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Antimicrobial Resistance and biofilm formation of Enterococcus spp isolated from human and pet animals

Hassan Falah Mohammed & Nagham Mohammed Al-Gburi
Zoonotic Diseases Unit, Coll. Vet. Med. Uni. Bagh. Iraq

Corresponding Author: Hassan Falah Mohammed; E-mail: hasan.falaah1204e@covm.uobaghdad.edu.iq

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ABSTRACT

Seventy three enterococci previously isolated from human,dogs,and cats, by culturing, microscopic characteristics, Vitek 2 compact system, and molecular methods using species-specific genes which *E.gallinarium* , *E.faecalis*,and *E.faecium*. The enterococci isolates were Gram-positive cocci, catalase, and gelatinase negative, all isolates were grown on 6.5% NaCl, and hydrolysis esculin,most of the isolates were γ hemolysis 46.38%, 21.92% were β-hemolysis, and 13.69% were α-hemolysis. Antimicrobial resistance profile indicated that human enterococci isolates were 100% resistant to Ceftriaxone, Clindamycin, and Gentamycin, while 93.54% resistant to Erythromycin, Azithromycin and Doxycycline, MDR were 100% with high-risk of the isolates, Dogs isolates were resistant 100% against Cefitrixone, Clindamycin, and Gentamycin, 75% to Azithromycin and Doxycycline, MDR were 100% with high risk, cats isolates were resistant to Cefitrixone, Erythromycin, Azithromycin, Doxycycline, and Gentamycin. Most of the isolates 74% could form biofilm using congo-red method and crystal violate assay methods, 77.77% of them were weak, and 22.22% were moderate. *E.gallinarum* were recorded 27.7% of them were moderate and 72.3% were weakly formed, *E.faecium* were 75% weak,and 25% were moderate. Whereas, *E.faecalis* 85.71% were weak and 14.28% were moderate. The study conclusion that *enterococci* from human and their pet animals were high antibiotic resistance, MDR and high risk,in addition producing biofilm with different category, so uman, dogs and cats might be considering the reservoirs of resistance and MDR *enterococci* to human, other animals and environment.

1. Introduction

Enterococci species (spp.) are naturally found in the gastrointestinal tract (GIT), oral cavity of both human and animals causing several infections including bacteremia, meningitis, endocarditis, intra-abdominal infections, wound infections, urinary tract infections (UTI), atherosclerosis and play a critical role in the riskiness or development of periodontitis, particularly in a suitable mouth environment (**Comerlato et al., 2020; Mendes *et al.,* 2020; Xiong *et al.,* 2021; H Elaywe, 2007**).

Recurrent and continues antimicrobial resistance (AMR) of opportunistic microorganisms is one of the serious public health risk, enterococci spp are often intrinsic resistant to antibiotics class routinly used in treatment such as Cephalosporins,Macrolids,Sulfonamides, β lactams, and Aminoglycosides. Furthermore, this genus can acquire resistant to several antibiotics horizontally by transfere genes, in the same time are able to transfer of AMR genes to other microorganisms, In addition, prevalence of MDR among enterococci spp has been reported worldwide, and is account as a major problems on public health (**Ali et al.,2018; Al-Shammary, 2019;Castano-Arriba *et al.*,2020**).

Enterococci spp has the ability to form biofilm,which contribute to their pathogencity and resistance to antibiotics, in a mature biofilm, the bacteria can resistance to antibiotics 1-1000 fold more than planktonic cells, several virulence factors participate in biofilm formation of enterococci , including adhesion factors(extracellular matrix protiens) linked to receptors on the surface of eukaryotic cells or adhesion to abiotic surfaces (**Hashem *et al.*,2017; Ghabraei *et al.*,2018 Mustafa et al., 2021**), and there is a correlation between biofilm production and both MDR and MAR index (**El-Zamkan *et al.*,2021**; **Oliva *et al.*,2021; Alzahrani *et al.*,2022**). Human can infected with enterococci especially that AMR by contaminated urin,feces,saliva; ingestion contaminated foods ,and via contact with infected human or animals, pet owners are usually in constant contact with these animals, in as well, the emergence of antimicrobial-resistant or virulent microorganisms in these pet animals act as a reservoir of AMR for humans, studies indicated that the mouth cavity of these animals could be an important

source of AMR, virulent and biofilm-forming enterococci strains and thus considered a major public health concern(**Bhardwaj *et al.*, 2020;Kim and Koo,2020; Atiyah & Alkhafaji, 2020; H Elaywe, 2007)**
The aim of this study was to investigate the characteristic and prevalence of antimicrobial susceptibility and biofilm production of *enterococci* spp isolated from human,dogs, and cats.

3. Methodology

Enterococci isolates

During the period from 2.NOV.2021-28.April.2022, clinical enterococci spp. (73) were isolated from oral, nasal and feces of dogs and cats when visiting the Baghdad veterinary teaching hospital this including *E.faecalis* (18), *E.faecium* (4) and *E.gallinarium* (20), and from owners of these animals, oral and nasal enterococci isolates including *E.faecium* (2) and *E.gallinarium* (20) and *E. faecalis* (9) from stool samples of other human, partial sequencing of the16s RNA were done on eight of these isolates and deposited in GenBank with the accession numbers ID:ON645240.1, ID:ON645241.1, ID:ON645242.1, ID:ON645243.1, ID:ON645244.1, ID:ON645245.1, ID:ON645246.1, and ID:ON645247.1. (**Mohammed and Al-Gburi., 2022;Mohammed(unpublished**).

Hemolysine activity ,Gelatinase and Nacl 6.5% Tolarence

The *enterococci* isolates were tested for ability to heamolysis of red blood cells by streaking of enterococci colonies on blood agar and incubation at 37C◦ for 24 hrs, developmed a halo zone around the colonies indicated positive production of hemolysin production, and the type of hemolysis were reported (**Coque *et al*.,1995**). Gelatin liquefaction test The gelatin ager was stabbing with heavy bacterial isolate nearly to the bottom of the tube. After the incubation at 37Cº for 18–24 hrs the tubes were placed in the refrigerator at 4ºC for 30 minutes when the resolidified indicated negative gelatin liquefaction . And the isolates were added to TSB contianing 6.5% Nacl and incubated in 37c° for 24-72 hrs, then subcultured on TSA and incubated for 24hrs in 37c° (**Procop *et al.*,2017**).

Antimicrobial Resistance, Multidrug Resistance (MDR) and Multidrug Antibiotic Resistance (MAR) Index

Antimicrobial resistance was done according to (**CLSI, 2020; EUCAST, 2021**). The bacterial suspensions 1-1.5x10^8 CFU/ml were prepared by picked seven to eight pure colonies of each isolated bacteria and suspended in sterile test tube containg four ml of sterile normal saline using (0.5 McFarland tube). A sterile swab was insert into bacterial suspension, then carefully pulled out, and spreading evenly over the surface on Mueller-Hinton agar, wait for 16 minutes. After that, the antimicrobial discs were placed on the agar by utilizing sterile forceps and pressed onto the medium. Then the plates were incubated at 37c for 24hrs, after incubation, the zone of inhibition around the antimicrobial disc was measured with millimeter(mm) unit with a metric ruler, and exhibited the isolates were interpreted as Sensitive (S) or Resistant (R) to specific antimicrobial, the antibiotics included: Amoxicillin (25µg), Azithromycin (15µg), Ceftriaxone (30µg), Chloramphenicol (10µg), Ciprofloxacin (10 µg), Clindamycin (2µg), Doxycycline (40µg), Erythromycin (15µg), Gentamycin (10µg), and Vancomycin (30µg). MDR was characterized as the bacteria resistance to three or more to three different classes of antimicrobial drugs (**Magiorakos *et al.*, 2012**). The MARI index for isolates were using the formula A/B: No. of antimicrobials resistant to which an isolate was resistant (A)/ the total No. of antimicrobials to which the isolate resistant (B)

Biofilm Formation

Enterococci isolates were streaking on congo red medium and incubated at 37C° for 24hrs. Developing black dry crystalline colonies indicated biofilm formation, dark colonies without dry crystalline colonies considered intermediate biofilm forming. While, pink colonies indicated non biofilm formation (**Freeman *et al*., 1989**). Crystal Violet biofilm was done according to **Stepanović *et al*. (2007)** with some modification. Enterococci isolate suspension 1x10 using McFarland tube (0.5) was prepared in normal saline normal saline. Then, from this suspension prepared 1x10^6 in trypticase soy broth containg glugose and sucrose .200µl of the suspension was distributed in three wells using 96 well microtiter plate, negative control well was only trypticase soy broth and incubated at 37c for 48hrs. After complete the incubation, the non adhering cells (planktonic) were removed. The microtiter plate was washed twice times by dipping in DW and shacked to remove the excess water, and left to dry in air then in hot oven 45c. the wells were stained with crystal violet 1% for 30 minutes, then the stain was removed by rinse with water and allowed to air dry, the stain bound to the adherent cells were resolublised with 200µl of 95% ethanol. The optical density (OD) was measured using plate reader at 570nm.

The results of biofilm formation using the following calculate, categorization done by the following calculate of OD cut off (ODc) which indicated the isolates as biofilm forming or not
One –ODC = average OD of negative control + (3x standard deviation of negative control)
Two - $OD_{isolates}$ = average OD of isolate – ODc
Biofilm formation were catigorised were categorized as follows:
Strong biofilm= 4 × ODc < OD
Moderate biofilm= 2 × ODc < OD ≤ × ODc
Weak biofilm= ODc < OD ≤ 2 × ODc
no biofilm= OD ≤ ODc

4. Results and Discussion

Characteristic of the isolates

The results showed that 47/73(46.38%) of the isolates were non produced heamolysis (γ-hemolysis), 26/73 (35.61%) were hemolysis; 10/73(13.69%) of isolates were α hemolysis, and 16/73(21.92%) were β-hemolysis. All *E. faecium* 6/6(100%) and *E. gallinarum* 40/40(100%) were γ-hemolysis, while *E. faecalis* strains were 16/27 (59.2%) β heamolysis, 10/27(37.1%) were α hemolysis, and 1/27(3.7%) were γ heamolysis. All strains were negative gelatinase activity. And all the strains were 73/73(100%) Nacl 6.5% tolarence after 24hrs.

Hemolysin is one of enterococcal virulence factors that is play an essential role in severity of infections. In current study, enterococci spp were produced hemolysis 35.61% were hemolysis,13.69% of isolates were α hemolysis and 21.92% were β-hemolysis, and the majorty of isolates were were non hemolysis 46.38%, a of **Gilmor and Hancock (2002)**, found that the hemolytic activity of *enterococci* (β and α) were range between (45-60%), the results were accordance with results found that the most enterococci were γ hemolysis followed by **β** and α hemolysis. In contrast, enterococci isolates were 25% β and α-hemolysis 22.2% (**Al-Rammahi *et al*.,2020**). *E. faecium and E. gallinarum* were found γ-hemolysis, while *E. faecalis* strains were 59.2% β hemolysis, 37.1% α, and 3.7% γ heamolysis. These results partially or totally in line with other researchers, **Salah *et al*.(2008)** reported *E. faecalis* were 25% produced haemolysin, and *E. faecalis* strains found 21.3% β-hemolysin higher than for *E. faecium* 8.3% (**Thanoon, *et al.,* 2019**). In a study, *E. faecalis* 33.3% *E. faecium* and *E.gallinarum* 0% were β hemolysis, *E. faecalis* 22.2%, *E. faecium* 28.6%, *E.gallinarum* 0% were α-hemolysis and *E. faecalis* 44.4%, *E. faecium* 71.4% and *E.gallinarum* 100% were γ-haemolysis (**Al-Rammahi *et al*.,2020**). Studies investigated the majority of intestinal bacteria were β hemolysis and there is different in commensals and clinical strains in their hemolytic activity, found higher hemolytic activity in commensal strains than in clinical isolates ranges between (54.1-65.96%), and attributed the rising rate of production of hemolysin to the constant presence of the bacteria (**Buhnik-Rosenblau *et al*., 2013 and Anderson *et al*., 2016).**

Gelatinase activity were negative in all spp isolates in this study, differences in the rates of gelatinase production were detected, *E.faecalis* isolates were the most capable of producing this enzyme, followed by *E.faecium*, and *E.gallinarum* isolates were lack their ability to gelatinase production (**Gilmor and Hancock 2002; Gulhan *et al.*, 2006 and Al-Azzawi., 2008**), gelatinase production was detected in 66.5% of enterococci isolates; *E. faecalis* 77.8%, *E. faecium* 56.1% and *E. gallinarum* 0% (**Al-Rammahi *et al*., 2020**). **Anderson *et al*. (2016)** showed gelatinase activity only 3.3% in commensal strains than the clinical isolates 46.6%.

Enterococci spp were 100% tolerant to 6.5% Nacl, and give positive growth when subcultured on medium, the turbitidy appear after 24hrs in *E. faecalis*, 36hrs in *E. faecium* and 27hrs in *E. gallinarum* that accordance with other studies **(Gomes et *al.*, 2008;Mozini *et al*., 2009; Zhang, *et al.,* 2017).**

Antimicrobial Susceptibility, MDR pattern and MARI

In human, the antimicrobial profile for isolated enterococci (31 isolates), all isolates were resistance 100% to Ceftriaxone, Clindamycin and Gentamycin, 93.54% resistant to Erythromycin, Azithromycin and Doxycycline. In contrast were 100% sensitive to Vancomycin, Amoxicillin, Ciprofloxacin and Chloramphenicol. *E.gallinarum* and *E.faecalis* were resistant 100% to six antibiotics which are Ceftriaxone, Erythromycin, Azithromycin, Clindamycin, Doxycycline and Gentamycin. *E.faecium* were resistance 100% to three antibiotics which are Ceftriaxone, Clindamycin, and Gentamycin Table (1).

Table 1 Antimicrobial Susceptibility Test of Human isolates

Antibiotics	*E.gallinarum* (20)		*E.faecium* (2)		*E.faecalis* (9)		Total	
	S %	R %	S %	R %	S %	R %	S %	R %
Ceftriaxone	0	100	0	100	0	100	0	100
Erythromycin	0	100	100	0	0	100	6.45	93.54

Azithromycin	0	100	100	0	0	100	6.45	93.54
Clindamycin	0	100	0	100	0	100	0	100
Doxycycline	0	100	100	0	0	100	6.45	93.54
Vancomycin	100	0	100	0	100	0	100	0
Amoxicillin	100	0	100	0	100	0	100	0
Ciprofloxacin	100	0	100	0	100	0	100	0
Gentamycin	0	100	0	100	0	100	0	100
Chloramphenicol	100	0	100	0	100	0	100	0

The present study showed none of the isolated were sensitive 100% to all antimicrobial agents tested. and the prevalence of MDR was 100% resistance and high resistance was to six antibacterial drug by 29/31((93.54%), *E.faecium* showed MDR 100% to three antibacterial drugs,while *E.gallinarum* and *E.faecalis* MDR showed 100% to six antibacterial drugs. The MARI of *E.gallinarum* and *E.faecalis* were 0.6 and *E.faecium* was 0.3 **Table (2)**.

Table 2 MDR and MARI patterns of Enterococcus spp

Enterococci spp	R0*	R3**	R6**	MDR	A.R	MARI
*E.gallinarum (*20)	0(0)	0(0)	20/20(100)	20/20(100)	6	0.6
*E.faecium (*2)	0(0)	2/2(100)	0(0)	2/2(100)	3	0.3
*E.faecalis (*9)	0(0)	0(0)	9/9(100)	9/9(100)	6	0.6
Total	0(0)	2/31(6.45)	29/31(93.54)	31/31(100)		

R0: sensitive for all classes of antimicrobial; **number of antimicrobial resistance of Enterococcus spp; A. R: number of antimicrobial resistance

The present data showed high resistance of isolates range from 93.54-100% to six antibiotics (Ceftrixone, Clindamycin, Gentamycin, Erythromycin, Azithromycin and Doxycycline, overall enterococci were absolute sensitive to Vancomycin, Amoxicillin, Ciprofloxacin and Chloramphenicol). The isolates were MDR and high risk, MRI were ranged from 0.2 to 0.6,when value lower than 0.2 is mean a low risk, and more than 0.2 mean high risk (**Magiorakos *et al*.,2012**). The present results are large concurring with other studies, *E. faecalis* isolated from nasal and perirectal were resistant to Ciprofloxacin 81.8%,69%; Erythromycin 90.9%,79.3%; Gentamicin (72.7%,79.3); Vancomycin (18.2%,10.3%) respectively. Whilest those *E. faecium* isolated from the same sites were resistant to Ciprofloxacin (66.7%,96.3%);Erythromycin (88.9%,96.3%);Gentamicin (79.3%,92.6%);Vancomycin(5.6%,11.1%) respectively(**Yameen *et al*.,2013**).As well as, 95.23% of *enterococci* isolates were resistant to amoxicillin, *E.faecalis* isolates were resistance 100% against Erythromycin this agree with (**AL-Gheethi *et al*. in 2013; StępieńPyśniak *et al*., 2016**). *E.faecalis* and *E.faecium* isolated from patients were resistant against Gentamicin 73.58%, 96.20% to ciprofloxacin, 50.94% to vancomycin, 98.11%) to clindamycin, 98.11% to erythromycin (**Ullah *et al*.,2015**). *Enterococci* isolated were resistance to 70.8% to Chloramphenicol and Ciprofloxacin , Doxycycline 68%, Erythromycin 64% and Vancomycin 41.7%, MDRwere observed in 75% of the isolates(**Yilema *et al*.,2017**). *E.faecalis* isolated from healthy human stool samples were resistant to Vancomycin 10.4%, Erythromycin and Gentamicin 96.9%,and Ceftriaxone 100%,and *E.faecium* was resistance to Vancomycin 16.7%, Erythromycin 88.9%, Gentamicin 77.8% and Ceftriaxone 100%;with high MDR of both spp(**Adesida *et al*.,2017**).

Enterococci spp (*E.faecalis* and *E.faecium)* from clinical samples including stool were resistant to Vancomycin 13% ,Erythromycin 91%, and ciprofloxacin 87% (**Muslem *et al*.,2021**), *E.faecalis* were resistant to Doxycycline 78.9% , Ciprofloxacin 26.6%; *E.faecium* isolates were resistance against Doxycycline 38.3%, Ciprofloxacin 9.7% . while both *spp* were 100% sensitive to Vancomycin (**Xiong *et al*.,2021**). *E.faecalis* from fecal human were resistance to Erythromycin 16.7%, Ciprofloxacin 25%, *E.faecium* were resistance to Erythromycin 10.3%, Ciprofloxacin 25.5%,different with this results and agree with that the isolates were 100% sensitive to Vancomycin (**Iseppi *et al*.,2020**). Current results agree partially with that results of (**Khdir and Mustafa,2020)** who found *E.faecalis* isolated from different clinical source showed 100% were resistant to Amoxicillin and Erythromycin, 81.81% and 68.18% of isolates were resistant to Gentamycin and Azithromycin respectively,and were susceptible for doxycycline 100%. *E.gallinarum* isolated from wound in human was resistant to Erythromycin, Vancomycin, Ciprofloxacin and sensitive to Chloramphenicol (**Eshaghi *et al*.,2015**), while *E.gallinarum* isolated from menngitis was resistance to Erythromycin and Clindamycin while, sensitive to Gentamicin (**Zhao *et al*.2018**).

Dogs isolates were 100% against Cefitrixone, Clindamycin and Gentamycin, 75% against Azithromycin and Doxycycline.while against Erythromycin was 62.5%.. *E.gallinarum* was resistant to Erythromycin 81.8%,against Azithromycin and Doxycycline was 100%. *E.feacium* was resistant to three antibiotic; Cefitrixone, Clindamycin and Gentamycin. *E.faecalis* absolute 100% resistant to Erythromycin , Azithromycin and Doxycycline. and the prevalence of MDR was 100% resistance and high resistance was to 6 antibacterial drug by 100%. The MARI of *E.gallinarum* and *E.faecalis* were 0.6 and *E.fasium* was 0.3 Table(3) and Table(4).

Table 3 Antimicrobial Susceptibility of Dogs isolates

Antibiotic	*E.gallinarum* (11)		*E.faecium* (4)		*E.faecalis* (1)		Total	
	S %	R %	S %	R %	S %	R %	S %	R %
Cefitrixone	0	100	0	100	0	100	0	100
Erythromycin	18.2	81.8	100	0	0	100	37.5	62.5
Azithromycin	0	100	100	0	0	100	25	75
Clyndamycin	0	100	0	100	0	100	0	100
Doxycycline	0	100	100	0	0	100	25	75
Vancomycin	100	0	100	0	100	0	100	0
Amoxicillin	100	0	100	0	100	0	100	0
Ciprofloxacin	100	0	100	0	100	0	100	0
Gentamycin	0	100	0	100	0	100	0	100
Chloramphenicol	100	0	100	0	100	0	100	0

Table 4 MDR and MARI patterns of Dogs isolates

Enterococcus spp	R3	R4	R5	R6	MDR	MARI
E.gallinarum (11)	0(0)	2/11(18.2)	9/11(81.8)	0(0)	11(100)	0.4,0.5
E.feacium (4)	4/4(100)	0(0)	0(0)	0(0)	4(100)	0.3
E.faecalis (1)	0(0)	0(0)	0(0)	1/1(100)	1(100)	0.6
Total	4/16(25)	2/16(12.5)	9/16(56.25)	1/16(6.25)	16(100)	

In cats , all isolates were absolute resistance (100%) to Cefitrixone, Erythromycin, Azithromycin, Doxycycline and Gentamycin, while the all isolates were absolute (100%) sensitive to others. and the prevalence of MDR was 100% resistance and all isolates were resistance to six antibacterial drug by 100%. The MARI of *E.gallinarum* and *E.faecalis* were 0.6 Table (5) and Table (6).

Table 5 Antimicrobial Susceptibility of Dogs isolates

Antibiotics	*E.gallinarum* (9)		*E.faecalis* (17)		Total	
	S %	R %	S %	R %	S %	R %
Cefitrixone	0	100	0	100	0	100
Erythromycin	0	100	0	100	0	100
Azithromycin	0	100	0	100	0	100
Clyndamycin	0	100	0	100	0	100
Doxycycline	0	100	0	100	0	100
Vancomycin	100	0	100	0	100	0
Amoxicillin	100	0	100	0	100	0
Ciprofloxacin	100	0	100	0	100	0
Gentamycin	0	100	0	100	0	100
Chloramphenicol	100	0	100	0	100	0

Table 6 MDR and MARI patterns of Cats isolates

Enterococcus spp	R6	MDR	MARI
E.gallinarum (9)	9/9(100)	9(100)	0.6
E.faecalis (17)	17/17(100)	17(100)	0.6
Total	26/26(100)	26(100)	

Present results agree or disagree with other researches, several reports comparable to current study that *E. faecalis* isolated from dogs and cats were resistant respectively to chiolrmphencol(85%,10); Erythromycin(51%,27%);Gentamicin(79%,21%); in contrast, *E.faecium* were resistant Chiolrmphencol (0%,5%); Ciprofloxacin(90%,10%); Erythromycin (18%,4%); Gentamicin (0%,0%) (**Jackson et al.,2009**). T**ürkyılmaz *et al*.,(2010)** detected resistances of *enterococci* isolated from dogs and cats to Clindamycin 100%, Erythromycin 69.2%,and were MDR 45.05%, *enterococci* spp isolated from fecal from dogs and cats were resistance 42-48% to Gentamicin, 33-45.3% to Erythromycin and sensitive to Vancomycin 100%, and detected MDR at rate 48.9% of *enterococci* isolates from cats (**Ghosh *et al.*,2012**; **Kataoka *et al.*, 2013).** *E.faecalis* and *E.faecium* from periodontal disease dog were sensitive 100% to Vancomycin, resistance against Chlormphenicol 10%, Erythromycin 20% (**Oliveira *et al*.,2016**).Whreas,those from work dogs showed 50% resistance to Ciprofloxacin, and all isolates were sensitive to Chloramphenicol, and Gentamicin and MDR rates were 98.5% (**Bang *et al*.,2017**).

Enterococci spp (*E.gallinarum, E.faecium* and *E.casseliflavus*) from fecal samples,dogs isolates were 83.3% resistant to Erythromycin, Chloramphenicol 33.3%, ciprofloxacin 66.6%, Gentamycin 8.3%, and 100% were MDR, cats isolates were resistant 75%,0%, 75% and 12.5% respectively, and 75% of isolates were MDR (**Bağcıgil et al.,2016**). *Enterococci* spp isolated from clinical samples of dogs were resistant gentamicin (71.3%), doxycycline (56.9%) ; MDR 93.1% (**Oguttu et al.,2021**). *E.faecalis* from cats fecal were resistance to Erythromycin 20.8%, Ciprofloxacin 41.7%, Vancomycin 25%; *E.faecium* resistant Erythromycin 25%, Ciprofloxacin 12.5%, Vancomycin 12.5%; while from dogs, *E.faecalis* were resistant to Erythromycin 25%, Ciprofloxacin 0%,and Vancomycin 0%; *E.faecium* were resistant to Erythromycin 25%, Ciprofloxacin 12.5%, Vancomycin 12.5% (**Iseppi et al.,2020**). *E.faecalis, E.faecium* and *E.gallinarum* from healthy dog were resistant to Chloramphenicol 41.2%, Gentamicin 68.6%, Ciprofloxacin 66.7%, Erythromycin 56.9%, Vancomycin 13.7%, clindamycin 84.3%; and 86.27% of these spp were MDR (**St ¸epie ´n-Py´sniak et al.,2021**). *E.faecalis* that detected from the dogs and cats with gastrointestinal tract infection were high resistant against Erythromycin 96%,Ciprofloxacin 93%, Gentamicin 29%, as well as MDR 78% (**Trościańczyk et al.,2021**).

The susceptibility profile in the current study was somewhat different than other studies. These differences may be the gradual vairation in resistance manner, in addition, the variations in antibiotics resistant bacteria in different geographic areas are relatively common because of the differing antibiotic drug prescribing practices, including overprescribing of antibiotic drugs (**Yilema *et al*.,201).** High antibiotic resistance in dogs may be attributed to that dogs treated with antimicrobials are usually colonized with antimicrobial resistant *enterococci* (**Kataoka *et al.*,2014**). companion animals such as dogs and cats generally live near their owners, and this suggests that these animals play a role as reservoirs of antimicrobial and MDR *enterococci* that can be transferred from these animals to human (**Ghosh et al., 2012; Jackson et al., 2009**;**Iseppi *et al*.,2020**).

Biofilm formation

The results of biofilm formation were similar in both congo red and crystal violet assay, of the 73 *enterococci* isolates 74% were positive biofilm formation, because of congo red assay could not distinguish strong from moderate and weak biofilm forms, the crystal violet assay was used to differentiate biofilm pattern. Human *enteroccoi* isolates give positive 77.41% were biofilm formation, dogs isolates 13/16(81.25%),and cats isolates were 65.38% biofilm formation. The distribution characteristics of the biofilm phenotype were as follows: the most enterococci were weak biofilm formation 77.77%; 79.16%,53.84%,94.11% human, dogs and cats isolates respectively, while 22.22% of isolates were moderate; 20.83%, 46.15%, and 5.88% human, dogs and cats isolates respectively Table (7) .

Table 7 Biofilm formation of enterococci isolates

isolates	Congo red	Crystal violet assay	Phenotype of biofilm	
	Positive no(%)	Positive no(%)	Moderate no (%)	Weak no(%)
Human (31)	24(77.41)	24(77.41)	5(20.83)	19(79.16)
Dogs(16)	13(81.25)	13(81.25)	6(46.15)	7(53.84)
Cats(26)	17	17(65.38)	1(5.88)	16(94.11)
Total (73)	54	54(74)	12(22.22)	42(77.77)

Of the 73 isolates, *E.gallinarum* were recorded higher a positive biofilm formed 90%, 27.7% of them were moderate and 72.3% were weak formed,followed by *E.facium* 66.66%, the distribution characteristics of the biofilm phenotype were as follows: 75% weak and 25% were moderate .Wherase, *E.faecalis* were 51.9% biofilm formed, 85.71% were weak and 14.28% were moderate table (8).

Table 8 Biofilm formation of enterococci spp

Spp(no)	**Positive No(%)**	Phenotype of biofilm	
		Moderate No(%)	**Weak No(%)**
E.gallinarum (40)	36(90)	10 (27.7)	26 (72.3)
E.faecalis (27)	14(51.9)	2 (14.28)	12 (85.71)
E.facium (6)	4(66.6)	1 (25)	3 (75)
Total (73)	54(74)	13 (24.07)	41 (75.92)

E.gallinarum isolated from human were positive biofilm 95% isolates, 78.95% were weak and 21.05% moderate; *E,faecalis* was 33.33%, 66.67% were weak 33.3% were moderate,and *E.facium* as 100% isolates were weak, dogs isolates showed that the *E.gallinarum* were 90.91%, 60% were moderate, *E,faecalis* 100% moderate , while *E.facium* were 50% moderate and weak ,and in cats isolates *E.gallinarum* were 77.78% positive biofilm formation, 100% were weak, *E,faecalis* were 58.8% biofilm formation, 90% of them was weak and 10% were moderate Figures (1), (2) and (3).

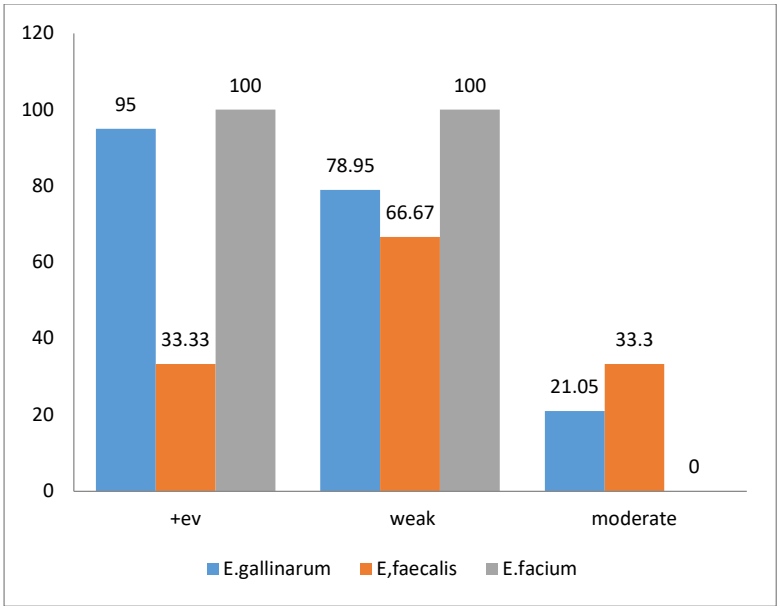

Figure 1 positive formation and phenotype of biofilm human enterococci spp

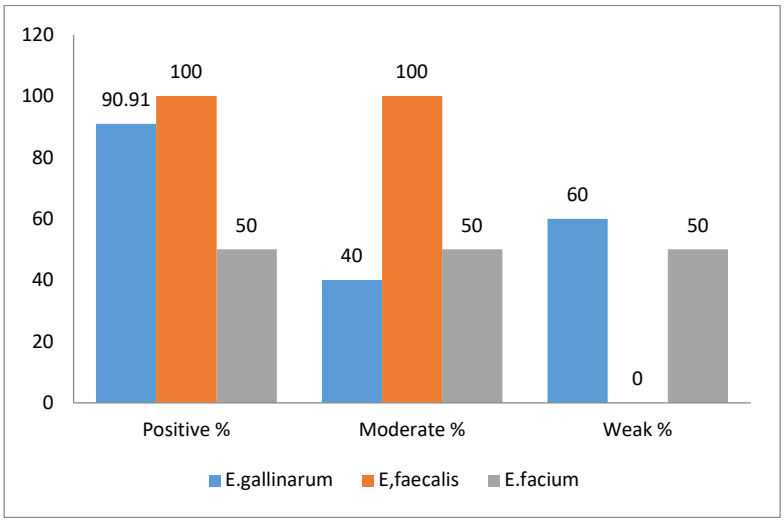

Figure 2 positive formation and phenotype of biofilm of cats enterococci spp

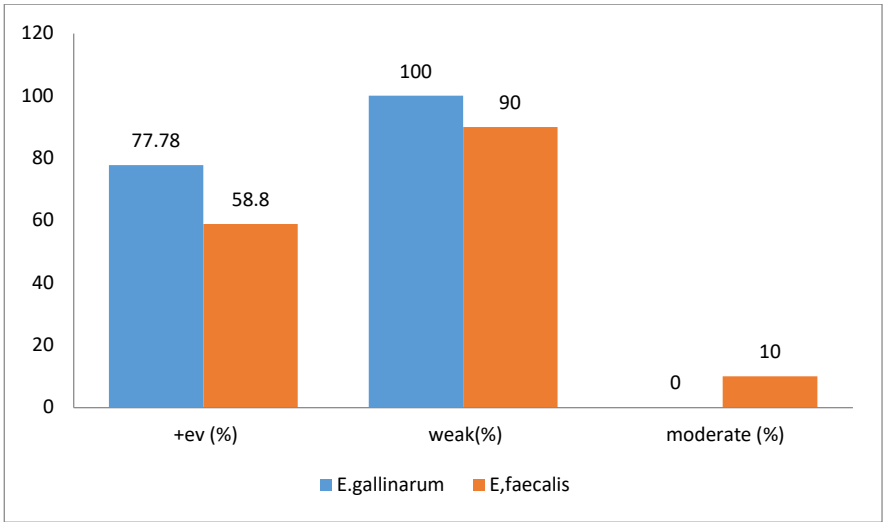

Figure 3 positive formation and phenotype of biofilm of cats enterococci isolates

The results demonstrates 74% of *enterocci* were biofilm production, the results were a higher than previous reported, *enterococci* isolates were biofilm formed 64.40%, 68% and 21.9% reported by (**Shridhar and Dhanashree,2019),** and lower than other studies, *enterococci* isolated from dogs were 100% biofilm producers,and 95% (**Oliveira *et al*.,2016;St ̨epie ́n-Py ́sniak *et al*.,2021**).

The most *enterococci* were weak biofilm formation and non of isolates give strong biofilm formation, which differs from previously recorded studies, isolated enterococci were strong biofilms at 34%,49% moderate and 17% were weak former (**Sindhanai *et al*.,2016**), another study reports 19.6% were weak,40% were strong ,and 35% were moderate biofilm formation (**Oliveira *et al*.,2016**). *Enterococci* clinical isolates were 42% moderate; 43% were weak biofilm-formers **(Hashem *et al*.,2017**). In a study, *enterococci* isolates formed biofilm at rate 73% ,18.9% of them were strong biofilm formed, 32.4% formed moderate and 21.6% were weak formed (**El-Zamkan and Mohamed,2021**).While **St ̨epie ́n-Py ́sniak *et al*.(2021)** detected 41.2% of *enterococci* were weak,37.3% strong and 21.5% were moderate biofilm producers .

Current study, as totally, *E.gallinarum* were a higher positive biofilm formed than other spp followed by *E.faecium* and *E.faecalis* . In reports, biofilm formed were detected in 27.5% *E. faecium* and 17.7% *E. faecalis* (**Shridhar and Dhanashree,2019**). *E. faecalis* was 25% weak,40% moderate,20% strong, *E. faecium* were 16.7% weak,16.7% moerate and 25% strong reported by(**El-Zamkan and Mohamed,2021**).

The current results are somewhat different from previous studies, many factors were found affects on formation and phenotype of the biofilm such as nutrient components of culture, virulence factors, time of incubation, and clinical states, it is

found that the glucose increased the biofilm growth of E.faecalis and the biofilm formation ability of this spp using 1% of glucose or sucrose has significantly greater biofilm formation and leading to produced high components of the biofilm matrix such as esp and eDNA and had more highly expressed virulence-related genes (**Ghabraei *et al*.,2018; Kim *et al*.,2020**). A highly considerable correlation was noticed between the ability of the *enterococci* isolates to production and potency biofilm formation and the presence of virulence factors . In addition, a statistical difference was observed between isolates from healthy and infected cases and biofilm formation (**Hashem *et al*.,2017**; **St ępie´n-Py´sniak *et al*.,2021).**

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