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Screening of Oxytetracycline and Tetracycline Residues in Pork Marketed in Guwahati City and its Adjoining Areas and Confirmation by Ultra-fast Liquid Chromatography-UV/Vis

Param Debbarma[1], Saurabh Kumar Laskar[2], Ankur Das[2], Sadhana Choudhury[2], Santosh Upadhyay[3], Nagendra Nath Barman[4], Dhruba Jyoti Kalita[5]

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ABSTRACT

Background: The use of antimicrobial agents in livestock production is inevitable and helpful in majority of their applications. However, their indiscriminate frequency of use has resulted in the deposition of antimicrobial residues in animal products and when that exceeds the maximum residue limits (MRLs) beyond permissible daily consumption put human health at risk. The present study aimed to detect Oxytetracycline and Tetracycline residues in the most popular meat (pork) marketed in and around Guwahati City (Kamrup Metropolitan) and its adjoining areas.

Methods: The preliminary screening of the antimicrobial residues in 261 pork samples was carried out by microbial inhibition assay using endospores of *Bacillus subtilis* MTCC 441 as test organism. A total of 80 pork samples were further screened by Ultra-Fast Liquid Chromatography system-UV/Vis (Model: Shimadzu Prominence LC-20AD, Detector- SPD-20A-UV/Vis; RP C18 Column: BDS Premium, 250 mm × 4.6 mm, 5 µm) to detect Oxytetracycline and Tetracycline residues.

Result: The preliminary screening revealed that none of the samples were positive for antimicrobial residues except 3 (three) which were doubtful to have traces of antimicrobial residues. On further screening, Oxytetracycline residues were detected in 2.5% of the samples, while none of the samples detected Tetracycline residue. The concentrations of the residues were 0.471 µg/g and 0.610 µg/g, respectively, which is well above the MRL value recommended by Codex Alimentarius. However, considering the detection of 2.5% Oxytetracycline residues and the absence of Tetracycline residue, it can be concluded that these antimicrobial compounds are not frequently misused in pig husbandry practices in these areas under study.

Key words: Antimicrobial residues, NER of India, Oxytetracycline, Pork and UFLC-UV/Vis.

INTRODUCTION

There are 3.9 million pigs in the North Eastern Region (NER) of India out of 9.06 million in the nation overall, of which, Assam alone hosts over 2.1 million pig population (20th Livestock Census, 2019, DAHD, GOI). It is well recognized in the country that people of the NER have a strong preference for foods of animal origin. Among the meat of different species, pork is the most commonly consumed meat in the region (Mahajan *et al.,* 2015). This preferential choice for pork by the indigenous people of NER is perhaps due to their close relationship with the Mongoloid people of neighbouring countries where pork is mostly preferred. Thus, pig husbandry is becoming a popular livelihood option for the majority tribal population of NER of India. The rearing system of pigs is mostly backyard and scavenging type, with one or a few pigs per family. In recent times, people have started practicing intensive/semi-intensive farming of pigs. Thus, to control disease outbreaks, the routine use of antimicrobial agents has become an integral part of the livestock production system in the region.

The use of antimicrobial agents in livestock production is helpful in the majority of their applications. However, their indiscriminate frequency of use has resulted in the deposition of antimicrobial residues in animal products and when that exceeds the maximum residue limits (MRLs) beyond permissible daily consumption put human health at risk

[1]Department of Livestock Products Technology, College of Veterinary Sciences and Animal Husbandry, R.K. Nagar, West Tripura-799 008, Tripura, India.
[2]Department of Livestock Products Technology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 001, Assam, India.
[3]AICRP on Post-Harvest Engineering and Technology, Department of Livestock Products Technology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 001, Assam, India.
[4]Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati- 781 001, Assam, India.
[5]Department of Veterinary Biochemistry, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 001, Assam, India.

Corresponding Author: Param Debbarma, Department of Livestock Products Technology, College of Veterinary Sciences and Animal Husbandry, R.K. Nagar, West Tripura-799 008, Tripura, India. Email: paramdebbarma.86@gmail.com

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(Samanidou and Nisyriou, 2008; WHO, 2014). Food allergy and toxicity are the direct effects of antimicrobial residue throughout the food chain (Beyene, 2016; Samanidou and Nisyriou, 2008). The long-term negative impacts on human health include an increased chance of teratogenicity, carcinogenicity and disturbance in the normal microbiota of the gut (Beyene, 2016; WHO, 2014). Other issues caused by the antimicrobial residue include the emergence of antibiotic-resistant bacteria, drug abuse and reproductive issues (Samanidou and Nisyriou, 2008; Timothy *et al.,* 2012; Hassan *et al.,* 2014; Beyene, 2016). The emergence of antimicrobial resistance microorganisms has reduced antibiotics' therapeutic efficacy, increasing death, morbidity and economic losses globally (Roberts *et al.,* 2009). Currently, at least 700,000 people worldwide die yearly due to antimicrobial resistance (AMR). The World Health Organization (WHO) estimates that without new and improved treatments, this number might increase to 10 million by 2050, emphasizing a health issue that is not of secondary importance. Because of inadequate detection capabilities and insufficient residue control mechanisms, developing nations are more likely to have residues in foods originating from animals (Patel *et al.,* 2022).

In recent years, antibiotic residues in meat, among other antimicrobial residues have become a growing concern in India (Waghamare *et al.,* 2020). Kumar *et al.* (2020), reported that Oxytetracycline is the preferred choice of antimicrobial for the treatment of pig diseases and is also used as a growth promoter in different pig farms of NER of India. Similarly, several authors reported the detection of Tetracycline group of drugs residues in pork sold in different markets of North-eastern states of India posing serious health concerns to the consumers (Roy and Gogoi, 2014; Gogoi and Roy, 2017; Gogoi *et al.,* 2017 and Roy *et al.*, 2019). Guwahati city in Assam state is one of the fastest growing cities in India and the largest metropolis and commercial centre of the North- eastern region of India which is home to nearly 1.0 million human populations (15th Indian Census, MHA, GOI). Hence, the present study was undertaken to monitor the current status of Oxytetracycline and Tetracycline residues in one of the most popular meats (pork) marketed in and around Guwahati city under Kamrup metropolitan district of Assam state and its adjoining areas.

MATERIALS AND METHODS

Location and period of the experiment

The experiment was conducted in the laboratory of All India Coordinated Research Project (AICRP) on Post-Harvest Engineering and Technology (PHET), Department of Livestock Products Technology, Assam Agricultural University, Khanapara, Guwahati, Assam for a period of 18 months w.e.f., April, 2021 to September, 2022.

Sources and collection of samples

Two hundred and sixty-one pork samples were randomly collected from 3 districts of Assam (Kamrup Metropolitan, Kamrup Rural and Morigaon) and 1 district of Meghalaya (Ri-Bhoi). Each sample were collected aseptically in a separate sterile plastic bag and transported under the chilled condition to the laboratory of the AICRP on Post-Harvest Engineering and Technology, Department of Livestock Products Technology, Assam Agricultural University, Khanapara, Guwahati, Assam and stored thereafter at -20°C, until used for analysis.

Bacterial culture

The bacterial strain used in the present study was a pure freeze-dried culture of *Bacillus subtilis* MTCC 441 procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR Institute of Microbial Technology, Chandigarh. The bacterial culture was activated in sterile nutrient broth and sub-cultured 3-times before use. The culture was stored at 4°C, until further use.

Instrumentation

The UFLC system (Model: Shimadzu Prominence LC-20AD, Detector-SPD-20A-UV/Vis) equipped with RP C18 Column (BDS Premium, 250 mm × 4.6 mm, 5 μm) was used for the study. A mobile phase of 0.01M Oxalic acid: Acetonitrile: Methanol (77:18: 5, v/v/v) at pH 2.0 was used. The flow rate was kept at 1.0 ml/min in an isocratic mode. The wavelength for the detector was set at 350 nm. The injection volume was 20 μl and the column thermostat was set at 35°C.

Bacteriological media, chemicals and reagents

Muller Hinton Agar, Nutrient Agar, Nutrient broth, Gentamicin disc (GEN), Oxytetracycline Dihydrate, Tetracycline Hydrochloride used in the study were procured from Hi-Media while, Acetonitrile, Methanol and HPLC Grade Water were from Qualigens. For analysis, 0.01 M oxalic acid (pH 1.6) and 0.1 M citric acid buffer were prepared in HPLC Grade Water, while, 0.01 M methanolic oxalic acid (pH 1.86) was prepared in methanol. 0.1 M EDTA-McIlvaine buffer was prepared by dissolving Di-sodium hydrogen phosphate (13.72 g), EDTA (32.62 g) and anhydrous citric acid (13.62 g) in 1000 ml of HPLC Grade Water and the pH was adjusted to 4.0 with extra citric acid solution. All these freshly prepared buffer solutions are filtered through 0.22 μm cellulose filter and were stored at 4°C until used.

Preparation of standard solutions

Stock solutions of the standard were prepared separately by dissolving 10 mg of the individual standard in 10 ml methanol (1000 μg/ml) and kept in amber coloured volumetric flask. A standard working solution of 3.0, 2.0, 1.0, 0.5 and 0.1 μg/ml was prepared separately by diluting the stock solution with methanol. As it is unstable at room temperature, so the standard solutions are prepared daily and stored at 4°C.

Preliminary screening of antimicrobial residues in pork

The preliminary screening of the antimicrobial residues in pork samples was performed as per the method developed by Wasch *et al.* (1998) with slight modifications.

Harvesting and preparation of bacterial spore suspension

A heavy inoculum of *Bacillus subtilis* MTCC 441 was streaked over a freshly prepared sterile nutrient agar plate. The plates were incubated at 30°C for 10 days to induce sporulation. After completion of the incubation period, colonies were collected in a 15 ml sterile centrifuge tube and the volume was adjusted up to 10 ml with normal saline. The tube was then heated in a water bath at 70°C for 10 minutes to destroy the vegetative cells. The heated suspension was thereafter centrifuged for 10 minutes at a speed of 3000 rpm and the transparent supernatant was discarded. An additional 10 ml of sterile normal saline was added to produce a pure suspension of endospores and the same procedure was repeated twice. Suspension turbidity was adjusted to match with 0.5 McFarland standard solution (≈1.5 × 10^8 CFU/ml) before use.

Preparation of the test plates

Muller-Hinton agar was prepared in a conical flask and sterilized by autoclaving at 15 psi for 15 minutes. After cooling to around 45°C, 0.1 ml inoculum of spore suspension (≈1.5 × 10^8 CFU/ml) was added to each of 100 ml agar. About 15-20 ml of the molten agar were put into sterile petri dishes and allowed to cool. Plates were either used on the same day of preparation or were kept at 4°C and used within a week.

Detection of antimicrobial residues in pork samples

The sampling of meat was done in frozen condition and disk-shaped slices of 8 mm diameter and 2 mm thickness were prepared. Two disks of a meat sample were placed on opposite ends of inoculated plates and incubated at 37°C for 24 h. Development of a transparent/clear zone/ ring ≥2 mm was considered positive. However, a zone of 1-2 mm was considered doubtful and a zone <1 mm was considered as negative. The area of the inhibition zone was calculated as $\pi r2$. The mean areas of both inhibition zones were calculated.

Confirmation of antimicrobial residues in pork samples by ultra-fast liquid chromatography (UFLC)

Detection of Oxytetracycline and Tetracycline residues in pork samples were carried out by UFLC system (UFLC: Shimadzu Prominence LC-20AD, Detector-SPD-20A (UV/ Vis; RP C18 Column: BDS Premium, 250 mm × 4.6 mm, 5 μm) as per the method described by Biswas *et al.* (2007) with slight modifications.

Sample preparation

The stored frozen meat samples were thawed and on removing the fascia and exterior fat, it was finely diced with scissors. Each sample was grounded into a fine paste using a pestle and mortar. The representative sample (10 g) was collected in a beaker and homogenized with an equal amount of HPLC grade water for 1.5 minutes in a tissue homogenizer at 7500 rpm.

Extraction, clean-up, filtration and analysis of the samples

Five grams of the representative meat sample was transferred in a beaker. It was carefully mixed with 3 ml of 0.1 M EDTA McIlvaine buffer (pH 4.0). The sample was then ultrasonicated by setting the amplitude at 20 microns for 15 cycles with a stop time of 30 seconds by maintaining a low temperature using crushed ice. To allow the extract to dissolve in the solvent, the sonicated sample was left undisturbed for 15 minutes. The sample was then transferred to a centrifuge tube and centrifuged at 10,000 rpm at 0°C for 15 minutes. The recovered supernatant was then filtered using Whatman filter paper no. 42. The filtrate was subsequently run through a C18 cartridge that had been pre-conditioned with 3 ml methanol and 3 ml water, eluted with 4.5 ml of 0.01 M Methanolic oxalic acid (pH 1.80) and then further filtered using 0.22 μm filter paper before being collected in a 2 ml HPLC autosampler vial. An aliquot of 20 μl was then injected into the UFLC system to analyse the residues.

RESULTS AND DISCUSSION

Preliminary screening of antimicrobial residues in pork samples

A total of 261 pork samples collected from 3 districts of Assam and 1 district of Meghalaya were preliminary screened by microbial inhibition technique using *Bacillus subtilis* MTCC 441 as a test organism to detect antimicrobial residues in the pork samples. The result of the preliminary screening is presented in Table 1 and the graphical presentation in Fig 1. The screening test revealed that none of the pork samples have antimicrobial residue which could inhibit the growth of the test organism, except 3 samples

Table 1: Preliminary screening of antimicrobial residues in pork samples.

Sources of samples	No. of samples screened	No. of positive samples	No. of doubtful samples
Kamrup Metro, Assam	95	ND	ND
Kamrup Rural, Assam	53	-	1
Morigaon, Assam	51	-	-
Ri-Bhoi, Meghalaya	62	-	2
Total	261	ND	3

ND-Not detected.

(1 from Kamrup rural and 2 others from Ri-Bhoi district) which were doubtful to have traces of antimicrobial residues.

The result of the present study was in the line of Wasch *et al*. (1998) who reported the detection of antimicrobial residues by microbial inhibition assay. The microbial inhibition assay or agar diffusion technique is a cheap screening method to detect antimicrobial residues when large numbers of samples are to be covered. They are essentially a qualitative screening test that detects any tissues/substance with the property of bacterial inhibition. But this method is less sensitive as it could not provide a clear zone of inhibition in 3 doubtful samples which agree with the observation of Javadi *et al.* (2011).

Detection of antimicrobial residues by Ultra-fast liquid chromatography (UFLC)

All the 80 pork samples collected from 3 districts of Assam (Kamrup metropolitan, Kamrup rural and Morigaon) and 1 district (Ri-Bhoi) of Meghalaya were screened by UFLC system to detect Oxytetracycline (OTC) and Tetracycline (TTC). All the positive samples were compared with recommended MRL values of Codex Alimentarius.

Detection of oxytetracycline (OTC) residues

The samples were screened by UFLC technique to detect OTC residues in pork. The result of the screening test by UFLC system is presented in Table 2 and the graphical representation in Fig 2. Out of 80 pork samples, 2 samples (2.5%) were positive for OTC residues. The concentrations of OTC residues in the positive samples were 0.471 µg/g and 0.610 µg/g, respectively and well above the MRL value recommended by Codex Alimentarius.

The use of OTC antibiotic in pig husbandry practices of the North-Eastern states of India was reported by Kumar *et al.* (2020). They reported that OTC is the most preferred choice of antibiotic for the treatment of pig diseases and also used as a growth promoter in different pig farms of the 3 North-Eastern states of India.

As in the present studies, Wasch *et al.* (1998) reported the detection of OTC residue in 0.38% of pork samples. Roy *et al.* (2019) also reported OTC residues in 2.32% of

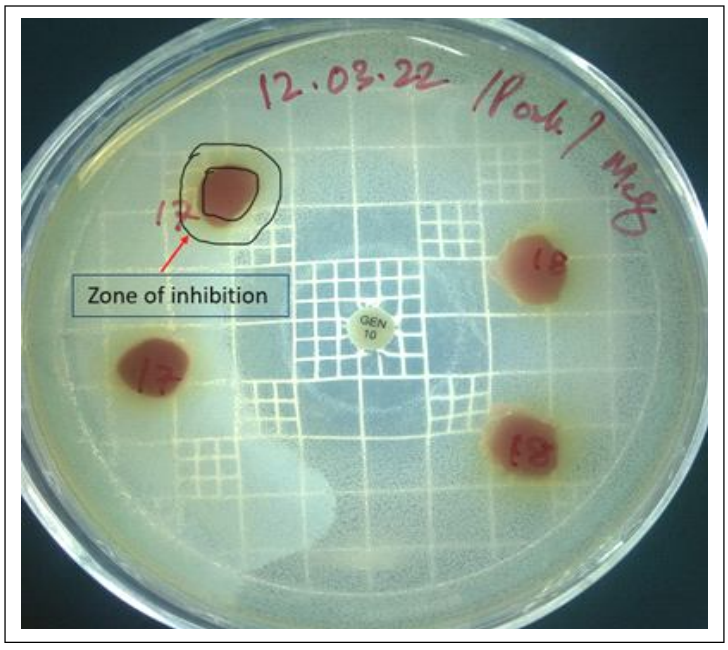
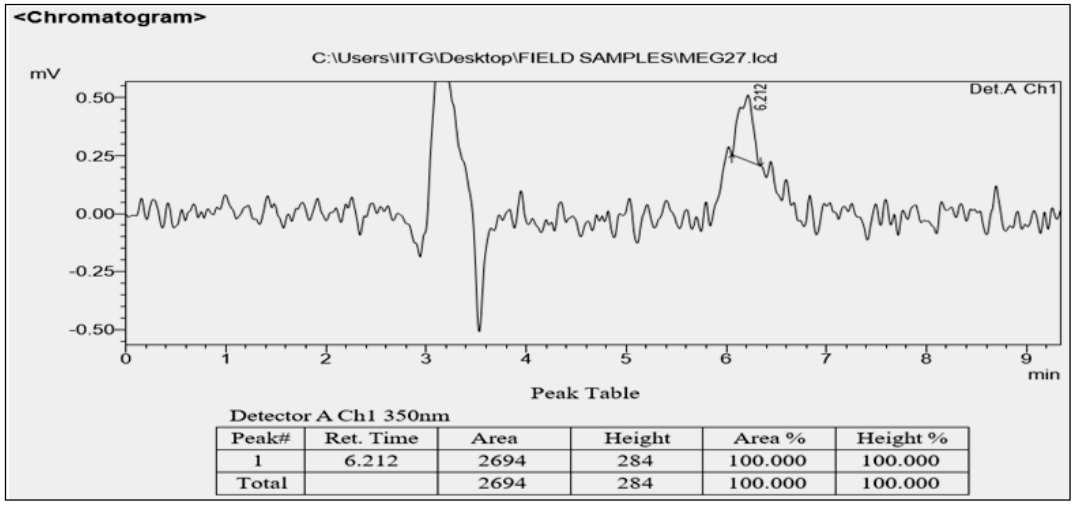


Fig 1: Inhibition zone (diameter<2 mm) of antimicrobial residues in doubtful pork sample.

Table 2: Detection of oxytetracycline (OTC) and tetracycline (TTC) residues in pork samples by UFLC/Vis.

Sources of samples	No. of sample screened	No. of positive samples detected		Concentration of the positive samples (µg/g)		No. of samples above MRL	
		OTC	TTC	OTC	TTC	OTC	TTC
Kamrup Metro,	20	ND	ND	ND	ND	ND	ND
Kamrup Rural, Assam	20	-	-	-	-	-	-
Morigaon, Assam	20	-	-	-	-	-	-
Ri-Bhoi, Meghalaya	20	2	-	0.471-0.610	-	2	-
Total	80	2	ND	0.471-0.610	ND	2	ND

ND-Not detected.



Peak Table

Detector A Ch1 350nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	6.212	2694	284	100.000	100.000
Total		2694	284	100.000	100.000

Fig 2: Chromatogram of oxytetracycline (OTC) residue in pork sample.

pork samples collected from various locations of Assam and the concentration of OTC residues in 2 pork samples was well above the MRL values recommended by Codex Alimentarius which is in close agreement with the results of the present studies. The obtaining of OTC residues well above the MRL values in the positive samples might be related to the administration of OTC prior to the slaughter of pigs *i.e.*, enough time for excretion of residues was not available and thus their flesh had recorded high OTC residues.

Detection of tetracycline (TTC) residues

The procured pork samples were screened by the UFLC technique to detect TTC residues in pork. The result of the screening test (UFLC system) is presented in Table 2. It was revealed that none of the pork samples was detected positive for TTC residues.

Contrary to the present findings, Roy and Gogoi (2014) reported the detection of TTC residue in 2% of pork samples which were collected from different roadside pork stalls of various locations in Assam. Likewise, Nheim *et al.* (2006) also reported the detection of TTC residue in 5.5% of pork samples in Hanoi, Vietnam.

A survey report by Kumar *et al.* (2020) however stated that TTC is not a choice of antibiotic neither for treating diseases in pigs nor as a growth promoter in pig farms of North-East India, which indirectly supports the results obtained of the present study.

CONCLUSION

Based on the results obtained in the study, it can be concluded that the presence of Oxytetracycline and Tetracycline residues in pork marketed in Guwahati city and its adjoining areas till date are reasonably good considering the detection of 2.5% Oxytetracycline residues and absence of Tetracycline residues in the present findings indicates that the antimicrobials are not frequently misused in pig husbandry practices in these four districts under study, however, studies with larger sample size involving samples from different markets of the districts would be more desirable to validate the present findings.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declares that there is no conflict of interest regarding the publication of this article.

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