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Prevalence of Gentamicin Residues in Milk Samples Collected from Dhaka Division of Bangladesh

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ABSTRACT

The aim of the study was to screen milk samples of Dhaka division for Gentamicin residues with "CHARM-II Radio Receptor Assay" to get a current scenario on the usage of the drug in Bangladesh. This screening study was performed following a validated method to analyse Gentamicin in milk sample. A cut-off value was set according to Commission Decision 2002/657/EC for method validation purpose. The validation parameters included detection capabilities (CCβ), test for applicability, specificity, cross-reactivity, and robustness. CCβ for Gentamicinwas established at 30 µg/kg (less than 0.5 MRL). Cut-off value was set at the CCβ level. F-calculated values less than F-critical value in one way ANOVA analysis for applicability and robustness tests accepts the fact that the data-sets being compared are significantly indistinguishable. Results from the screening study reveals absence of Gentamicin in the collected milk samples., which indicates that either application of Gentamicin is properly monitored in the dairy farms, or, the drug residue level is present below the CCβ level. Test results for all other validation parameters demands that the method is 100% group specific, and applicable to detecting residues of the aforesaid four antimicrobials in egg from different poultry species in Bangladesh.

Key words: **Gentamicin, Milk, Validation, CCβ, Cut-off, MRL, Charm-II**

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INTRODUCTION

Antimicrobial drugs are commonly utilized in animal for both prevention and treatment, as well as growth boosters at subtherapeutic doses [1]. "Pharmacologically active chemicals (whether active principles, recipients, or degradation products) and their metabolites that persist in consumables acquired from animals to whom the veterinary medical products in question have been administered" is how the European Union (EU) defines residues [2]. Veterinary drug residues are metabolites or degradation products of pharmacological compound found in the foods of animals that have undergone drug treatments or growth stimulants. MRL is the maximum level or concentration of a medicine or chemical that is considered non-hazardous by regulatory organizations and is allowed in or on food or feed intended for animal or human consumption at a specific time [3]. Different antimicrobial agents have their own MRL.

Overuse of antibiotics in animal have some serious consequences. Consumers' health is jeopardized when milk and other dairy products contain drug residues above the MRL [4]. Although high-quality milk and other dairy products are essential for overall health, those products with antibiotic residue have negative health consequences, such as allergic reactions, toxicity etc [5]. Finally, the most alarming consequence is the antibiotic resistance.

Different antimicrobial agents have been used in milk producing animal such as beta-lactam, tetracycline, sulfonamide, gentamicin, neomycin etc. Gentamicinis aantibiotic of aminoglycosidegroup [6]. This antibiotic mainly has antimicrobial activity against gram negative bacteria. It disrupts the protein synthesis of bacteria. The EU and Codex MRL for gentamicin are 100 µg/Kg and 200 µg/Kg respectively. Withdrawal time of gentamicin in milk is reported to be at 132 hours in case of intramammary treatment of 500mg gentamicin for consecutive 5 days while withdrawal time of 36 hours was also reported in case of intravenous treatment of gentamicin at 5 mg/kg BW for consecutive 5 days [7].Gentamicinhas some side effects such as inner ear problems, kidney problems etc.

Several analytical methods have been used in gentamicin determination from milk such as LC-MS/MS, HPLC, ELISA, RIA etc [8 -11]. Among these LC-MS/MS, HPLC are time consuming and costly method whereas radioimmuno receptor assay (RIA) is easy and cheap [12].

In our study, we validated CHARM II RIA method according to EC/2002/657 for screening of gentamicin residue in milk samples. Fianally, we analyzed gentamicin in different milk samples from Bangladesh.

MATERIALS AND METHOD

Chemicals, Standard solutions, and Equipment

Multi-Antimicrobial standard (containing 30 ppb gentamicin when reconstituted with 100ml negative control) was supplied with the Charm II kit. Reconstituted positive control standard was held refrigerated or on ice for up to 48 hours. Zero control standard supplied with kit was reconstituted 100 ml of 40^{o}C distilled water. Chemicals were of analytical grade.

Kit and Instrument

CHARM II Kits (GTBL Tablet reagents) for analyzing gentamicin were purchased from Charm Science Inc., which were in tablet form. Other consumables equipment and reagents like MSU Multi-Antimicrobial Concentrate Standard, Zero control standard, Scintillation fluid (Opti-Fluor O) were also purchased from Charm Science Inc.

The instruments were Charm II Intronic incubator, scintillation counter of Charm Sciences Inc were purchased from Charm Science Inc.

Matrix

Raw cow milk samples were collected from different regions of Dhaka division on around 2020-21. Besides, pasteurized milks were also collected from local markets of these regions. A total of 79 milk samples were collected, among which 69 were raw milk samples and other 10 were pasteurized milk. After collection these samples were stored at -20^{0}C for not more than two months.

Gentamicin CHARM II test procedure

A test tube filled with ¾ milk sample is centrifuged 3 minutes at 3400 rpm and is cooled to 4^{o}C. A white tablet, binder for antibiotic, is added to a new test tube. Then 300±100 µl water is added and mixed for 10 seconds. After that 5±0.25 ml previously centrifuged sample or control from below fat layer is added.

Then the yellow tablet, radio-labelled tracer, is added and is immediately mixed by swirling up and down for 15 seconds. The tube is then incubated at 35±2°C for 3 minutes. After that the tube is centrifuged for 3 minutes at 3400 rpm. Then themilk is poured off and the fat is removed and wiped dry with cotton swabs. After that 300±100 µl water is added and the pellet is broken up by mixing with water. Finally, 3±0.5 ml scintillation fluid is added, and the tube is shaked to form uniform cloudy appearance. The tube is then entered into scintillation counter and the CPM (Count per minute) is counted for one minute. If the sample CPM is greater than the control point, the sample is negative. That means there is lower or absence of antibiotic than tracer tablet. If the sample CPM is less than the control point, the sample is positive. That means there is high amount of antibiotic in sample than tracer tablet.

Validation protocols

For Abridged validation protocol the main parameters are CCβ (Detection Capability), and, Cut-off factor. Other validation parameters such as applicability, robustness, specificity and cross reactivity were also investigated.

Detection
CapabilityThe detection capability of an analyte is defined as the lowest concentration at which the analyte can be detected by a test method with equal or less than 5% positive error (error). The number of samples required for the approval of a screening method depends on some degree of statistical confidence for the approval of a screening strategy, which requires the result and relationship between the target concentration and the maximum residue limit (MRL) of the targeted analyte, according to European guidelines CRL 20/1/2010 [13, 14].
Consider the following scenario:
(i) If the Screening Target Concentration is set at 1/2 of the MRL or less, 20 "Screen Positive" Control Samples with at least one or no falsepositive result are sufficient to show that the chosen CCβ is less than or equal to the half MRL;
(ii) If the Screening Target Concentration is set between 50% and 90% of the MRL, at least 40 "Screen Positive" Control Samples (with no more than 2 falsepositive findings) are required to demonstrate that the selected CCβ is less than the MRL.
(iii) More "Screen Positive" Control Samples may be required if the screening test's sensitivity is such that the Screening Target Concentration approaches the MRL (just 10% below the regulatory/action limit).In this situation, a maximum of 60 replicates are required (with no more than three false-positive findings) to demonstrate that CCβ is suitable for the task.
In this study, the screening target concentration was 30 µg/kg which is less than half MRL of gentamicin. So 20 blank samples and 20 spiked samples at screening target concentration were analyzed for detection of CCβ.

Determination of cut-off level
To validate the screen method, the cut-off level (the response at or above which the samples are marked as screen positive) is necessary. The cut-off level in our study was calculated by taking a 5% error into account. The threshold value T and cut-off factor (Fm) were computed using the responses of blank and spiked samples in this method.To determine the cut-off level, the matrix blank samples should be spiked at half the Regulatory/Action Limit.The positivity limit T is matrix-specific, as is the cut-off factor Fm.

$$T = B - 1.64 \times SDb \quad \text{…….(1)}$$

$$Fm = M + 1.64 \times SDs \quad \text{…..….(2)}$$

Where,
B = Mean optical density of blank samples;

SDb = Standard deviation of the optical densities (ODs) $_{blank}$;
M = Mean optical density of spiked samples;
SDs = Standard deviation of the ODs $_{(spiked\ tests)}$;

The spiking concentration (screening target concentration) where Fm≤B is obtained as the method's limit of detection after calculating "Threshold value" T and "Cut-off factor" Fm. When the cut-off factor Fm is less than or equal to the mean of blank sample responses, the limit of detection is valid. It is also essential to define the rate of false positives. T<Fm<B indicates a false-positive rate of more than 5%. If Fm<T is used, the false positive rate is less than 5%.

Specificity (false-positive rate), and Cross-Reactivity
For the validation test, various types of blank control milk samples from various cows were used. Following comparison with the zero control standard provided with the CHARM II kits, all of the milk samples were found to be antibiotic-free. On five consecutive days, a minimum of twenty blank milk samples and twenty spiked milk samples were examined (minimum 4 blank and 4 spiked samples per day). The cross reactivity of gentamicin test kits was also tested by dosing negative blank samples with antibiotics from four separate groups at high concentrations and then testing them with gentamicin charm II test methods.

Robustness
The kit's robustness was tested by altering the analysis time. The samples were spiked with gentamicin (30 µg/kg). Following the addition of the
scintillationfluid, a sample reading was collected. Following that, the same sample was examined again aft er 24 hours and the results were compared.

Applicability
The applicability of test kits from different milks such as raw milk, pasteurized mik and condensed milk were tested. Whether the test kit gives same result or different result.

RESULTS AND DISCUSSION

Evaluation of Charm-II kits for Gentamicin.
The distribution of CPMs of blank and spiked samples with the threshold value (T), and, Cut-off factor (Fm) for Gentamicin test kit of Charm-II is illustrated in Fig.1. A comparison of different performance characteristics (T and Fm), applicability, specificity and cross-reactivity of the test method were helpful to make the following choice. T and Fm were calculated from the CPMs of the samples, and, the detection capability (CCβ) were set at the concentrations of 30 µg/kg following a validation procedure.

As guided by the Commission Decision 2002/657/EC and the European guidelines for the validation of screening methods (CRL 2010), the CCβ will be validated only if the cut-off factor Fm < B. The assay is valid only if Fm < T, which suggests the positive error should be equal or less than 5%. If T < Fm, the false-negative rate is greater than 5%.

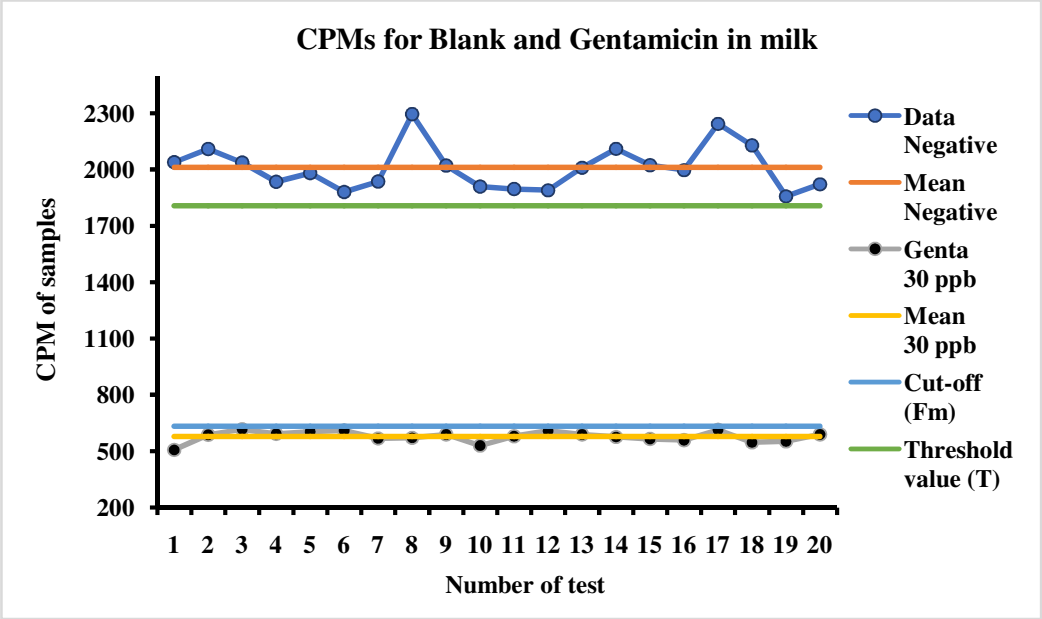

Figure 1: Graphical Presentation of the distribution of CPMs of blank and spiked milk samples. Milk samples were spiked with Gentamicin (GEN) at the concentration of 30 µg/kg. The result is presented for the Charm-II kit of Gentamicin with the threshold value (T) and the cut-off value (Fm).

The summary of the test result is presented in Tab.1, where the observed Fm, T, and, B values for Gentamicin test assay are 624.51, 1816.55, and, 2011.75 respectively which indicates that our test method is valid, and, the selected concentration as CCβ is fit for the test methods.

TABLE 1: DETERMINATION OF T, FM, AND, EVALUATION OF CCB FOR GENTAMICIN (GEN) IN MILK.

Tested Antibiotic	**Gentamicin**
Number of Blank samples analysed, (Nb)	20
Mean of Blank, (B)	2011.75
Standard Deviation of Blank Samples, (SDb)	119.03
Coefficient of variation of Blank Samples, CVb (%)	5.917
Threshold value, T = (B - 1.64XSDb)	1816.55
Number of Spiked samples analysed, (Ns)	20 at 30 µg/Kg
Mean of Spiked Samples, (M)	577.90
Standard Deviation of Spiked Samples, (SD)	28.42
Coefficient of variation of Spiked Samples, CV (%)	4.918
Cut off, Fm = (B + 1.64XSD)	624.51
Number of False Positive Samples	0/20
Number of False Negative Samples	0/20
Validation of theTest Assay (Fm < T)	"Fm < T" is Justified
Validation of the CCβ (Fm < B)	"Fm < B" is Justified
EU-MRL =>	100 µg/Kg
Justified CCβ =>	30 µg/Kg

Validation

Applicability of CHARM-II test for screening Gentamicin in different milk matrices. The applicability test was performed by spiking milk samples from different sources and conditions (eg; Raw milk, Pasteurised milk, Powdered milk, and, Condensed milk) with the targeted analyte (Gentamicin = GEN) at same concentration (30 µg/kg), and this test method was able to detect the GEN from all the milk samples types. Result from one way ANOVA analysis demonstrates that for all the type of milk matrix, "F-calculated" values were lower than the "F-critical" value, which suggests that these tests could be applied to detect the targeted analyte from any type of milk matrix (Tab-2).

TABLE 2: APPLICABILITY OF CHARM-II GENTAMICIN KITS

Matrix (n=6)	Raw milk	Pasteurised milk	Powder milk	Condensed milk
Mean CPM of Blank Samples	2008.3	1915.3	2097.8	2009.8
Standard Deviation of Blanks, SDb	281	381	264	260
Coefficient of variation of Blanks, CVb (%)	10%	13%	9%	9%
F calc		1.499803	2.826078	0.000564
F crit		4.964603		
Mean CPM of Spiked (30 µg/Kg) Samples	1551	1660	1765	1696
Standard Deviation of Spiked Samples, (SD)	205	43	103	100
Coefficient of variation of Spiked Samples, CV (%)	13%	3%	6%	6%
F calc		0.009731	0.036926	0.001924
F crit		4.964603		
Gentamicin EU-MRL		100 µg/Kg		

Detection capabilities (CCβ) and specificity. The detection capabilities CCβ and specificity/false positivity of the tests was checked by taking scintillation counts of minimum 20 blank samples, and 20 spiked samples spiked with Gentamicin (GEN) at 30µg/Kg. Each tests were run for five consecutive days, and, the concentration at which the CPMs overlapping with the blank were rejected. Finally, the concentrations at which the CPMs of spiked samples didn't overlap with the blank samples CPMs were chosen as presented on Tab-3. The test result shows that the test methods had 100% specificity with less than 5% false positive error, and the finally chosen concentrations (30µg/Kg) is set as CCβ for Gentamicin test.

TABLE 3: Results Of Charm-Ii Test Kits For Gentamicin For Blank (Specificity) And Doped (Detection Capabilities) Milk

Test on	No. of Test	GEN (Blank)	GEN (30µg/Kg)
Day 1	1	2039	508
	2	2110	587
	3	2038	616
	4	1935	592
Day 2	5	1982	604
	6	1880	610
	7	1937	569
	8	2295	572
Day 3	9	2022	589
	10	1909	530
	11	1897	579
	12	1891	609
Day 4	13	2011	589

	14	2112	577
	15	2023	565
	16	1998	559
Day 5	17	2245	613
	18	2129	548
	19	1859	553
	20	1923	589
Mean =		2011.75	577.90
SD =		119.026	28.421
CV% =		**5.917%**	**4.918%**

Notes: GEN = Gentamicin

Cross-Reactivity. For cross-reactivity test it was found that samples spiked with other than Gentamicin were detected as negative for the test method of Gentamicin test kit. (Tab-4). This Gentamicin test kit did not show any interferences from drug families of tetracyclines, sulfa drugs, and, beta lactams at high concentrations (100 X MRL) though the test kit is not sensitive enough to detect Gentamicin according to the operator manual of CHARM-II test kits.

Robustness. The robustness test was performed to study the effect of variation in reading time interval for the processed samples. This test was performed on both blank and spiked samples (spiked with 30 µg/kg GEN), and were analyzed immediately after mixing (0 h) and after 24 hrs. From Tab-5, it is evident that there was no significant difference in the CPMs for both readings after 0 or 24 hrs (ANOVA, F-calculated < F-critical) confirming the robustness of the test methods, in regard to variation in reading time intervals of the processed samples.

TABLE 4: CROSS-REACTIVITY OF THE CHARM-II TEST KITS FOR GENTAMICIN IN MILK (IN DUPLICATE TESTING).

Analyte used	**SMZ**	**Chlortetracyclin**	**Pen G**	**Erythromycin**
Analytes MRLs	10 µg/Kg	100 µg/Kg	10 µg/Kg	50 µg/Kg
Analytes Conc (100 X MRL)	1,000 µg/Kg	10,000 µg/Kg	1,000 µg/Kg	5,000 µg/Kg
CPM 1	1870	1791	1807	1820
CPM 2	1901	1778	1755	1823
Result	Negative	Negative	Negative	Negative

Notes: Pen G = Benzylpenicillin; SMZ = Sulfamethazine;

Table 5: Robustness Test For Gentamicin Charm-II Assay

Samples Type	**Negative Samples**		**Spiked Samples (30µg/Kg)**	
Reading on	Day 1	After 24 hrs	Day 1	After 24 hrs
CPM for test 1	1915	1911	610	612
CPM for test 2	2128	2120	658	665
CPM for test 3	2076	2069	598	592
CPM for test 4	2147	2153	621	611
CPM for test 5	2287	2293	572	567
Mean CPMs	2110.6	2109.2	611.8	609.4
SD	128.635	794.861	31.994	36.0597
CV(%)	6%	38%	5%	6%
F-calculated	0.000263		0.012524	
F-critical	5.31766		5.31766	

Screening for Gentamicin Residues in milk Samples.

A total of 79 different milk samples were collected from different region of Dhaka division. These collected samples includes raw milk (69), pasteurized milk (10). The summary of the screening results is presented in Tab-6, which indicate absence of Gentamicin residues in the collected milk samples.

TABLE 6: STATUS OF GENTAMICIN RESIDUES FOUND IN MILK IN DHAKA DIVISION.

Milk Type	*Total Samples*	*Negative Sample*	*Suspected Samples*	*Suspect%*
Raw milk	69	69	0	0%
Pasteurized milk	10	10	0	0%
Total count =	**79**	**79**	**0**	**0%**

Analysis of Screening Results. Following the data presented in Tab-6, it is easy to perceive that among the tested milk samples none were found contaminated with Gentamicin (GEN) residues, or, at least they contained GEN residues lower than the CCβ level. Our study data indicates that the presence of Gentamicin residues are not common in milk, and, It is because either this drug is not used frequently for any treatment purposes in cattle's in those specific areas, or, this drug (GEN) is used under full monitoring.

Conclusions:

The statistical analysis for the method validation protocol showed that the test methods designed to detect antibiotics in milk matrices with CHARM-II system are applicable to detect Gentamicin from different types of milk matrices, and, this test method not being time-consuming like ELISA or HPLC, can be applied for routine monitoring of these antimicrobial drugs in our country, and also for international trade purpose. Only suspect samples need to be further analyzed with a time-consuming and expensive confirmatory method.

Our findings from the screening of raw and pasteurized milks showed undetectable levels of Gentamicin residues which represents no use of Gentamicin in the dairy farms. However, a strong regulation system is highly required, so that the misuses of any other antimicrobial drugs can be controlled.

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