Combined Antimicrobial Effects Against Gram Negative Bacteria Isolated From Broilers

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ABSTRACT

Developed multidrug resistant bacteria (MDR) in the field of poultry industry lead to the wide use of combined antimicrobials against enterobactericeae infections. This study deal with benefit drug interactions for the use in broiler industry. A total of 75 samples were collected from visceral organs of diseased broilers. Fivty isolates of enterobactericeae were recovered and biotyped as 32 *E. coli*, 12 *Salmonellae* and 6 *Klebsiellae*. Antibiogram revealed frequent sensitivity to colistin, cefotaxime and gentamycin. Meanwhile, all isolates were resistant to erythromycin and *Nigella sativa* oil. The isolates with intermediate sensitivity to any of the tested antimicrobial were examined for combined interactions of synergism. Fractional inhibitory concentration of two combined antimicrobials was detected. There was synergism between colistin with floriphenicol, ciprofloxacin, doxycyclins and amoxicillin. In addition, to synergism between gentamycin with cefotaxime, doxycyclin and erythromycin. Fractional inhibitory concentration of two anti-microbials with synergism was less than 0.50. *Nigella sativa* oil showed no synergism with any one the tested antimicrobials. It could be concluded that MDR enterobacteria with resistance to all antimicrobials could be susceptible to several of two combined antimicrobials.

INTRODUCTION

Drug resistant pathogenic enterobacteria in the field of poultry industry became more widely spread in Egypt. Multidrug resistant bacteria are also developed all over the world. Therefore, it was extremely important to find new antimicrobial agents or new ways that are effective for the treatment of infectious diseases caused by drug-resistant bacteria *(1)*. Synergism between antimicrobials is the benefit drug interaction and its investigation is the request of many prctioners and specialists for treatment of polymicrobial infections *(2)*. Several studies confirmed synergism between cephalosporins with aminoglycosides, beta-lactam with ciprofloxacin and penicillins with cephalosporins against resistant Gram negative bacteria *(3-9)*.

Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with plant extracts against different multidrug-resistant (MDR) bacterial pathogens *(10)*.

Nigella sativa (*N. sativa*), commonly known as black seed, belongs to the botanical family of Ranunculaceae which is one of the most usable medicinal herbs nowadays. The antimicrobial activity of *N. sativa* was further established against several species of pathogenic bacteria *(Staphylococcus aureus, Pseudomonas aeruginosa)* and pathogenic yeast (*Candida albicans*). Trials to find benefit drug interactions between *Nigella sativa* and antimicrobials were investigated *(10)*.

Therefore this study was planned to get ride pathogenic MDR enterobacteria especially that resistant to all antimicrobials depending on benefit drug interaction between antimicrobials.

MATERIAL AND METHODS

Bacerial isolation and biotyping

A total of 75 specimens were aseptically collected from visceral organs (heart blood, liver, gall bladder, spleen, lung and oviduct) of diseased broilers, The latter were turned from farms located in Sharkia and Ismailia governorates in period between 2010 till 2011. Bacterial isolates were submitted to biochemical tests using the API 20E system (BioMe´rieux, Marcy l'Etoile, France).

Nigella sativa oil extraction

Ethanolyic extract of *N. sativa* seeds was extracted as previously done *(8)*. Briefly, 100 ml of 70% ethanol were added to 250 g. crushed *N. sativa* seeds, macerated seed allowed to proceed for 12 -24 hr, excess water was removed using rotatory evaporator machine, then the extract was filtrated throughr 45µl filter. The extract was kept at 4°C until used. The total volume of *N. sativa* oil extracted from 250 gm black seeds was 20 ml.

Disc diffusion antimicrobial susceptibility testing

The isolates were subjected to plate antibiotic sensitivity testing according to the National Committee for Clinical Laboratory Standards (NCCLS) *(9)*. Briefly, an inoculums density equal to 0.5 Macfarland of each bacterial isolate was spread onto the surface of Muller Hinton agar, antibiotic discs of colistin, chloramphenicol, ciprofloxacin, cefotaxime, gentamycin, amoxicillin, doxycyclin, streptomycin, erythromycin were fixed onto the medium surface. Medium plates were incubated at 37°C for 18 hrs. Inhibition zones were measured.

Fractional inhibitory concentration (FIC)

Minimal inhibitory concentration (MIC) for each antimicrobial was detected by broth microdilution method as previously described *(9)*. Briefly, serial double fold dilution each antibiotic in 2x Muller hinton infusion. Equal volume of inoculums density 1 x 10^6 C.F.U of each bacterial isolate was added. Tubes were incubated at 37°C for 18 hrs, MICs were recorded. Two antimicrobial combination was done *(10)* according to checkerboard titration by adding 0.5, 1.0, 2.0 MIC of the first drug to 0.5, 1.0, 2.0 MIC of the tested 2^{nd} drug respectively. Inoculum bacterial density 1 x 10^6 C.F.U was added and mixed then incubated for 24 hrs. at 37 °C, MIC of each drug in combination was determined. After calculation of MIC of each antibiotic alone and in combination FIC was calculated as follow:-

FIC index for each drug = MIC of antibiotic in combination / MIC of antibiotic in alone

FIC of the two drugs = (FIC index of 1^{st} drug) + (FIC index of the 2^{nd})

Synergism is defined as FIC $\leq$ 0.5, additive as FIC > 0.5 and $\leq$ 1, indifference as FIC > 1 and $\leq$ 2 and Antagonism is defined as FIC > 2.

RESULTS

Fifty Bacterial isolates were recovered from75 broilers specimens. The isolates were biotyped as *Escherichia coli*, *Salmonella* and *Klebsiella*. All isolates were examined by disc diffusion antimicrobial susceptibility test. Sensitivity to each antimicrobial was tabulated (Table 1). The isolates were frequently sensitive to colistin, cefotaxime and gentamycin, meanwhile all isolates were resistant to erythromycin and *Nigella sativa* oil.

Table 1.Sensitivity percentages of fifty bacterial isolates to each antimicrobial agent

Antimicrobial agent	Sensitivity percentages
Colistin	29/50(58%)
Cefotaxime	23/50(46%)
Gentamicin	23/50(46%)
Chloramphenicol	14/50(28%)
Ciprofloxacin	13/50(26%)
Streptomycin	9/50(18%
Doxycycline	7/50(14%)
Amoxicillin	6/50(12%)
Erythromycin	0/50(0.0%)

The most resistant isolates of E.coli, Salmonella species Klebsiella species, were selected and tabulated (Table 2). These isolates were resistant at least to three antimicrobials. In addition, the isolates with intermediate sensitivity to any of the tested antimicrobial were included. The antimicrobials with intermediate sensitivity against some of tested isolates were colistin, chloramphenicol, ciprofloxacin, cefotaxime, gentamycin, amoxicillin, doxycyclin, streptomycin, and erythromycin,. The isolates were resistant to amoxicillin, doxycyclin, erythromycin and *Nigella seed* oil.

Table 2.Results of disc diffusion of different antimicrobials against bacterial isolates

Isolate type	Isolate code	CTX	AML	CIP	CN	CT	DO	C	St	E	N.S
E.coli	N31	S	-	-	S	IM	-	IM	-	-	-
	N33	S	-	IM	-	IM	-	S	-	-	-
	N34	IM	-	-	IM	S	S	S	-	-	-
Salmonella species	N12	-	-	-	-	IM	-	-	-	-	-
	N45	S	IM	S	S	IM	IM	S	-	-	-
	N46	S	-	S	IM	S	IM	S	-	-	-
	N40	-	-	-	IM	S	-	-	IM	-	-
	N42	-	-	-	-	-	-	-	-	-	-
Klebsiella species	N18	IM	-	-	IM	-	-	-	S	IM	-

AML = amoxicillin F = florfenicol CT = colistin CTX = cefo-taxime DO = doxycycline CN = gentamicin CIP= ciprofloxacin E = erythromycin St = streptomycin N.S = *Nigella sativa* oil - = resistant. S=sensitive IM= intermediate sensitive

MIC of each antimicrobial showing intermediate sensitivity against these was included in Table (3). MIC of *Nigella sativa* oil against most of the tested bacteria was $\leq$ 120 µg /ml. MIC of two combined antimicrobials was examined against bacterial isolates for calculation of FIC of two antimicrobials. Colistin was combined with floriphenicol, ciprofloxacin, doxycyclins, amoxicillin. Gentamycin was combined with cefotaxime, streptomycin and erythromycin, these combin-ations showed synergism except the combination of gentamycin with streptomycin that revealed additive effect against an salmonella isolate (code N42). *Nigella sativa* oil showed no synergism with any one of the antimicrobials with intermediate sensitivity against tested isolates. FIC of two antimicrobials with synergism ranged from 0.25 to 0.50.

Table 3. Fractional inhibitory concentration (FIC) index showing drug interactions of combined antimicrobials against bacterial isolates

Bacterial Isolate	Isolates code No.	Combined Antimicrobials	MIC(µg/ml) of each antimicrobial	MIC of 2 Combined antimicrobials	FIC of two antimicrobials	Drug interaction
E. coli	N.31	CT/F	8 , 16	2/4	0.50	S
	N.33	CT/CIP	8 , 2	2/0.5	0.50	S
	N.34	CN/CTX	8 , 16	1/2	0.25	S
		CT/DO	8, 16	2/4	0.50	S
	N.12	CT/AML	8, 64	2/16	0.50	S
		CT/CIP	8, 8	2/2	0.50	S
	N.45	CT/DO	8, 8	2/2	0.50	S
		CT/AML	8, 16	2/4	0.50	S
Salmonella species	N.46	CN/DO	8, 8	2/2	0.50	S
	N.40	CN/S	8, 16	4/8	1.0	Ad
	N.42	CN/CTX	32, 128	8/32	0.50	S
Klebsiella species	N.18	CN/E	8, 16	1/2	0.25	S

AML = amoxicillin F = florfenicol CT = colistin CTX = cefotaxime DO = doxycycline CN = gentamicin CIP= ciprofloxacin E = erythro-mycin S = streptomycin MIC= minimal inhibitory concentration S=synergism Ad=additive

DISCUSSION

Field prctioners used to combine anti-chemotherapeutics for treatment of resistant bacterial infections, due to frequency of MDR enterobacteria especially in the field broiler industry. Over use of antimicrobials as feed additives ***(14)***, in addition to the treatment of infections without antimicrobial sensitivity testing lead to developed resistance against infections of *Enterobacteriaceae*. Therefore Synergism between current used antimicrobials against field bacterial isolates was examined.

The recovered isolates of *Escherichia coli*, *Salmonella* and *Klebsiella* were frequently sensitive to aminoglycosides (colistin and genta-mycin) and cephalosporin (cefotaxime), meanwhile the isolates were less sensitive to chloramphenicol, streptomycin, doxycycline, amoxicillin (Table 1), comparable results were similarly stimulated ***(15)*** which might be due long and over use of these drugs. Ciprofloxacin could affect only 13/50 (26%) of the examined isolates, meanwhile higher sensitivity was previously recorded which might be due to Ciprofloxacin abuse in Egypt.

Nigella sativa oil produced non clear inhibitory zones in disc diffusion tests on the examined isolates. MIC of the oil against most of the tested bacteria was ≤120 µg /ml, meanwhile lower MIC 6 µg/ml was previously recorded by thymohydroquinone (main component of *Nigella sativa*) against *Escherichia coli, Pseudomonas aeruginosa, Shigella flexneri, Salmonella typhimurium, Salmonella enteritidis* ***(10)***. Resistance of Gram-negative bacteria to *Nigella sativa* oil is due to an effective permeability barrier, comprised of the outer membrane ***(16)***.

An *E.coli* and *Salmonella* isolate (Table 3) were sensitive to colistin combined with amoxicillin, similar synergestic effects between colistin with erythromycin, clindamycin and penicillin against *Escherichia coli, Klebsiella sp., Proteus mirabilis, Serratia marcescens, Enterobacter aerogenes and Pseudomonas aeruginosa* were recorded ***(17)***.

Synergism between gentamycin and cefotaxime against Gram negative bacteria was proved in several works *(3-9)*. synergistic interactions was detected in colistin with floriphenicol, cefotaxime, ciprofloxacin, doxycyclins (Table 3), meanwhile there was no enhanced antibiotic activity after adding ciprofloxacin to colistin *(18)* which might be due to occurrence synergism between antimicrobials against some isolates. *E.coli* isolate (code N12) is the only intermediate sensitive to colistin (Table 2), the isolate became susceptible to several two combined antimicrobials.

Synergistic interactions of colistin with floriphenicol, doxycyclins require further inestgations against more isolates of Gram negative bacteria.

It could be concluded that, some *Escherichia coli,* and *salmonella* with intermediate sensitivity to colistin could be highly sensitive by synergism of colistin with doxycyclins, amoxicillin and ciprofloxacin in addition synergism between gentamycin with cephotax. MDR enterobacteria with resistance to all antimicrobials could be susceptible to several of two combined antimicrobials.

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الملخـــــص العربى

ارتباط بعض المضادات البكتيرية لقضاء على البكتيريا السالبة لصبغ الجرام والمعزولة من بدارى التسمين

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دفعتنا المقاومة الميكروبية المتطورة لاستخدام المضادات الحيوية المرتبطة للتغلب على الميكروبات الجوف معوية المنتشرة فى مجال صناعة الدواجن. تخص هذه الدراسة التفاعلات المفيدة بين المضادات الحيوية فى صناعة بدارى التسمين. تم تجيمع ٧٥ عينة من الأعضاء الداخلية للدواجن المريضة وتم عزل ٥٠ ميكروب جوف معوي وتشمل ٣٢ عينة لميكروب الإى كولي, ١٢ عينة لميكروب السالمونيلا, ٦ عينات لميكروب الكلبسيلا. وبعد إجراء اختبارات الحساسية لوحظ حساسية هذة الميكروبات للكوليستين وللجينتاميسين والسيفوتكسيم وكانت مقاومة للإريثروميسين وزيت حبة البركة. العزلات المتوسطة الحساسية للمضادات الحيوية اختبرت حساسيتها للمضادات الحيوية المرتبطة المفيدة وتم تحديد موشر قياس الارتباط Fractional inhibitory concentration. هناك ارتباط محفز مفيد بين الكوليستين مع كل من الدوكسيسيكلين و الأموكسيسللين و الفلورفنيكول و الإنروفلوكساسين وكذلك لوحظ تفاعلات محفزة synergism للجينتاميسين مع كل من السيفوتكسيم و الدوكسيسيكلين و الإريثروميسين وقد كان موشر قياس الارتباط اقل من ٢/١. لم يوضح زيت حبة البركة تفاعلات مفيدة مع المضادات الحيوية المختبرة. نستخلص من هذا العمل ان الميكروبات المتعددة المقاومة للمضادات الحيوية اصبحت حساسة للمضادات الحيوية المرتبطة