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MOLECULAR DETECTION AND ANTIMICROBIAL ACTIVITY OF *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM* ISOLATED FROM URINARY TRACT INFECTION (UTI) PATIENTS, ANIMALS AND POULTRY

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ABSTRACT

Microbiological and polymerase chain reaction (PCR) methods was used to detect the occurrence of *Enterococcus faecalis* and *Enterococcus faecium* among 95 urine samples collected from patients suffering from urinary tract infection (UTI) recurrence admitted to outpatient clinics of private and governmental hospitals in Sohag city and 102 fecal and cloacal swabs collected from reared animals and / or poultry in some patient´s households. The results revealed that *E. faecalis* and *E. faecium* was detected in 13 (13.7%) patients, among them 11 patients were reared animals and / or poultry in their households, also isolated from 15 (14.7%) out of 102 fecal and cloacal samples of animals and poultry reared in 11 patient´s households. Referring to antimicrobial resistance and presence of *esp* gene among all enterococcal strains, *E. faecalis* and *E. faecium* isolates of both human and animals possess resistance to some antimicrobials with clinically importance for human therapy and *esp* gene was detected in 11 (84.65) out of 13 *E. faecalis* and *E. faecium* strains isolated from UTI patients and in 9 (60%) out of 15 *E. faecalis* and *E. faecium* strains isolated from the reared animals and poultry. This study suggests that reared animals and poultry, miss use of antimicrobials and presence of *esp* gene considered a risk factors for UTI recurrence caused by enterococci in human.

Key words: *E. faecalis, E. faecium*, urinary tract infection, antimicrobial activity

INTRODUCTION

Recent years have witnessed increased interest in enterococci not only because of their ability to cause serious infection like endocarditis, bacteremia, intra-abdominal and urinary tract infection (UTI), but also because of their increasing resistance to many antimicrobial agents (Desai *et al.,* 2001). In humans, as well as in other mammals and birds, enterococci are mainly found in the gastrointestinal tract as commensals but may become opportunistic pathogens in individuals with serious diseases whose immune systems are compromised and in patients who have been hospitalized for prolonged periods or who have received broad-spectrum antimicrobial therapy (Gonzalo *et al.,* 2013). Antibiotics may promote colonization and infection with multidrug resistant enterococci by at least two mechanisms; First, many broad spectrum antibiotics have little or no anti-enterococcal activity, and administration commonly leads to overgrowth of susceptible or resistant enterococci. Second, most antibiotics substantially reduce the normal resistance of the intestinal tract to colonization by exogenous organisms (Miller *et al.,* 2014). Therefore, the selective pressure caused by the intensive use of antimicrobial agents in human and veterinary medicine, contributed to the emergence and wide spread of resistance mechanisms in bacteria of different ecosystems (Lebreton *et al.,* 2013). Furthermore, anti- microbial-resistant enterococci in animals are likely to serve as a reservoir from which resistance genes are transferred to enterococci in humans, either through human consumption of food of animal origin, by direct contact between animals and humans, or via the environment. (Heuer *et al.,* 2006).

Enterococcal surface protein encoded by the chromosomal *esp* associated with increased virulence, colonization and persistence in the urinary tract (Shankar *et al.,* 2001), and biofilm formation which could lead to resistance to environmental stresses, and adhesion to eukaryotic cells of the urinary tract (Borgmann *et al.,* 2004). Therefore, disruption of the *esp* gene impairs the ability of *E. faecalis* to form biofilms (Latasa *et al.,* 2006). In addition, *E. faecium* strains that carry the *esp* gene have higher conjugation rates than strains that do not possess this gene. The aim of this study was to detect the extent of *E. faecalis* and *E. faecium* in UTI patients and their

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reared animals and poultry and detect some virulence factors of enterococci as antimicrobial resistance and *esp* gene presence.

MATERIALS AND METHODS

1- Sampling

- **Human samples**

Between September 2014 and December 2015 a total of 95 urine samples were collected from patients suffering from urinary tract infections admitted to outpatient clinics of private and governmental hospitals in Sohag city, Egypt. Urine samples were immediately transported to the laboratory in the faculty of veterinary medicine, Sohag University for microbiological isolation and identification of *Enterococcus faecalis* and *Enterococcus facium.* All patients were asked about whether they reared animals and / or poultry in their houses, infection recurrence and antimicrobial used.

- **Animals and poultry samples**

52 rectal and 50 cloacal swabs were collected from different animals (18 sheep, 21 goats and 13 cattle) and /or poultry (35 chicken and 15 duck) reared in 11 patient's households whose urine samples give positive results for *Enterococcus faecalis* and *Enterococcus facium*. The number of recteal or cloacal swabs collected from animals or poultry from each household is ranged from 3 to 5 samples for each animal and / or poultry species.

2- Isolation and identification of Enterococci

The samples were inoculated into enterococcus selective broth and incubated at 37ºC for 24 hrs, a loopful from incubated tubes was streaked onto KF Streptococcal agar (TM media, India) and incubated at 37ºC for 48 hrs, red colonies presumptive to be Enterococci were transferred to nutrient agar slants for further identification of *Enterococcus species* according to Morrison *et al.* (1997) and Manero and Blanch, (1999).

3- Molecular detection

- **Genomic DNA extraction**

DNA was extracted from all isolates of enterococci using the QIAamp DNA mini kits (QIAGEN, Germany, No. 69504) in accordance with the manufacturer's instructions.

- **Detection of *E. faecalis* and *E. facium***

Multiplex PCR for detection of D-alanine-D-alanine ligase (*ddl*) of *E. faecalis* and *E. faecium* was done as described by Dutka-Malen *et al.* 1995 with modifications as the following; initial denaturation step at 94°C for 5 min; 30 cycles of amplification (denaturation 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min); and a final extension at 72°C for 5 min. The PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide and transilluminated under UV light. 100 pb DNA ladder (Norgen biotek, Canada) was used as a marker.

- **Detection of *esp* gene**

PCR was carried out to detect *esp* gene among all isolates of *Enterococcus faecalis* and *Enterococcus facium* of UTI patients and their reared animals and poultry as reported by Vankerckhoven *et al.* (2004) but conditions have been optimized for *esp* gene, as initial activation step at 95°C for 15 min, followed by 30 cycles at 94°C for 1 min, annealing at 56 °C for 1 min. and extension at72 °C for 1 min followed by final extension cycle at 72 °C for 10 min.

Sequence of primers used for detection of *E.faecalis, E.faecium* and *esp* gene.

Primer	Sequence	Size of PCR product (bp)	Reference
ddl E. faecalis	5'-ATCAAGTACAGTTAGTCT-3' 5'-ACGATTCAAAGCTAACTG-3'	941	Dutka-Malen *et al.* (1995)
ddl E. faecium	5'-TAGAGACATTGAATATGCC-3' 5'-TCGAATGTGCTACAATC-3'	550	Dutka-Malen *et al.* (1995)
esp	5′ AGATTTCATCTTTGATTCTTGG ′3 5′ AATTGATTCTTTAGCATCTGG ′3	510	Vankerckhoven *et al.* (2004)

4- Antimicrobial sensitivity test

The disk diffusion method of antimicrobial sensitivity test was performed according to Clinical and Laboratory Standards Institute (CLSI) guideline (2009) using antimicrobial discs of (Oxid, UK). The all isolated strains of *E. faecalis* and *E. facium* recovered from UTI patients and their reared animals were tested against Amikacin (AK) 30µg, Amoxycillin / clavulanic acid (AMC) 30 µg, Ciprofloxacin (CIP) 5 µg, Vancomycin (VA) 30 µg, Spiramycin (SP) 100 µg, Gentamicin (CN) 120, Ceftriaxone (CRO) 5µg, Nitrofurantoin (F) 300 µg, Tetracycline (TE) 30 µg and Neomycin (N) 30µg.

RESULTS

Table1: PCR detection of *E. faecalis* and *E. faecium* among UTI patients.

	Examined samples No./95		Animals and birds breeders		Non breeders	
	No	%	No	%	No	%
E. faecalis	8	8.4	7	7.4	1	1.1
E. facium	5	5.3	4	4.2	1	1.1
Total	13	13.7	11	11.6	2	2.1

Table 2: PCR detection of *E.faecalis* and *E.faecium* among animals and poultry in patient's households.

	Examined samples No./102		Sheep No./18		Goat No./21		Cattle No./13		Chicken No./35		Duck No./15	
	No	%	No	%	No	%	No	%	No	%	No	%
E. faecalis	7	6.9	1	5.6	1	4.8	2	15.4	3	8.6	1	6.7
E. facium	8	7.8	2	11.1	2	9.5	2	15.4	1	2.9	1	6.7
Total	15	14.7	3	16.7	3	14.3	4	30.8	4	11.4	2	13.3

Table 3: Antimicrobial profile of *E.faecalis* and *E.faecium* isolated from UTI patients.

Antimicrobial	***E.faecalis* No./8**						***E.faecium* No./5**						**Total No./13**					
	S		**I**		**R**		**S**		**I**		**R**		**S**		**I**		**R**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
AK	1	12.5	1	12.5	6	75	1	20	1	20	3	60	2	15.4	2	15.4	9	69.2
AMC	6	75	1	12.5	1	12.5	4	80	0	0	1	20	10	76.9	1	7.7	2	15.4
CIP	5	62.5	1	12.5	2	25	3	60	1	20	1	20	8	61.5	2	15.4	3	23.1
VA	6	75	1	12.5	1	12.5	1	20	2	40	2	40	7	53.9	3	23.1	3	23.1
SP	1	12.5	4	50	3	37.5	2	40	2	40	1	20	3	23.1	6	46.2	4	30.8
CN	0	0	2	25	6	75	0	0	2	40	3	60	0	0	4	30.8	9	69.2
CRO	2	25	2	25	4	50	0	0	4	80	1	20	2	15.4	6	46.2	5	38.5
F	5	62.5	1	12.5	2	25	2	40	1	20	2	40	7	53.9	2	15.4	4	30.8
TE	0	0	2	25	6	75	0	0	2	40	3	60	0	0	4	30.8	9	69.2
N	6	75	1	12.5	1	12.5	4	80	1	20	0	0	10	76.9	2	15.4	1	7.7

Amikacin (AK), Amoxycillin/ clavulanic acid (AMC), Ciprofloxacin (CIP, Vancomycin (VA), Spiramycin (SP), Gentamicin (CN), Ceftriaxone (CRO), Nitrofurantoin (F), Tetracycline (TE)، Neomycin (N)

Table4: Antimicrobial profile of *E.faecalis* and *E.faecium* isolated from animals and poultry.

Antimicrobial	*E.faecalis* No./7						*E.faecium* No./8						Total No./15					
	S		I		R		S		I		R		S		I		R	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
AK	3	42.9	1	14.3	3	42.9	4	50	1	12.5	3	37.5	7	46.7	2	13.3	6	40
AMC	5	71.4	1	14.3	1	14.3	7	87.5	1	12.5	0	0	12	80	2	13.3	1	6.7
CIP	4	57.1	1	14.3	2	28.6	5	62.5	2	25	1	12.5	9	60	3	20	3	20
VA	0	0	2	28.6	5	71.4	3	37.5	2	25	3	37.5	3	20	4	26.7	8	53.3
SP	1	14.3	2	28.6	4	57.1	4	50	2	25	2	25	5	33.3	4	26.7	6	40
CN	0	0	1	14.3	6	85.7	0	0	0	0	8	100	0	0	1	6.7	14	93.3
CRO	5	71.4	0	0	2	28.6	4	50	2	25	2	25	9	60	2	13.3	4	26.7
F	6	85.7	0	0	1	14.3	6	75	1	12.5	1	12.5	12	80	1	6.7	2	13.3
TE	0	0	1	14.3	6	85.7	0	0	0	0	8	100	0	0	1	6.7	14	93.3
N	3	42.9	2	28.6	2	28.6	3	37.5	3	37.5	2	25	6	40	5	33.3	4	26.7

Table 5: Frequency distribution of *esp* gene among *E.faecalis* and *E.faecium* isolated from human, animals and poultry.

	Human						Animals and poultry					
	E.faecalis No./8		*E.faecium* No./5		Total No./13		*E.faecalis* No./7		*E.faecium* No./8		Total No./15	
	No	%	No	%	No	%	No	%	No	%	No	%
***esp* gene**	7	87.5	4	80	11	84.6	6	85.7	3	37.5	9	60

Table 6: Frequency distribution of *esp* gene among antimicrobial resistant strains of *E.faecalis* and *E.faecium* isolated from human, animals and poultry.

Antimicrobial	Human						Animals and poultry					
	E.faecalis			*E.faecium*			*E.faecalis*			*E.faecium*		
	Resistant isolates	*esp* gene	%	Resistant isolates	*esp* gene	%	Resistant isolates	*esp* gene	%	Resistant isolates	*esp* gene	%
AK	6	6	100	3	2	66.7	3	3	100	3	2	66.7
AMC	1	1	100	1	1	100	1	1	100	0	0	0
CIP	2	1	50	1	1	100	2	2	100	1	1	100
VA	1	0	0	2	2	100	5	5	100	3	1	33.3
SP	3	2	66.7	1	1	100	4	4	100	2	1	50
CN	6	5	83.3	3	3	100	6	5	83.3	8	3	37.5
CRO	4	3	75	1	1	100	2	2	100	2	2	100
F	2	1	50	2	2	100	1	1	100	1	1	100
TE	6	5	83.3	3	3	100	6	6	100	8	3	37.5
N	1	0	0	0	0	0	2	2	100	2	2	100

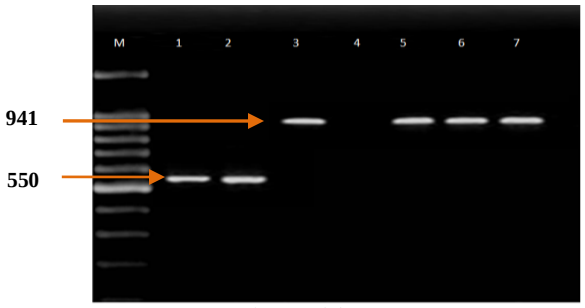

Figure (1)

Figure (1): Agarose gel electrophoresis of multiplex PCR amplification products using specific *ddl E. faecalis* primer of *E.faecalis* and *ddl E. faecium* primer of *E.faecium*. Lane M: 100 bp ladder as molecular DNA marker, lane1and lane 2: positive *E. faecium*, lane 3, lane 5, lane 6 and lane 7: positive *E.faecalis*, lane 4: Negative

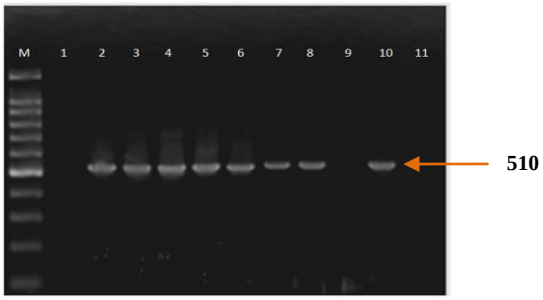

Figure (2)

Figure (2): Agarose gel electrophoresis of PCR amplification products using specific *esp* gene of *E.faecalis* and *E. faecium*. Lane M: 100 bp ladder as molecular DNA marker, lane 2, lane 3, lane 4, lane 5, lane 6, lane 7, lane 8 and lane 10: positive *esp* gene, lane 1, lane 9 and lane 11: Negative *esp* gene.

DISCUSSION

Microbiological and PCR analysis of 95 urine samples collected from patients suffering from UTI recurrence revealed detection of *E.faecalis* and *E.faecium* in 13 (13.7%) patients; *E.faecalis* was slightly higher than *E.faecium* with percentage of 8.4% and 5.3% respectively (Table1). In comparison with Gonzalo *et al.* (2013) and Sharifi *et al.* (2013) who recorded higher percentage of infection; enterococci in the present study not reflect the true incidence of infection but it definitely suggest the increased frequency of their isolation from UTI patients and this variation may be due to the difference in the study population.

The majority of patients infected with enterococci in the present study reared animals and / or poultry in their households (Table1) which enhance the probability of zoonotic transmission of enterococci from animals to human. From the results in Table 2 it is clear that *E. faecalis* and *E. faecium* were isolated by microbiological and PCR methods from 15 (14.7%) out of 102 fecal and cloacal samples of reared animals and poultry in 11 patient's households with highest percentage in cattle (30.8%), followed by sheep (16.7%), goat (14.3%), ducks (13.3%) and chicken (11.4%), therefore close contact with animals may play a role in enterococcal infection but exposure to infection outside the household environment cannot be excluded, so further epidemiological studies are needed to investigate the risk factors of enterococcal infection.

Arias *et al.* (2010) illustrated that enterococci possess intrinsic or acquired resistance to several antimicrobials, such as glycopeptides, b-lactams, and fluoroquinolones, and can exhibit high levels of resistance to aminoglycosides, leading to drastically reduced therapeutic options for patients infected with enterococci, owing to the lack of antimicrobial policy and the massive use of antibiotics both in the human health care system and agriculture. Furthermore, several antimicrobial agents that are used in animals belong to the same class of antimicrobial agents of clinically important for human therapy, thus antimicrobial resistant enterococci may frequently be transferred from animals to humans either by ingestion of contaminated food or from the environment. (Heuer *et al.*, 2006).

Results in Table 3 revealed that *E. faecalis* and *E. faecium* isolates recovered from UTI patients exhibited higher resistance to the most common antimicrobials such as amikacin, gentamicin and tetracycline with percentage of (69.2%) followed by Ceftriaxone (38.5%), spiramicin and nitrofurantoin (30.8%), vancomycin and ciprofloxacin (23.1%), amoxycillin/ clavulanic acid (15.4%), while lower resistance to neomycin (7.7%) associated with lower use of this antibiotic in human therapy. Furthermore, 10 (76.9%) out of 13 *E. faecalis* and *E. faecium* strains isolated from clinical human samples were multidrug resistant to at least three or more unrelated antimicrobials led to recurrence of infection among the infected patients, this results goes parallel to Sharifi *et al.* (2013). Table 4 showed that *E. faecalis* and *E. faecium* strains isolated form fecal and cloacal samples of reared animals and poultry in 11 patient's households were frequently resistance to the similar antimicrobials which used for human medicine with highest proportion for gentamicin and tetracycline (93.3%) followed by vancomycin (53.3%), amikacin and spiramicin (40%), Ceftriaxone and neomycin (26.7%), ciprofloxacin (20%), nitrofurantoin (13.3%) and amoxycillin/ clavulanic acid (6.7%). In addition, multidrug resistant to at least three or more unrelated antimicrobials were detected in 13 (86.7%) out of 15

strains of *E. faecalis* and *E. faecium* recovered from reared animals and poultry in 11 patient's households. Therefore, concerns about public health issues evoked by exchanging antimicrobial-resistant and virulent enterococci between animals and human beings have increased (Ghosh *et al.,* 2011).

Harada *et al.* (2005) elucidate that although *esp* gene in enterococci was initially found only in hospital derived human isolates of enterocicci, the *esp* gene was later observed in human and animal isolates in community settings. Results in Table 5 showed that 11 (84.6%) out of 13 strains of *E. faecalis* and *E. faecium* isolated from UTI patients possess *esp* gene, this result is lower than that recorded by Vankerckhoven *et al.,* 2004 and Sharifi *et al.,* 2013 and higher than that reported by Strateva *et al.,* 2016. In contrast, Shanker *et al.,* 1999 who revealed failure of detection of *esp* gene in *E. faecium*. In addition, 9 (60%) out of 15 strains of *E. faecalis* and *E. faecium* isolated from reared animals and poultry in patient´s household were harbor *esp* gene, this result is higher than the results obtained by Klibi *et al.,* 2014. In contrast, Kown *et al.,* 2012 and S˘eputiene *et al.,* 2012 who detect *esp* gene in *E. faecalis* only. The presence of multidrug resistance among our study may be related to the higher incidence of *esp* gene in the resistant isolates of *E.faecalis* and *E.faecium* in both UTI patients and their reared animals and poultry (Table 6), since the presence of this gene promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance (Moreno *et al.,* 2006).

CONCLUSION

The emergence of antimicrobial resistance enterococci among UTI patients and their reared animals and poultry emphasizes the need to investigate their ecology, epidemiology and virulence.

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الكشف الجزيئى والمقاومة الميكروبية للانتيروكوكاى فيكالز والانتيروكوكاى فاكيم المعزولة من مرضى المسالك البولية والحيوانات والدواجن

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تهدف هذه الدراسة الى معرفة مدى تواجد ميكروب الانتيروكوكاى فيكالز والانتيروكوكاى فاكيم ومدى مقاومتها للمضادات الميكروبية الشائعة الاستخدام وكذلك تواجد جين الضراوة (*esp*) فى ٩٥ عينة بول مجمعة من المرضى المترددين على العيادات الخارجية للمستشفيات الخاصة والحكومية بمدينة سوهاج والذين يعانون من تكرار عدوى المسالك البولية ، كذلك من ١٠٢ عينة براز وزرق من الحيوانات والدواجن المقتناة داخل منازل بعض المرضى ، وقد تبين من فحص عينات البول وجود ميكروب الانتيروكوكاى فيكالز والانتيروكوكاى فاكيم فى ١٣ (١٣.٧%) مريض ، أيضا فى ١٥ (١٤.٧%) عينة براز و زرق من الحيوانات والدواجن المقتناة فى منازل المرضى. وأوضحت النتائج أن الانتيروكوكاى فيكالز والانتيروكوكاى فاكيم المعزولة من عينات المرضى وكذلك من حيواناتهم مقاومة للمضادات الميكروبية الشائعة الاستخدام فى علاج المرضى ووجود جين الضراوة (*esp*) في ١١ (٨٤.٦%) من الانتيروكوكاى فيكالز والانتيروكوكاى فاكيم المعزولة من عينات المرضى وكذلك فى ٩(٦٠%) من الانتيروكوكاى فيكالز والانتيروكوكاى فاكيم المعزولة من الحيوانات والدواجن المقتناة فى منازل المرضى. وتعزى هذه الدراسة الى أن تربية الحيوانات والدواجن وسوء استخدام المضادات الحيوية ووجود جين الضراوة (*esp*) عوامل خطورة لتكرار حدوث عدوى المسالك البولية للمرضى.