13. STANDARD OPERATING PROCEDURE (SOP) FOR DETERMINATION OF ANTIBIOTIC RESIDUE IN FARMED SHRIMP BY ELISA IN FISH PROCESSING UNITS

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Introduction:

Inappropriate use of antibiotics in aquaculture leads to the twin problems of antibiotic residues in shrimp meat and the development of antimicrobial resistance in microorganisms associated with aquatic animals.The government of India, by the Gazette Notification, has prohibited the use of antibiotics such as chloramphenicol, nitrofurans (furaltadone, furazolidone, furylfuramide, nifuratel, nifuroxime, nifurprazine, nitrofurantoin, nitrofurazone), neomycin, nalidixic acid, sulphamethoxazole and glycopeptides in aquaculture farms, hatcheries, feed manufacturing units, pre-processing or processing units of shrimps, prawns or any other variety of fish (GOI, 2002).The presence of residues of antibiotics or their metabolites in food is potentially harmful to the consumers. Nitrofurans are genotoxic and carcinogenic. Chloramphenicol causes aplastic anaemia in humans. Stringent food safety regulations have been put in place by the national and international food safety agencies regarding the presence of antibiotics residues/ antibiotic metabolites in fish meat.Antibiotic residue testing has become mandatory for farmed fish destined to export markets.In India, the maximum residue level (MRL) in fish meat was notified for four antibiotics namely tetracycline, oxytetracycline, trimethoprim and oxolinic acid (EIC, 2002; FSSAI, 2011).European Union has established a minimum required performance limit (MRPL) of 1 µg/kg (ppb) for nitrofuran metabolites (furazolidone-AOZ, furaltadone-AMOZ, nitrofurantoin-AHD and nitrofurazone-SEM) and 0.3 µg/kg for chloramphenicol in aquaculture products intended for human consumption (EC directive 2003/181/EC).In the event of detection of antibiotics or their metabolites (above MRL/ MRPL) in fish products, the faulty products are considered as unfit for human consumption and are either rejected or destroyed.The presence of antibiotics in farmed shrimp exported from India was reported by the EU's Rapid Alert System for Food and Feed (RASFF), the US's Food and Drug Administration (USFDA), and Japan's Ministry of Health, Labour and Welfare. Several

analytical methods are employed for the quantitative detection of chloramphenicol in seafood such as HPLC,GC, immunoassays (RIA, CLIA, ELISA, etc.) Chromatographic techniques such as GC and HPLC coupled with MS offer greater sensitivity of detection. These techniques were more laborious and require highly specialized technicians and expensive instruments. Hence, enzyme-linked immunosorbent assay (ELISA) has been recommended as a screening test to test the aquaculture products before procurement (pre-harvest testing) and at raw material receiving stage (prior to processing) in fish processing units.If the fish samples are positive inELISAscreening, then the samples are to be confirmed by the HPLC-MS (High Performance Liquid Chromatography-Mass Spectrometry All the antibiotic residue screening ELISA methods are Competitive-ELISA owing to its more specificitycompared to other ELISA methods. ThisSOP mainly deals with the collection of samples from the farm and at the raw material receiving section of the fish processingunit.

i) How many samples to be collected:

The collection of shrimpsfor the ELISA testing purposeis based on the quantity of the raw material.

- In the case of pre-harvest testing, a minimum of 500g of sample (whole shrimp) has to be collected from each aquaculture pond.

- A sampling at the raw material section of the fish processing unit is performed at a scale of one Kilogram (Kg) for every one ton (1000kg) of raw material received.

How to collect the sample:

- Farm-level: Netting for the collection of shrimps should be done at 4 to 5 positions of the aquaculture pond.Netting has to be done at all four corners and the centre of the pond. About 100 to 200g of shrimp need to be collected at each netting site.

- Processing unit level: Calculate the total amount of shrimp sample to be collected based on the total quantity of raw material (1 kg for every 1000kg. E.g., If 4 tons of raw material is received then you have to collect 4 Kgs of shrimps). Shrimp samples must be collected from the insulated boxes/ crates located at the four corners and from the centre of the insulated vehicle.

How to analyse the sample:

Extract the antibiotics/metabolites from the shrimp meat sample following the kit manufacturer's instruction. Test the extracted sample for the presence of antibiotics in ELISA. Always use the series of positive standards provided with the kit. One positive control (spiked) and one negative control (previously tested by LC-MS-MS) have to be always tested to avoid false-positive and false-negative results. A spiked sample can be prepared by adding the required quantity of the spiked standard provided with the kit (usually 10 ppb) to the composite shrimp meat sample. Spiking is done at the level of MRPL or MRL. The use of spiked samples indicates the extent of antibiotic recovery and also the performance of the test.

Required ppb / Spike standard ppb = ml of spike standard to be added to 1g of meat

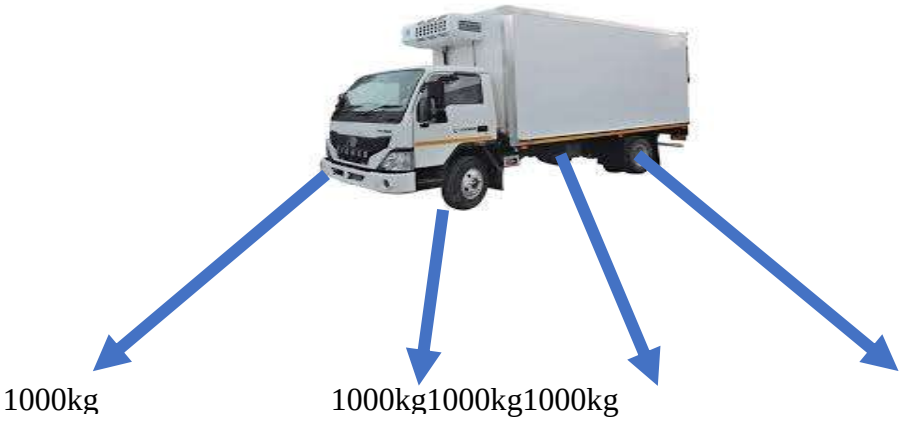
E.g., 10 ppb of spike standard; required ppb is 0.3; ml of spike solution required = 0.3/10 =0.03 ml or 30 microlitres per gram of shrimp meat.

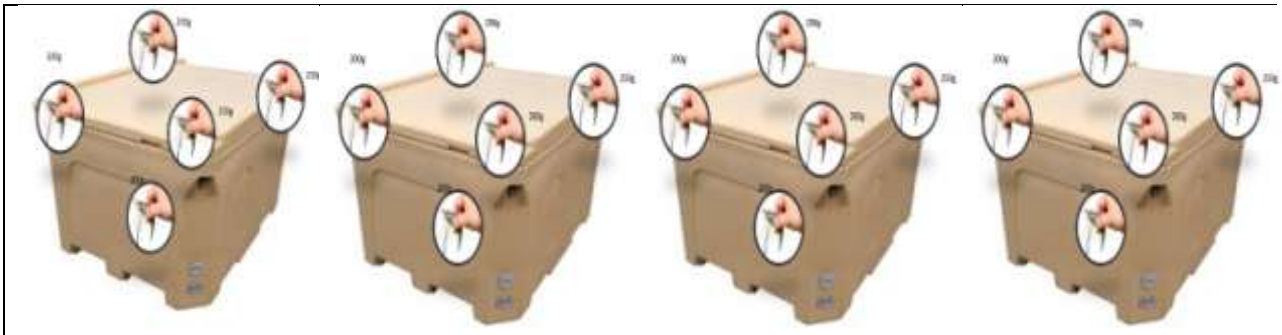
For preparing 3g of shrimp meat sample with 0.3 ppb of chloramphenicol: Take 90 microlitres of the 10ppb spike standardand add it to 3g of composite shrimp meat sample and mix thoroughly.

Illustrated procedure for the receiving of 4-ton shrimp material for testing of antibiotics in ELISA

4 tons (4000kg)

How to take Pooled samples:

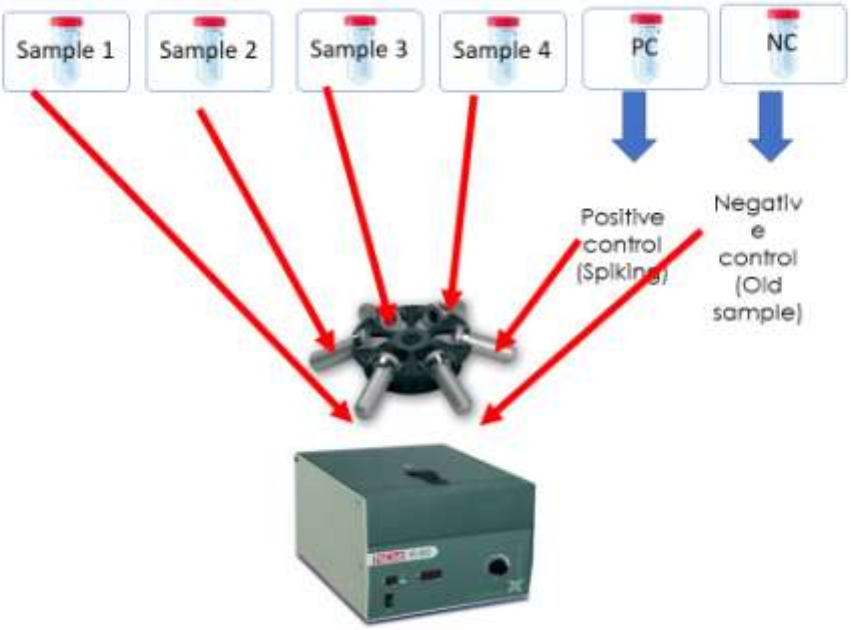




(Sample 1)	(Sample 2)	(Sample 2)	(Sample 3)
Take 200 gram of shrimp from all four corners and mix grind well then	**Take 200 gram of shrimp from all four corners and mix grind well then take**	**Take 200 gram of shrimp from all four corners and mix grind well then take**	**Take 200 gram of shrimp from all four corners and mix grind well then take**

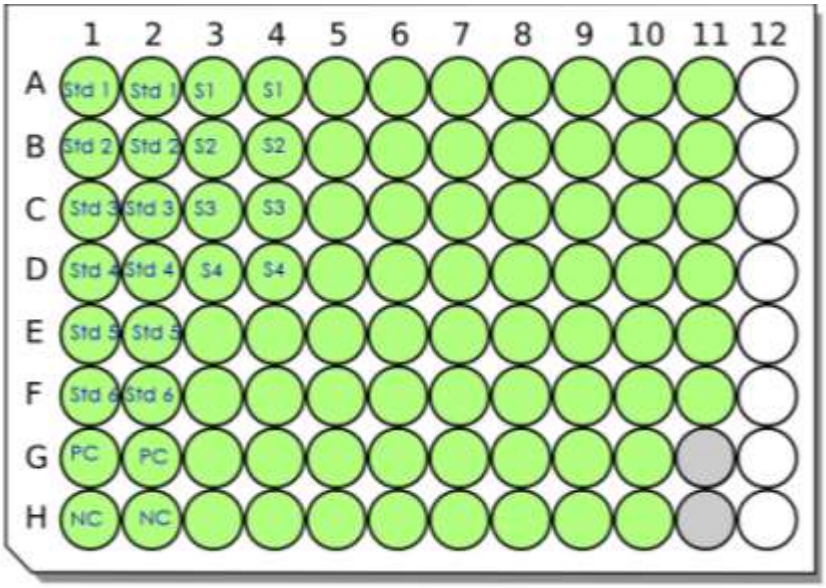
take 3 g for analysis	**3 g for analysis**	**3 g for analysis**	**3 g for analysis**

Test sample and control samples:



ELISA Plates:

Samples have to be tested as per the kit manufacturer'sinstructions. All standards (Std1, Std 2, Std 3, Std 4, Std 5 and Std6), positive control (spike sample; PC), negative control (NC) and each sample (S1, S2, S3, S4)have to be tested in duplicate wells.



Std 1: Standard 1; Std 2: Standard 2; Std 3: Standard 3; Std 4: Standard 4; Std 5: Standard 5; Std 6: Standard 6; S1: Sample No.1; S2: Sample No.2; S3: Sample No.3; S4: Sample No.4; PC: Positive Control (Spiked); NC: Negative Control (Un-spiked)

Determination of cut-off value

Important points to be remembered:

1. Extraction step is the most critical process to avoid false-negative results. Extractions conditions (pH, temperature, time etc.) prescribed by the kit manufacturers must be strictly followed.
2. Plastic centrifuge tubes should not be used during the evaporation of the solvent using nitrogen gas. Antibiotics willfirmlyattach to the walls of the plastictube and will not detach while adding the buffer. So, this will give a false-negative result. Hence, a glass centrifuge must be used in this step.
3. Spiking is needed for every chloramphenicol analysis and spiking with anyone nitrofuran metabolite (AOZ or AMOZ or AHD or SEM) is sufficient for nitrofuran analysis. No need to spike all the nitrofuran metabolites.
4. One positive and one negative control is sufficient even more than the 4 samples.
5. One more centrifuge (instrument) is needed if the one-time sampling will be more than 4 samples for easy handling procedure.
6. If the sample is are positive, then it has to be confirmed by LCMS-MS.

References:

EC (2003). Minimum Required Performance Limits, Commission decision of 13 March 2003 regards the setting up of minimum required performance limits (MRLs) for certain residues in food of animal origin. EC directive 2003/181/EC

EIC (2002). Compendium of Fish and Fishery Products Export Inspection Council, Ministry of Commerce and Industry, Govt. of India, S.O. 729 (E) dated 21st August 1995; Subsequently amended vide No. Order S.O.722 (E) dated 10^{the} July 2002

FSSAI (2011) Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011 (version 3, 31/08/2018), Ministry of Health and Family Welfare, Govt. of India

GOI (2002). The Gazette of India Extraordinary, dated 10th July 2002, vide order No.729 (E) of the Ministry of Commerce and Industry, Government of India.

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