -lactam group of antibiotics and results showed compliance with AOAC approved microbial receptor assay (Charm Assay) and microbial inhibition based test kit. The indicator strain of *B. cereus* 66 was screened for MIC against sixteen antibiotics belonging to β -lactam and non β -lactam groups to ensure their non-interference in working performance of microbial based assay.

Based on outcome of these research findings, it may be concluded that the developed microbial assay has a commercial potential in monitoring β -lactam group of antibiotic in milk, however, further work is needed to transform the lab scale study for industrial application which is in progress.

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