Kafkas Univ Vet Fak Derg
19 (3): 407-412, 2013
DOI: 10.9775/kvfd.2012.7905

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Simultaneous Detection of Six Different Groups of Antimicrobial Drugs in Porcine Oral Fluids Using A Biochip Array-Based Immunoassay

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Makale Kodu (Article Code): KVFD-2012-7905

Summary

The objectives of this study were 1) to determine whether a biochip array-based immunoassay could be used to detect 6 different group antimicrobials in porcine oral fluids collected under experimental conditions; 2) to determine the feasibility of antimicrobial detection in clean versus dirty oral fluid samples; and 3) to determine if the assay could be used to detect chlortetracycline in oral fluids of swine ingesting a routine diet containing chlortetracycline. Biochip array-based immunoassay is currently used for simultaneous detection and quantitation of different group six antimicrobials in milk, urine, meat, honey, and feed. The assay had not been investigated for potential use in oral fluids. Following evaluation of different extraction procedures, a centrifugation method was chosen. Results showed that of the six target drugs, norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline could be detected in both clean and dirty oral fluid samples. However, tetracycline was not well recovered in dirty samples. Chlortetracycline from tetracycline group was detected in all oral fluid samples collected from the field at concentrations ranging from 176.0-698.6 ppb. In conclusion, this assay can detect all target antibacterials from different groups in clean and dirty oral fluid samples with the exception of tetracycline which was not well-recovered from in dirty samples.

Keywords: *Antimicrobials, Biochip array-based immunoassay, Oral fluid, Swine*

Domuz Ağız Sıvısında Altı Farklı Grup Antimikrobiyal İlacın Aynı Anda Biochip Array-Based Immunoassay ile Tayini

Özet

Çalışmanın amacı Biochip array-based immunoassay yöntemi ile 6 farklı grup antibakteriyel ilacın deneysel şartlarda toplanmış domuz ağız sıvısında tespit edilip edilemediğini; temiz ve kirli domuz ağız sularında bu antimikrobiyallerin tespit edilebilirliğini ve bu metotla, rasyonlarında sürekli olarak klortetrasiklin bulunan domuzların ağız sıvılarında klortetrasiklinin tespit edilebilirliğini belirlemektir. Bu metod, aynı anda altı farklı grup antimikrobiyalin süt, idrar, et, bal ve yem numunelerinde miktarlarının belirlenmesinde kullanılmaktadır, ancak ağız sıvılarında tespit edilebilirlikleri henüz araştırılmamıştır. Yapılan farklı ekstraksiyon çalışmalarından sonra bir santrifüj metodu seçildi. Bu metodla hedef ilaç olan norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklin temiz ve kirli domuz ağız sıvısında tespit edildi. Ancak, kirli ağız sularında tetrasiklinin geri kazanımı oldukça düşük olarak elde edildi. Klortetrasiklin çiftlikteki domuzların ağız sıvılarında 176.0-698.6 ppb arasında tespit edildi. Sonuç olarak, bu test ile kirli ağız sıvısında tetrasiklin aranması dışında, altı farklı gruptaki hedef antibakteriyel ilaç domuzların ağız sıvısında kolaylıkla tespit edilebilmektedir.

Anahtar sözcükler: *Antimikrobiyaller, Biochip array-based immunoassay, Ağız sıvısı, Domuz*

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INTRODUCTION

Oral fluid is composed of saliva, gingival cervical fluids contained in the dentogingival sulcus, mucosal transudate, cell detritus, bacteria and food remains [1]. Human saliva is composed of 98% water. The remaining amount is made up of other compounds, such as electrolytes (Na, K, Ca, Mg, hydrogen carbonates, and phosphates), mucus (composed mainly of mucopolysaccharides and glycoproteins), antiseptic substances (hydrogen peroxide, IgA), and various enzymes (α-amylase, lysozymes, lingual lipase) [2]. Saliva also contains many other proteins such as histatin and polypeptides with antibacterial and antifungal properties [2].

Following oral or parenteral administration, antimicrobials can be transported from blood to saliva by simple diffusion and/or active transport mechanisms [3,4]. Depending upon the degree of ionization, antimicrobials that are weak bases may reach high concentrations in saliva [5].

Antimicrobial residues are of food safety concern. To protect health of consumers it is necessary to test pigs for potential antimicrobial residues on the farm before they are put on the market. Simultaneous analysis of different groups of antimicrobials is a difficult task but is highly desirable in diagnostic laboratories. A biochip array-based immunoassay test (BABIT) 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. The test can be used to simultaneously quantify multiple analytes from a single sample [6].

It has been demonstrated that oral fluids can be used as a matrix to detect some drugs in domestic animals [7]. Significant research has also been conducted on the use of oral fluid as a diagnostic medium for detection of some viral infections [7-9]. Recently, there is a report [10] on the detection of antibacterials such as ceftiofur and oxytetracycline in swine oral fluids. In this study [10], ceftiofur and oxytetracycline were qualitatively detected in swine oral fluid as positive or negative using a pen-side competitive ELISA. In veterinary diagnostic medicine, oral fluid specimens could potentially be used for detection of some drugs for food safety applications. At present, commonly used matrices for this purpose are milk, meat, urine and serum. There are some advantages in using oral fluids compared to traditional matrices. Collecting oral fluid specimens is animal friendly (less stressful) and non-invasive [7]. Besides, oral fluids can be collected from a single animal or from group of animals. Oral fluid is not a common diagnostic specimen for analysis of antimicrobials in domestic animals and in veterinary medicine. Therefore continued research is needed to standardize collection methods of oral fluids from swine [7]. The quality of oral fluids collected depends on sampling method, number of pigs in the pen and cleanliness of the pen.

The impact of sample quality on antimicrobial test results in domestic animals warrants continued research. There is also a need for further research on tests which can simultaneously detect multiple antimicrobials in a single oral fluid sample. The objectives of this study were: 1) to determine whether a biochip array-based immunoassay could be used to detect 6 specific antimicrobials simultaneously in porcine oral fluids collected under experimental conditions; 2) to determine the feasibility of antimicrobial detection in clean versus dirty oral fluid samples; and 3) to determine if the assay could be used to detect chlortetracycline in oral fluids of swine ingesting a routine diet containing chlortetracycline.

MATERIAL and METHODS

Animals and Animal Care, and Feeding Conditions

Clean oral fluid samples were collected from pigs housed in research facilities in the College of Veterinary Medicine at Iowa State University (Ames, IA), and all studies were approved by the Institutional Animal Care and Use Committee. The samples were collected from 8 pens of 15 pigs each. The oral fluid used in the methods development part this study was from a pooled sample containing oral fluid from each of the 8 pens. The animals were conventionally produced pigs, weighed approximately 14 kg (30 lbs), and contained both male and female pigs. The pigs were fed an antimicrobial-free diet. The field collection of oral fluid was from 20 pens of 25 animals per pen in a commercial finisher swine barn. The pigs weighed approximately 34 kg (75 lbs) and were placed in the finisher barn 3 days prior to oral fluid collection. There were a total of approximately 1200 pigs in the barn but only a pen with 20 pigs were enrolled in the study. These pigs were fed a commercial diet containing chlortetracycline (approximately 440 g/ton) and tiamulin (tiamulin hydrogen fumerate approximately 38g/ton). Animal were fed *ad libitum*, and no parenteral antimicrobial treatments were administrated to these pigs. Oral fluids were collected on January 06, 2012.

Sample Collection

Oral fluid samples were collected from pigs as described in a recent study [10] by hanging 100% cotton rope (Wep Ringing Suplly, Inc., Lake Barrington, IL, USA) in each pen for a minimum for 20 min in the morning. Oral fluids were extracted by wringing the ropes through a manual wringer. Oral fluid from each pen were pooled into a 50-ml Falcon plastic tube and frozen at -20°C until analysis. Sample quality as a colour can range from very clean to very dirty *(Fig. 1)*.

Biochip Array-Based Immunoassay Test Procedure

Oral fluid 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). All assays were done according to AM II manufacturer's instructions [6]. Biochips

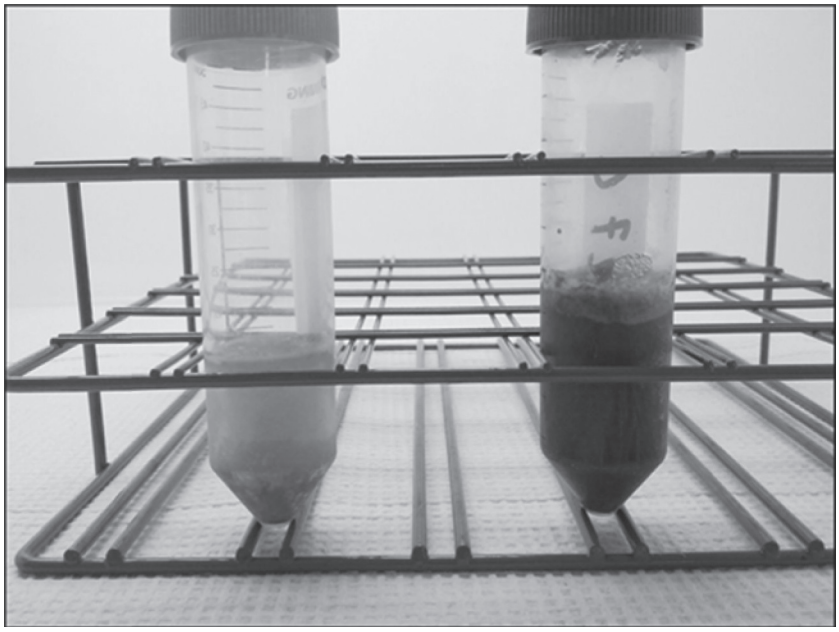

Fig 1. Clean and dirty porcine oral fluids

Şekil 1. Temiz ve kirli domuz ağız sıvısı

were equilibrated to room temperature for approximately 30 min prior to opening. After extraction, 100 µl of "assay diluent" was pipetted into the biochip wells. 100 µl of calibrator or samples was pipetted into the wells and gently taped all edges of the handling tray to mix reagents. Biochips were incubated for 30 min at 25°C on a thermo-shaker (Randox Laboratories Ltd., Crumlin, UK) 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°C and 370 rpm on the thermo-shaker. Reagents were discarded to the waste container using a sharp flicking action of the handling tray. 2 quick wash cycles were immediately carried out with "diluted wash buffer" (wash buffer) with approximately 350 µl for each well. 4 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. 250 µl "working signal reagent-EV805" was added to each well and covered to protect from light in the thermoshaker. After exactly 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.

Extraction Method Development for Oral Fluids of Pigs

Antibacterial standards and chemicals: In the present study, 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 chlor-tetracycline hydrochloride (Sigma-Aldrich, Seelze, Germany) were used as antimicrobial standards. Ethylenediamine tetra acetic acid (EDTA) disodium salt dehydrate (Fisher Scientific, New Jersey, USA), sodim sulfate (Fisher Scientific, New Jersey, USA) and acetic acid (glacial) (Fisher Scientific, New Jersey, USA) were used for extraction in Method 3.

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.

Extraction studies in oral fluid: Different extractions methods for antibiotics in oral fluids were investigated, including filtration, dilution, precipitation, and centrifugation. A summary of each extraction method investigated follows:

- *Method 1. Filtration and dilution:* The manufacturer's (Randox) suggested sample protocol involves filtration of 3 ml of oral fluid with 0.45 µm syringe filter (TITAN, Nylon 0.45 µm). Then, 100 µl of the filtrate is dilute with 900 µl of washing buffer.

- *Method 2. Dilution-filtration and dilution-centrifuge:* 100 µl oral fluid samples at two different spike levels were diluted with 900 µl washing buffer (n=2) or deionized water (n=2), vortexed (FISHERbrand, Vortex Genie 2™, USA), filtered through a 0.45 µm syringe filter, and the filtrate was analyzed. In *dilution-centrifuge,* 100 µl oral fluids that at two different spike levels were diluted with 900 µl washing buffer (n=2) or deionized water (n=2) and centrifuged (Eppendorf, 5417 C, USA) at 5.000 rfc for 10 min at room temperature, and the supernatant was analyzed.

- *Method 3:* 0.5 ml of 0.3 M EDTA and 4 ml acetonitrile/1%

acetic acid was added to 1 ml of oral fluid, and sample was vortexed for 1 min. Then 0.4 g sodium sulfate was added and vortexed, followed by centrifugation at 2.500 rfc for 20 min at room temperature. The supernatant was evaporated and reconstituted with 1 ml of washing buffer. Two different spike levels (2 and 4 ppb for streptomycin, and 1 and 2 ppb for the other antimicrobials) were performed (n=6).

- *Method 4. Centrifugation*: This involved a slight modification of the AM II urine method described in the manual. Briefly, 1 ml of oral fluid sample and 1.5 ml eppendorf tube was used. The 1 ml oral fluid sample was centrifuged at 5.000 rfc for 10 min at room temperature. 50 µl of centrifuged oral fluid was collected and diluted with 450 µl washing buffer, the dilution factor was 10. The experiment involved testing different centrifuge speeds (5.000, 10.000, and 15.000 rfc) (n=6). Assay ranges, recovery and precision values of clean and dirty oral fluids were performed according to Australian Pesticides and Veterinary Medicines Authority Guidelines [11]. Samples were spiked for determination of assay ranges, percent recovery and precision. Assay range studies were carried out between 0.25 and 12 ppb for norfloxacin, florfenicol, tylosin, and tetracycline; 0.25 and 20 ppb ceftiofur, and 0.5 and 100 ppb for streptomycin in clean and dirty oral fluid samples. Recovery was studied at 1.0, 1.2 and 1.5 ppb levels for tetracycline; at 1.5, 1.7 and 2 ppb levels for norfloxacin, florfenicol and tylosin; at 2.0, 2.5 and 3.0 ppb for ceftiofur, and at 10.0, 15.0 and 20.0 ppb levels for streptomycin. Precision was carried out six times at 1.0 ppb for tetracycline, at 2.0 ppb for norfloxacin, ceftiofur, florfenicol and tylosin at 20.0 ppb concentrations for streptomycin.

For field samples, chlortetracycline (representing tetracyclines as a group) concentrations in 20 porcine oral fluid samples were determined using the centrifugation method (Method 4). There was a color difference between clean and dirty oral fluid samples. The test specificity was 51% for chlortetracycline according to the kit manual [6], and recovery rates (n=3) averaged 55% for these oral fluid samples. The dilution factor was 80 (10 times are coming from method 4, and 8 times made dilution before analysis). All the results were calculated according to these factors.

Statistical Method

Descriptive statistics were performed using the Minitab Statistical Program [12].

RESULTS

The procedure recommended by the manufacturer (Method 1) did not work for both clean and dirty pig oral fluids because the oral fluids could not pass through the filter without dilution. As for method 2 (dilution and filtration), the oral fluid could be filtered, but with difficulty, and there was huge variability between results (between 13% and 500%). Results from Method 3 indicated very high recovery rates (between 790% and 5.000%) except florfenicol results (between 74% and 295%). As such, the first 3 extraction procedures did not work. Method 4, which was a modification of the urine extraction procedure, worked well. We investigated 3 centrifugation speeds, (5.000, 10.000 and 15.000 rfc) for both clean and dirty oral fluid samples, and results indicated that 5.000 rfc recovery values were slightly better than the other speeds, and this speed was adopted by the researchers.

Standard curves were prepared using AM II Kit calibrators. Assay ranges were 0-9.8 (norfloxacin), 0-20.7 (ceftiofur),

Table 1. *Assay ranges, recovery, precision and control results of norfloxacin, ceftiofur, florfenicol, streptomycin, tylosin and tetracycline in clean and dirty oral fluids of pigs (as ppb), and AM II Control and mix standard results (as ppb)*

Tablo 1. *Domuzların temiz ve kirli ağız sıvılarında norfloksasin, seftifor, florfenikol, streptomisin, taylosin ve tetrasiklinin ölçüm aralığı, geri kazanımı, kesinlik (doğruluk) ve control 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 %Recovery	1	2.81 (140%)	4.25 (212%)	2.54 (127%)	2.65 (64%)	1.64 (82%)	0.84 (42%)
COF control	1	0.01	0.02	0	0	0.04	0.07
DOF control	1	1.62	0.61	0.14	0	0.02	0.27
COF assay ranges	1	0.5-6	0.5-9	0.25-3	2.0-75	0.25-5	0.5-1.5
DOF assay ranges	1	0.5-8	0.5-10	0.25-4	1.0-75	0.25-6	2.0-6
COF %Recovery (Mean)	1	140±7.54	130±6.24	122±8.00	71±3.46	78±9.53	171±50.26
DOF %Recovery (Mean)	1	42±7.23	117±12.74	93±12.74	69±3.51	55±1.15	5±0.57
COF Precision (Mean), %RSD	1	2.71±0.14 (5.2%)	2.74±0.19 (6.9%)	2.50±0.12 (4.9%)	13.44±0.70 (5.2%)	1.55±0.07 (4.4%)	1.21±0.07 (6.0%)
DOF Precision (Mean), %RSD	1	1.33±0.18 (13.3%)	2.74±0.25 (9.2%)	2.45±0.19 (7.7%)	14.54±0.24 (1.6%)	1.22±0.09 (7.4%)	0.09±0.05 (57.0%)

** 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,* ***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,* ***COF:*** *Clean Oral Fluid,* ***DOF:*** *Dirty Oral Fluid,* ***DF:*** *Dilution Factor,* ***Norflox:*** *Norfloxacin,* ***Florfen:*** *Florfenicol,* ***Strep:*** *Streptomycin,* ***Tetra:*** *Tetracycline,* ***±:*** *Standard Deviation*

0-4.8 (florfenicol), 0-54.9 (streptomycin), 0-4.5 (tylosin), and 0-4.0 (tetracycline). R^2 values that determined with AM II calibrators were 0.998, 0.999, 0.999, 0.997, 0.998, and 0.993 for norfloxacin ceftiofur, florfenicol, streptomycin, tylosin, and tetracycline, respectively. Results for assay ranges, recoveries, precisions and controls of clean and dirty oral fluid's centrifuge method (Method 4), and AM II Control and mix standard results are presented in *Table 1*. Results of field assays for chlortetracycline concentrations determined in 20 oral fluid samples collected from a pig farm are between 176.0 ppb and 698.6 ppb, with a mean level of 421.3±210.6 ppb.

DISCUSSION

It turned out that developing a suitable method for simultaneous extraction of six different antimicrobials in oral fluid was challenging. The challenge with Method 1 was filtration of the oral fluids; oral fluid samples did not filter well. The oral fluid of pigs is too thick for filtration through a 0.45 µm syringe filter. In Method 2, following dilution the filtration process worked most of the time, but the recovery values were unreliable. The reasons for this are unclear, but probably antimicrobials in the oral fluid did not filter well enough thru 0.45 µm syringe filter. Method 3 did not work well either as recovery values were very high. The reasons for the extremely high recoveries are not clear. Results were acceptable for the Centrifugation method (Method 4), as recovery values were generally acceptable for both for clean and dirty oral fluid samples with the exception of the tetracycline. Recovery of tetracyclines in dirty oral fluid was low *(Table 1)*. Because of the overall performance, this extraction method was chosen for analysis of six antimicrobials with the BABIT. This extraction method is simple as it does not require adding any chemical. Simply, 1 ml of oral fluids is used for analysis. Because of its simplicity, and because it is easy to collect this sample size from pigs singly or as group, this method was adopted and is recommended.

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. This confirms that the reagent kits were working well. However, recovery values were different for individual antimicrobials. For example, for norfloxacin (140%), ceftiofur (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 tetracyclines. Dirty oral fluid samples had slightly higher, but insignificant control values for norfloxacin, ceftiofur, florfenicol and tetracycline. Reasons for this are not clear, but could likely be caused by some interference in this matrix for these antibiotics. It is interesting that both clean and dirty oral fluid samples did not contain any streptomycin *(Table 1)*. Assay ranges for dirty and clean oral fluids were generally similar except for tetracycline. For tetracyclines, the assay range of dirty oral fluid (2.0-6.0 ppb) was wider than the clean oral fluid assay range (0.5-1.5 ppb) *(Table 1)*. Tetracycline recovery was very low (5%) in dirty oral fluid *(Table 1)*. Although reasons for this are not clear, it is likely that tetracyclines easily bind to the matrix ingredients such us calcium, magnesium, aluminum, and iron [13]. It is possible that constituents in dirty oral fluid samples bind to the tetracycline group. This may be valid for norfloxacin as well because recovery for this antimicrobial in dirty oral fluid samples was low (42% versus 140%). Besides these exceptions, recovery values for streptomycin, ceftiofur and tylosin in clean and dirty oral fluid samples were close *(Table 1)*.

In the field samples, concentrations of chlortetcycline in 20 pig oral fluid samples were between 176.0 ppb and 698.6 ppb, with a mean level of 421.3±210.6 ppb. The highest level was approximately 4 times higher than the lowest level. There could be several reasons why there was so much variability in concentration of chlortetracycline in oral fluids collected from pigs fed a diet containing 440 ppm chlortetracycline, including improperly mixed feeds, time of last meal, etc. Overall, chlortetracycline was present in oral fluids at approximately 1.000 times less in oral fluid of pigs than in feeds. In this study, we did not collect blood samples from these pigs. This was not the objective of this study. However, these results indicate that tetracyclines could pass to oral fluids of pigs fed feeds containing tetracyclines. Therefore, oral fluids can be used to detected tetracyclines in pig oral fluid using this novel biochip array-based immunoassay. Oxytetracycline and ceftiofur in oral fluids of swine was also reported in a previous study [10]. However their assay was qualitative, with results reported as either positive or negative, using a pen-side competitive ELISA. In addition, if the ceftiofur pass from the blood to oral fluid as mentioned the previous study [10], ceftiofur might also be detected easily with the BABIT in field conditions. The average recovery was 55% in the oral fluids collected from pig farm. The recovery values could for tetracycline could vary between 5% and 171% *(Table 1)* depending on the samples properties, eg very clean versus dirty samples *(Fig. 1)*. More research is therefore needed to validate this assay and to determine factors affecting recovery of different antimicrobials in oral fluids.

In general, results suggest that this Biochip technology can be used to simultaneously detect and quantify the antimicrobials evaluated in this study with the exception of tetracyclines in dirty samples. Overall, the technology is can work for clean oral fluid samples like those collected under controlled research conditions, in clean facilities. However, this technology will be of value if it can be used under typical field conditions. Field oral fluid samples are reflective of the environment in which the pigs are housed. Therefore, sample cleanliness should be considered during assay development of this technology especially for tetracyclines.

In conclusion, oral fluids can be used to detect and/or monitor certain antibacterials in domestic animals. This study has demonstrated feasibility for the use of a biochip array based immunoassay for simultaneous detection and quantitation of (name the drugs) in porcine oral fluids. More field studies are recommended to characterize the technique under field conditions before it can be adopted for field applications.

Acknowledgements

The authors thank YÖK (Turkish Higher Education Council) for support provided for this study, and Dr. John Johnson, Dr. Jeffrey J. Zimmerman, and the faculty and staff of the Iowa State University Veterinary Diagnostic Laboratory for advice and technical support.

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