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Residual Studies of Cefquinome and Tylosin against Experimentally Induced *Echerishia Coli* Infection In Chicken Using HPLC

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Abstract

ANTIBIOTIC resistance is a worldwide problem either for human or animals. Antibiotics combination is an effective way to combat this resistance. The current work studied the effect of cefquinome and tylosin combination against *E. coli53.*Fifty-eight broiler chickens were separated into 5 groups. Group I and II were used as control negative and positive respectively. Group III, IV and IV were all infected with *E. coli 53* but cefquinome treated, tylosin treated and the combination; respectively. Cefquinome and tylosin were extracted from tissues matrix and detected by validated reversed phase high performance liquid chromatography (RP-HPLC) technique. The technique used determined cefquinome and tylosin with low quantification limits 30.3 ppb and 24.6 ppb; respectively. Moreover, the method achieved high recovery percentage reached 99.4% for cefquinome and 99.1% for tylosin. The minimum inhibitory concentration (MIC) was 0.025mg/L for cefquinome against E. *coli 53* while tylosin recorded a resistance level which was 16mg/L. Fraction inhibitory concentration (FIC) index was measured as 0.85 judged the combination effect as additivity. The tissues residues recorded higher levels of cefquinome and tylosin in Group V than Group III and IV; respectively resulted in prolongation of the withdrawal time of cefquinome for kidney and liver tissues to 5 days after cessation of the combination administration (Group V). These results revealed the additive manner of cefquinome and tylosin combination against *E. coli 53* resulted in more recorded levels of tylosin especially for kidney and liver and elongation of the withdrawal period of cefquinome.

Keywords: Cefquinome, tylosin, bacterial resistance, antibiotics combinations.

Introduction

Birds and animals' digestive tracts are home to the adaptable gram-negative bacteria *Escherichia coli*, also referred to as *E. coli*. Avian Pathogenic *Escherichia Coli* (APEC) can lead to colibacillosis, a serious illness that affects broilers and layers as well as other poultry production systems, even though many strains are innocuous. E. coli may be classified as a main or secondary pathogen depending on a variety of conditions that affect its virulence [1]. The public health concern of APEC is the growing antimicrobial resistance in their strains, as it can pass their resistance genes to human causing serious diseases through horizontal gene transfer which makes treatment difficult [2]. Moreover, wide use of antibiotics can cause antibiotic resistance and raises public health burdens [3]. Antibiotics combination is one of the most effective ways to overcome antimicrobial resistance (AMR) [4]. Cephalosporins are a vital component in the battle against bacterial infections. The β-lactam ring, which is essential to these β-lactam antibiotics' antibacterial action, is part of their shared chemical structure. Veterinarians like them because of their excellent safety record in animals. Cefquinome, a fourth-generation cephalosporin created especially to use in animals, stands out among them. It has an extensive range of antibacterial activity and works well against both

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gram-positive and gram-negative bacteria [5 and 6]. It affords a MICs against black swans *E. coli ranged* from 0.03 to 8 µg/mL, with MIC50 and *MIC90* of 0.06 and 0.5 µg/mL, respectively [7]. So, it is considered as a good choice for Enterobacteriaceae infections especially *E.coli.*

In veterinary medicine, tylosin is a bacteriostatic feed additive and macrolide antibiotic. It works against a wide variety of Gram-positive species while only a small number of Gram-negative ones [8]. Some antibiotics modulates susceptibility towards other antibiotics by increased MICs [9]. So, trial combination study of cefquinome with tylosin to investigate cefquinome effect on tylosin restricted effect on *E.coli 53* infection.

Each of cefquinome and tylosin was detected in different matrices by different methods. For mention; cefquinome detection in various matrices using high-performance liquid chromatography (HPLC) has been extensively researched, focusing on its quantification in biological and food matrices. The methodologies developed emphasize the importance of sample preparation, matrix effects, and validation to ensure accurate results across different applications. Different methods were reported as in milk and its products [10]; plasma [11] and rabbit serum and tissues [12]. Moreover, many studies analysed tylosin in different matrices as milk [13], meat [14] and tissues [15 and 16] by RP-HPLC. These methods focus on optimizing extraction, clean-up, and detection processes to ensure accurate and reliable quantification of tylosin residues.

There are no previous records on using one HPLC method for detection of both cefquinome and tylosin in tissues matrix. So, this study directed an interested step to determine the two antibiotics combination in tissues matrix by a validated RP-HPLC method. Besides, the main idea of this study is to investigate the impact of cefquinome and/or tylosin combination against *E.coli 53* infection with a special concern to their residue's behaviour.

Material and Methods

Standards, drugs and chemicals

Reference standard of cefquinome sulfate (VETRANAL80.9%) and tylosin tartarate, analytical standard, were taken from Sigma Aldrich Chemical Co. (St.Louis, MO). Commercial Cefquinome sulphate (Cobactan 2.5%), and tylosin tartarate (Tylovet 100%) were purchased from Pharma Sweed-Egypt Company. Acetonitrile of HPLC grade and all analytical reagents were purchased from Riedel-de Haen (Seelze, Germany).

Bacterial strain

E. coli O53 was obtained from bacteriology unit, animal health research institute, Benha. One ml of *E. coli* suspension, containing 3×10^8 CFU.

Experimental animals and design

The present study was carried out at Animal Health Research Center, El-Dokki. Fifty-eight broiler chickens were obtained from a private commercial hatchery, fed on drug free-ration and supplied with water ad-libitum before and during the experiments. Birds were sectioned into 5 groups. Group I (5 chicken) served as control negative as non-infected. Group II (5 chicken) served as control positive as infected with *E. coli O53* orally at a dose of $3*10^8$ CFU for each chicken [17]. Group III (12 chicken) were given *E. coli O 53* orally to induce infection at a dose of $3*10^8$ CFU for each chicken and treated with intramuscular injection of cefquinome (2mg/ Kg bw) once daily for 3 consecutive days [18]. Group IV (12 chicken) were given *E. coliO 53* orally to induce infection at a dose of $3*10^8$ CFU for each chicken and treated with oral administration of tylosin (50mg/ Kg bw) once daily for 5 consecutive days [19]. Group V (12 chicken) were given *E. coli O53* orally to induce infection at a dose of $3*10^8$ CFU for each chicken and treated with intramuscular injection of cefquinome (2mg/ Kg bw) once daily for 3 consecutive days in combination with oral administration of tylosin (50mg/ Kg bw) once daily for 5 consecutive days.

Antibiotic was administered to groups after appearance of symptoms. Thereafter, muscle, liver, and kidney tissues were gathered after the dissection of birds to quantify cefquinome and tylosin residuesat 1, 3, 5 and 7 days after the administration of the final dose of drugs. Tissue samples were kept at -20°C till the examination by RP-HPLC method. The effectiveness of both cefquinome and tylosin alone and combined will also be assessed using MIC, MBC and FIC.

Standards preparation

Cefquinome and tylosin standards were dissoluted in deionized water to get a stock solution with a concentration of 1000 parts per million (ppm). Blank tissues from control group (muscle, liver and kidney) were used to prepare different concentrations of working standards varying from 10 to 1000 parts per billion (ppb). The quality control (QC) threshold was designated at 100 ppb in accordance with established protocols [20].

Tissues residues analysis and chromatographic conditions:

Tissues (kidneys, liver and muscles) were homogenized and extracted as AbdElhafeez and Fadel [12]. Samples were mixed with M ammonium acetate buffer (TAC5) pH 5 adjusted with isooctane followed by shaking and centrifugation steps. The buffer layer was subjected to solid phase extraction (SPE) and evaporated then eluted by 500µl of isocratic mobile phase consisted of acetonitrile–0.04 M Na HPO4 pH 2.4 (33:66 v / v) [21].

Samples were injected for HPLC by 10µl and separated by a Supelco C18 column with dimensions of 5 µm, 250 mm × 4.6 mm id. Column temperature was set at 40^{o}C. Multiwave detector (AGILENT, Japan) was used at 280 nm. The Chemstation software was utilized for method control and data analysis. This method was validated by following the USP guidelines [20], which encompassed the validation of parameters including linearity, precision, recovery, as well as detection and quantification limits (DL and QL).

Antimicrobial activity of antibiotics

Investigation of antimicrobial sensitivity test (AST), minimum inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) of cefquinome and / or tylosin against E. coli O53

The broth microdilution method was used to determine AST and MIC for *E.coli O53* strain which recommended by the Clinical and Laboratory Standards Institute [22] against cefquinome and tylosin. Cefquinome breakpoint was prepared in a dilution range 0.0125 to 8 mg/L and 1 to 32mg/L for tylosin [23]. Two-fold dilutions of antibiotic were used in broth with $3*10^8$ CFU/ml of *E. coli O53*. Growth control tube was tested by broth free from antibiotic. The tubes were incubated at 37°C for 24h. MIC was the lowest concentration of the antibacterial agent that inhibited bacterial growth after 24hours of incubation on the basis of turbidity. To determine the MBC, all the tubes with no visible bacterial growth in the MIC test were sub cultured. The plates are incubated at 37˚C for 24 hours. The lowest concentration with non-visible bacterial growth was termed as the MBC, representing 99.5% killing of the original inoculum [24].

Fractional inhibitory concentration (FIC) index for interaction evaluation between cefquinome and tylosin

Checkboard method assed cefquinome and tylosin combination. Cefquinome at a concentration corresponding to 1/2 MIC was used with tylosin concentrations ranged from 1/32 MIC to two times of MIC (2×MIC) and vice versa [25]. The fractional inhibitory concentration (FIC) was calculated using an equation described by [26]. The FIC index is determined by adding FIC A and FIC B, where each FIC is the minimum inhibitory concentration (MIC) of the drug combination divided by the MIC of the drug alone. If the FIC index is less than 0.5, the interaction is considered synergistic, meaning the combined effect is greater than the sum of the individual effects. An FIC value between 0.5 and 4 indicates an additive or indifferent interaction, where the combined effect equals the sum of the individual effects. If the FIC index is greater than 4, the interaction is classified as antagonistic, meaning the combined effect is less than the sum of the effects of each agent alone.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS Inc., version 20.0) was utilized to analyze the dataset (Chicago, Illinois). Two-tailed T-test was implemented for the comparison between groups. The data were represented using the standard deviation (SD) and the mean. P-values that were lower than 0.05 were deemed statistically significant [27].

Results

Clinical signs

Chickens showed dullness, dropping of wings, loss of appetite, ruffled feathers and greenish brown diarrhea after 24 hours in all infected groups. The symptoms alleviated gradually after cefquinome administration in Group III rather than Group IV (Tylosin) which appeared no relief of signs. Group V showed slight relief of the clinical signs with 16.6% mortalities (2/12birds).

Minimum inhibitory concentration (MIC) of Cefquinome and Tylosin against E.coli O53 and calculation of their FIC index

The minimum inhibitory concentration (MIC) of cefquinome against *E. coli O53* was 0.025 mg/L, while tylosin had an MIC of 16 mg/L. This suggests that cefquinome was more effective than tylosin against this strain. However, when both antibiotics were combined, they showed an additive effect against *E. coli O53*, as demonstrated by an FIC index of 0.85.

Tissue residues concentrations assessment by RP-HPLC

The results of the method validation indicated a robust linearity for both analytes (> 0.999), accompanied by elevated recovery percentage that spanned from 90.9% to 99.4% for cefquinome and from 92.7% to 99.1% for tylosin. Low detection limits (DL) and quantification limits (QL) were ascertained for cefquinome at 10.1ppb and 30.3 ppb, respectively, while for tylosin, they were determined to be 8.2ppb and 24.6 ppb. Furthermore, the method exhibited selectivity and sensitivity, characterized by distinct retention times for both cefquinome and tylosin, with no observed impurities interference (Fig. 1).

On day 3 after cessation of drug administration; tylosin level in kidneys and liver tissues of the combination group (Group V) recorded significantly higher levels than Group IV continuously to day 5 for kidney tissues only. Meanwhile; liver and muscles tissues hadn't any detected tylosin residues from day 5. On the other hand, there was no significant change in cefquinome levels between groups in the tested tissues as illustrated in Table 1

According to EU regulation [28], maximum residual limits (MRLs) for cefquinome in kidneys, liver and muscles tissues were 200ppb,100ppb and 50 ppb; respectively. So, the safe human consuming of muscles tissues in groups III and V was on day 3 after cessation of cefquinome administration. Meanwhile, it was safe for liver and kidneys tissues on day 5.

Regarding EU regulation [28] guidelines concerning tylosin MRLs to be100ppb for kidneys, liver and muscles; the withdrawal time in this study for tylosin was on day 3 for Group IV and day 5 for Group V except for muscles tissues which should to be safe for human consumption on day 3.

Discussion

To increase the efficiency of antibiotics against microorganisms, antimicrobial combinations are employed. When the effectiveness of the combination equals the total of the effects of the separate antibiotics, this is known as an additive effect. When the combination is more successful than the sum of the separate effects, this is known as a synergistic effect, and it frequently results in a more effective course of therapy. Conversely, when the combination is less successful than the separate effects, an antagonistic impact occurs, which might impede therapy and necessitate re-evaluating the antibiotic approach [29; 30 and 31].

In clinical antimicrobial therapy, the synergy principle-which states that the total impact of an agent is larger than the sum of its individual effects-is frequently used to strategically mix multiple drugs. This strategy can increase effectiveness against infections, especially those brought on by bacterial strains that are resistant. Some combinations, on the other hand, may be made to be hostile or even additive; the latter is occasionally employed to slow the emergence of resistance. Striking a fine balance between reducing the likelihood of resistance and optimizing therapeutic results [32]. Combination of bacteriostatic and bactericidal antibiotics to overcome the antibiotics resistance faced a great challenge which resulted mainly in an antagonism effect [33].

E.coli has a great resistance to antibiotics due to its highly, this threatens poultry industry. The current study reported high resistance of *E.coli O53* to tylosin unlike sensitivity record of cefquinome which in the line with the experimental trial of [34] in the treatment mastitis induced by *E.coli*. Conversely, Gregova et al. [35], reported 22% resistance of E.coli strains to cefquinome. High resistance was recorded by [36 and 23] to tylosin. Similarly, *Escherichia coli*is an intrinsically tylosin resistant [37].

Confirmation of an RP-HPLC technique for detection of cefquinome and tylosin in tissues matrix is a challenged goal in this study. The method produced low quantification limits 30.3 ppb and 24.6 ppb for cefquinome and tylosin; respectively. Moreover, the method achieved high recovery % reached 99.4% for cefquinome and 99.1% for tylosin the same in this study but in tissues matrix, with recoveries ranging from 73.4%to 99.4% [11; 38 and 39]. On the other hand, tylosin detected by other UV-HPLC methods in animal tissues with a recovery % 79.9% [40] and in pig tissues (fat, kidney, liver, and muscle) ranging from 70% to 85% [15]. These data ensured the reproducibility and the economic side of the current validated HPLC method.

Residues of tylosin in tissues of broiler chicken were higher in kidneys and liver than in muscles. These data agree with those reported by Soliman and Sedeik [19]. On the other hand, the current findings revealed that the highest concentrations of cefquinome were found in the kidneys, followed by the liver, in infected treated broilers, while the lowest levels were observed in muscle tissue. This indicates that cefquinome is primarily excreted through the kidneys [18 and 41]. These results are consistent with previous studies in chickens [18; 42 and 43] and in rabbits [12].

Drug interactions rely on either stimulating or inhibiting metabolism, and the inhibition is caused by inhibition of enzymes such as cytochrome P450 (CYP) [44]. Cytochrome P450 (CYP) represents a large group of heme-containing drug-metabolizing enzymes (DMEs) that are mainly produced in the liver, gastrointestinal tract, lungs, and kidneys. This is especially noteworthy in farm animals, particularly cattle, as these globally significant food-producing animals are continually exposed to xenobiotics, many of which are of human origin, throughout their lives (e.g., drugs, pesticides, and environmental pollutants) or natural (e.g., mycotoxins, plant secondary metabolites) xenobiotics. The accumulation of residues in tissues fit to be eaten and the occurrence of potentially harmful drug–drug interactions may be harmful to consumers as well as the animal itself [45 and 46].

Commonly used medications may interact negatively with macrolide antibiotics, typically via changing metabolism through complex formation and cytochrome P-450 IIIA4 (CYP3A4) inhibition in the liver and enterocytes [47]. This inhibition might be the reason behind the prolongation of cefquinome levels in Group V which treated with both cefquinome and tylosin. It was explained that the1stof this group assumed to be the 3th day for cefquinome residues noted the drug regimen of administration in this experiment. It was more obvious when noted the absence of cefquinome on day 3 from the muscles and on day 5 from liver, kidneys and muscles in Group III.

Conclusion

Cefquinome was effective in treatment of *E.coli53* infection. Meanwhile, tylosin failed to overcome the tested infection. Cefquinome-tylosin combination possessed an antagonistic effect on the treatment of *E.coli 53 infection.* The combination led to increase tylosin concentration especially in kidney and liver. Moreover, the combination had a negative effect on cefquinome residual levels in the tissues reflected on elongation of its withdrawal period.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

The experiment followed the Research Committee of the Agriculture Research Centre and Animal Health Research Institute (ARC-IACUC), Ministry of Agriculture, Giza, Egypt. The approval number was ARC-AHRI/132/24.

TABLE 1. Residue concentrations in different broiler tissues after Cefquinome and /or tylosin repeated treatment.

Groups/Days		**Unit (PPb)**			
		Group III (Cefquinome)	**Group IV (Tylosin)**	**Group V**	
				Cefquinome	**Tylosin**
1st	**Kidneys**	930±1.3	692.1±1.5	860±0.9	695.3±1.4
	Liver	532±0.5	431.6±1.2	604.2±0.9	453.1±1.1
	Muscles	222.2±0.2	201.2±1.1	220.2±0.2	203±0.9
3rd	**Kidneys**	375.2±0.12	100.01±0.8	403.3±0.1	190.8±0.7*
	Liver	132.1±0.4	84.3±0.7	110.4±0.3	100.5±0.8*
	Muscles	ND	63.3±0.3	ND	60.3±0.8
5th	**Kidneys**	ND	50.6±0.2	ND	90.1±0.6*
	Liver	ND	ND	ND	ND
	Muscles	ND	ND	ND	ND
7th	**Kidneys**	ND	ND	ND	ND
	Liver	ND	ND	ND	ND
	Muscles	ND	ND	ND	ND

ND: Not detected; Data was expressed as mean ±SD. * Significant at $p < 0.05$ using t- test.

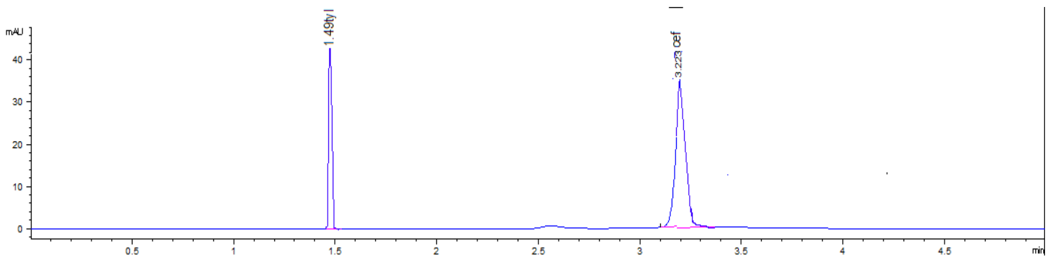

Fig. 1. HPLC chromatogram for tylosin and cefquinome in muscle at a concentration of 50 ppb with retention times of 1.49 and 3.23, respectively.

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دراسة متبقيات السيفكينوم مع التيلوزين في الدواجن المصابة تجريبيا بالاكولاي الايشيريشية بأستخدام جهاز الكروماتوجرافي العالي الاداء

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الملخص

مقاومة المضادات الحيوية مشكلة عالمية تؤثر على الإنسان والحيوان على حد سواء. يُعتبر الجمع بين المضادات الحيوية طريقة فعالة لمكافحة هذه المقاومة. دَرَسَت الدراسة الحالية تأثير الجمع بين السيفكوينوم والتيلوزين ضد الإشريكية القولونية 53. تم فصل ستة وأربعين فرخ لاحم إلى خمس مجموعات .استُخدمت المجموعة الأولى والثانية كمجموعة ضابطة سالبة وموجبة على التوالي. أُصيبت المجموعات الثالثة والرابعة والخامسة جميعها بالإشريكية القولونية 53 ولكن عُولجت بالسيفكوينوم والتيلوزين والجمع بينهما على التوالي. تم استخلاص السيفكوينوم والتيلوزين من مصفوفة الأنسجة وكشفهما باستخدام تقنية الكروماتوغرافيا السائلة عالية الأداء ذات الطور المعكوس المُعتمدة(RP-HPLC) . حددت التقنية المستخدمة السيفكوينوم والتيلوزين بحدود تقدير منخفضة بلغت 30.3 جزء في المليار و24.6 جزء في المليار على التوالي .علاوة على ذلك، حققت الطريقة نسبة استرداد عالية وصلت إلى 99.4 %للسيفكوينوم و99.1% للتيلوسين. كان الحد الأدنى للتركيز المثبط (MIC) 0.025ملغ/لتر للسيفكوينوم ضد الإشريكية القولونية 53 بينما سجل التيلوزين مستوى مقاومة بلغ 16 ملغ/لتر. تم قياس مؤشر التركيز المثبط الجزئي (FIC) بـ 0.85 مما يُقيم تأثير الجمع بينهما كتأثير تجميعي. سجلت بقايا الأنسجة مستويات أعلى من السيفكوينوم والتيلوزين في المجموعة الخامسة مقارنة بالمجموعتين الثالثة والرابعة على التوالي، مما أدى إلى إطالة فترة الانسحاب للسيفكوينوم في أنسجة الكلى والكبد إلى خمسة أيام بعد وقف إعطاء الجمع بينهما (المجموعة الخامسة). كشفت هذه النتائج عن الطبيعة التجميعية لجمع السيفكوينوم والتيلوسين ضد الإشريكية القولونية 53 مما أدى إلى تسجيل مستويات أكثر من التيلوسين خاصة في الكلى والكبد وإطالة فترة الانسحاب للسيفكوينوم.

الكلمات الدالة: السيفكوينوم ، التيلوزين، مقاومة البكتريا، جمع المضادات الحيوية.