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Simultaneous Detection of Six Different Groups of Antimicrobial Drugs in Milk, Meat, Urine, and Feed Matrices

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Abstract: Antimicrobials are widely used for animal health in food-producing animals. Therefore, antimicrobial residues are of food safety concern. Simultaneous detection of different antimicrobial residues in the same matrice is important. Therefore, the objective of this study was to detect six different group antimicrobials in milk, meat, urine, and feed matrices under experimental conditions, and to evaluate its use for routine analysis. Biochip array-based immunoassay is currently used for simultaneous detection and quantitation of different groups of six antimicrobials in milk, urine, meat, honey, and feed. Results showed that of the six target drugs, norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline could be detected in milk, meat, urine, and in feed matrices. In conclusion, this assay can effectively detect all target antibacterials from different groups in milk, meat, urine, and feed matrices, and this assay can be used for routine detection of these antibacterial residues in the stated matrices.

Key Words: Antimicrobials, biochip array-based immunoassay, milk, meat, urine, feed.

Süt, Et, İdrar ve Yemde Altı Farklı Grup Antimikrobiyal İlacın Biochip Array-Based Immunoassay ile Aynı Anda Tespiti

Özet: Antimikrobiyaller besin üretiminde faydalandığımız hayvanlarda yaygın olarak kullanılmaktadır. Bu nedenle, bu besinlerde bulunabilen antimikrobiyal kalıntıları insan sağlığı açısından önemlidir. Farklı antimikrobiyal kalıntılarının aynı numunede ve aynı anda birlikte tespiti önemlidir. Bu nedenle, bu çalışmanın amacı Biochip array-based immunoassay yöntemi ile altı farklı grup antibakteriyel ilacın süt, et, idrar ve yemde deneysel şartlarda tespit etmektir. Bu test, aynı anda altı farklı grup antimikrobiyalin süt, idrar, et, bal ve yem numunelerinde miktarlarının belirlenmesinde kullanılmaktadır. Bu metodla hedef ilaçlar olan norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklin süt, et, idrar ve yemde tespit edildi. Sonuç olarak, bu test ile yukarıda belirtilen altı farklı gruptaki hedef antibakteriyel ilaç süt, et, idrar ve yemde etkili bir şekilde tespit edilebilmektedir. Bu metod, belirtilen numunelerde bu antibakteriyel ilaçların kalıntılarının rutin analizinde kullanılabilir.

Anahtar Kelimeler: Antimikrobiyaller, biochip array-based immunoassay, süt, et, idrar, yem.

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Introduction

Antimicrobials are widely used for animal health by intramuscular, intravenous or oral route as feed supplementation. The residue problem arises when treated animals are slaughtered without recourse to withdrawal periods for the specific drug. This situation is more likely to occur with prolonged administration and over dosage of antimicrobial agents. Antimicrobial residues are of food safety concern. To protect health of consumers it is necessary to test on foods obtain food producing-animals for potential antimicrobial residues on the farm before they are placed on the market. In most countries, veterinary medicine is allowed to use only those agents that are officially registered and approved. The Codex Alimentarius and Joint FAO/WHO programme have been developing standars concerning the residues in foods since 1985[10]. In Europe, the estabilishing of MRL level in the Europen Union (EC) is regulated by the Council Regulation (EEC) 2377/90[4]. In EU, monitoring of veterinary drug residues, including antibiotics, has been governed by Council Directive[3] since 1996. This Directive describes the required frequency and level of sampling, the investigation procedures, the necessary documentation to be produced, and the appropriate action to be taken in the case of non-compliance.

The reasons for monitoring veterinary drugs residues in feed, urine, foodstuffs and foods of animal origin include the ethical ones (preventing undesired exposition of health consumers to therapeutical doses of drugs in food), hygienic (protection against possible harmful effects of the residues on the consumer's health), technological (preventing the disruption of the fermentation processes), and ecological[5,9,10]. There are some reports about occurrence of antimicrobial residues in foods and biological specimens such as meat, milk, feed and urine in some previous studies[2,6,8,11,13,14].

There are various methods such as Thin Layer Chromatography, Charm Tests, ELISA, Liquid Chromatography, and Biosensors for the antimicrobial agent detection for milk, meat, feed and urine[1,7,10]. However, simultaneous analysis of different groups of antimicrobials is a difficult task but is highly desirable in diagnostic laboratories. A biochip array-based immunoassay test (Antimicrobial Array II, Randox Laboratories Ltd., Crumlin, UK) that can quantitatively analyze for quinolones, ceftiofur, thiamphenicol, streptomycin, tylosin and tetracyclines, simultaneously in selected matrices was recently developed and has been used for analysis of honey, milk, tissue, urine, and feed matrices[15] . The test can be used to simultaneously quantify multiple analytes from a single sample[15]. The Biochip technology can also use for analysis of oral fluid[12].

The objectives of this study were to detect simultaneously six specific antimicrobials in milk, meat, urine, and feed matrices using a biochip array-based immunoassay, and to evaluate its use for routine analysis.

Materials and Methods

Samples

Beef cattle meat and cow milk (whole and skim milk) samples were obtained from a market, and porcine urine and feed samples were collected from a pig farm housed in Ames, Iowa State, USA.

Antibacterial standards and chemicals

Norfloxacin (FLUKA, Buchs, Switzerland), florfenicol (FLUKA, Buchs, Switzerland), tylosin tartrate (FLUKA, Buchs, Switzerland), ceftiofur (Sigma-Aldrich, Seelze, Germany), streptomycin sulfate salt (Sigma-Aldrich, Seelze, Germany), tetracycline (Sigma-Aldrich, Seelze, Germany), and chlortetracycline hydrochloride (Sigma-Aldrich, Seelze, Germany) were used as antimicrobial standards.

Preparation of standards

Stock standard solutions were prepared as 10 mg/ml. Tylosin, tetracycline, florfenicol in methanol (Fisher Scientific, New Jersey, USA), norfloxacin in acetone (Fisher Scientific, New Jersey, USA), ceftiofur in deionized water (Water Aries High Purity Water System, West Berlin, Germany): acetonitrile (Fisher Scientific, New Jersey, USA) (7:3), and streptomycin in deionized water were dissolved. Dilutions from stock solutions were made with the washing buffer of AM II Kit.

Biochip array-based immunoassay test procedure

The samples were tested using Antimicrobial Array II (AM II) Evidence Investigator Test Kit and the AM II Control was used as a control (EV 3524 and EV5337, Randox Laboratories Ltd., Crumlin, UK). Extraction of the samples and all assays were performed according to AM II manufacturer's instructions[15]. Biochips were equilibrated to room temperature for

approximately 30 min prior opening. After extractions, 100 µl of 'assay diluent' was pipetted into the biochip wells. 100 µl of calibrator or sample was pipetted into the wells and all edges were taped gently to handling tray to mix reagents. Biochips were incubated for 30 min at 25 ^{o}C on a thermoshaker (Randox Laboratories Ltd., Crumlin, UK) at 370 rpm. 100 µl of working strength conjugate was slowly mixed before use and pipetted into the wells. Biochip wells were incubated for 60 min at 25 ^{o}C and 370 rpm on the thermoshaker. Reagents were discarded using a sharp flicking action of the handling tray. Two quick wash cycles were carried out with 'diluted wash buffer' (wash buffer) with approximately 350 µl for each well. Four additional wash cycles were used; for each cycle all edges of the handling tray were gently taped approximately 10-15 sec, then biochips were left to soak in wash buffer for 2 min. After the final wash, all the wells were filled with wash buffer and left to soak until directly prior to imaging. Two hundred and fifty microliters of 'working signal reagent-EV805' was added to each well and covered to protect from light in the thermoshaker. After 2 min (+/- 10 sec), the carrier was placed into the Evidence Investigator (Randox Laboratories Ltd., Crumlin, UK). Captures of images were automatically initiated as defined by the dedicated software.

Results

Assay ranges, control and recovery results of norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline in meat, milk, urine and feed samples (as ppb) are presented in Table 1.

Discussion

As shown in Table 1, assay ranges of the AM II Kit was generally between 0 and 54.9 ppb depending on the antimicrobial. AM II control background results for this study was similar to manufacturer's background control results, which confirms that the reagent kits were working well. However, recovery values were different for individual antimicrobials. For example, for norfloxacin (140%), ceftiofur

Table 1. Assay ranges, control and recovery results of norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline in meat, milk, urine and feed samples (as ppb), and AM II Control and mix standard results (as ppb).

Tablo 1. Et, süt, idrar ve yemde norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklinin ölçüm aralığı, control ve geri kazanımı sonuçları (ppb olarak), AM II kontrol ve karışık standart sonuçları (ppb olarak).

Sample	DF	Norflox	Ceftiofur	Florfen	Strep	Tylosin	Tetra
Assay ranges* (AM II Kit)	1	0 - 9.8	0 - 20.7	0 - 4.8	0 - 54.9	0 - 4.5	0 - 4.0
AM II controls	1	1.11 (1.21)**	2.48 (2.5)**	0.56 (0.63)**	6.22 (7.31)**	0.46 (0.54)**	0.37 (0.45)**
Mix std %Rec	1	2.81 140%	4.25 212%	2.54 127%	2.65 64%	1.64 82%	0.84 42%
Washing buffer (AM II Kit)	1	0.11	0.12	0.18	0.57	0.06	0.21
Deionized water	1	0.11	0.10	0.16	0.44	0.03	0.27
Meat control	20	2.40	0	0	16.71	0.53	3.26
Meat %Rec (n=3, Mean)	20	105%	83%	87%	95%	73%	30%
Skim milk control	20	0	0	0	1.01	0	0
Skim milk %Rec (n=2, Mean)	20	121%	57%	37%	78%	44%	>40%
Whole milk control	20	0	0	0	1.08	0	0
Whole milk%Rec (n=2, Mean)	20	122%	52%	39%	68%	40%	>40%
Urine control	10	0	0	0	1.28	0	22.97***
Urine %Rec (n=2, Mean)	10	63%	51%	39%	58%	33%	>40%
Feed control	40	12.13	0	0	16.88	22.26***	0
Feed %Rec (n=2, Mean) With extraction buffer	40	143%	113%	59%	93%	53%	42%
Feed %Rec (n=2, Mean) Without extraction buffer	40	134%	101%	55%	23%	50%	130%

* Assay ranges that determined with AM II calibrators according to AM II Manual.

** AM II Control result: Antimicrobial II Controls were assigned with HPLC by Randox.

***Positive controls

Mix std: Six antibacterial standards were prepared (4 and 2 ppb for strep. and others, respectively) with diluted washing buffer from stock solution and analyzed.

Std: Standard, Rec: Recovery, DF: Dilution Factor; Norflox: Norfloxacin; Florfen: Florfenicol; Strep: Streptomycin; Tetra: Tetracycline.

(212%), and florfenicol (127%) higher than 100%; for streptomycin (64%), tylosin (82%), and was least for tetracycline (42%). This test therefore likely has higher sensitivity for norfloxacin, ceftiofur and florfenicol, and lower sensitivity for streptomycin and especially tetracyclines. Streptomycin control results were generally higher than the other antimicrobial control results except tylosin in feed control and tetracycline in urine control results. Tylosin and tetracycline levels detected in feed and urine control samples, respectively, were positive. This could be explaned by.the information obtained from both farms, pigs had infection, and therefore fed with tylosin containing feed in one farm, and with tetracycline containing feed in the other farm.

The antibacterials studied in meat, milk, urine and feed samples were clearly detected by this test. However, recoveries of florfenicol in skim and whole milk, and urine; recovery of streptomycin in feed (without extraction buffer); recoveries of tylosin in skim and whole milk, and urine; recoveries of tetracycline in meat and feed (with extraction buffer) were low (<50%) (Table 1).

In general, results suggest that this Biochip technology has a potential to be used for simultaneous detection and quantification of the antimicrobials evaluated in this study. Simultaneous analysis of different groups of antimicrobials is a difficult task but is highly desirable in diagnostic laboratories. A biochip array-based immunoassay test (Antimicrobial Array II) that can quantitatively analyze for quinolones, ceftiofur, thiamphenicol, streptomycin, tylosin and tetracyclines, simultaneously in select matrices was recently developed and has been used for analysis of honey, milk, tissue, urine, and feed matrices. Therefore, the Biochip technology is preferable than other analysis methods[2,8,11,14] used for indicated samples for certain antimicrobials.

In conclusion, meat, milk, urine and feed of domestic animals can be routinely used to detect and/or monitor certain antimicrobials. This study has demonstrated feasibility for the use of a biochip array based immunoassay for simultaneous detection and quantitation of (name the drugs) in meat, milk, urine and feed.

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