# Virulence determinants and antimicrobial resistance in *Enterococcus faecalis* isolated from hatcheries

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## 1. Abstract

Hatcheries have the power to spread antimicrobial resistant (AMR) pathogens through the poultry production chain. Enterococci are commensal intestinal bacteria and opportunistic pathogens in humans and animals. The aim of this study was to assess the occurrence of enterococci in broiler breeder hatcheries in 3 different governorates in Egypt "Sharqia, Beheira, and Dakahlia" with regard to the manifested diseases. The isolation results revealed that *E. faecalis* occurrence in the examined samples were 5.4% (65/1200) from pipped chicks' yolk sacs and 5.8% (69/1200) from live chicks' organs. The antibiotic susceptibility testing of the recovered isolates showed high resistance to vancomycin, oxytetracycline and high sensitivity for penicillin, difloxacin and amikacin. The genetic screening for some virulence and antibiotic resistance genes indicated the high incidence of *gelE* and *asa1* virulence genes between the recovered isolates in correlation with the high incidence of *ermB* and *blaZ* antibiotic resistance genes. In conclusion, hygienic measures in breeders' hatcheries are recommended to avoid the spreading of resistance and virulent *E. faecalis* as it represents a public health threat in addition to its deleterious effects on poultry production.

**Key words:** Antimicrobial resistance; *Enterococcus*; Hatchery; Poultry; Virulence.

## 2. Introduction

Genus *Enterococcus* contains 6 species (*E. avium*, *E. cecorum*, *E. durans*, *E. faecalis*, *E. faecium*, and *E. hirae*) that have been associated with diseases in poultry. *Enterococcus faecalis* (*E. faecalis*) is part of the normal intestinal microflora of animals and humans and it has been found to be among the dominating intestinal microflora of day-old chicks [1].

However, *E. faecalis* is also considered an opportunistic pathogen with the potential to cause clinical infections. In chickens, it is often associated with septicemia, endocarditis, and salpingitis, and may subsequently lead to amyloid arthropathy and amyloidosis [2, 3].

*E. faecalis* affects avian species of all ages; however, the majority of serious infections have been associated with embryos and young birds [4].Chicks are particularly susceptible to infections during the first week of life and substantial mortality may occur due to omphalitis (yolk sac infection) and septicemia [4].

Previous studies have demonstrated an increase of *E. faecalis* as a cause of mortality in broiler parent flocks [2, 3]. It has been speculated that this increase could be due to the banning of

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antibiotic growth promotors in 1998–1999 since they were primarily affecting the Gram-positive bacteria [5]. The presence of strains producing extended-spectrum β-lactamases (ESβLs) or *ampC* β-lactamases in food products is a cause for particular concern because these phenotypes are usually accompanied by a low susceptibility to other classes of antibiotics.

*E. faecalis* has the ability to acquire new genetic traits, as the virulence genes are often found on horizontally transmittable pathogenicity islands and this feature may increase the virulence of *E. faecalis*, enabling it to colonize new areas in the host and maybe new hosts [6]. The spectrum of virulence factors identified in *E. faecalis* isolated from lesions in chickens did not differ from that of humans. The knowledge of virulence factors of *E. faecalis* would be beneficial for the detection and limitation of pathogenic *E. faecalis* spread in poultry production, by early treatment of parental and production flocks, as well as prophylactic control, such as by treatment with probiotic bacteria [4].

Colonization of *E. faecalis* in newly hatched chicks occurs by both vertical and horizontal transmission including environmental sources [4, 7]. Vertical transmission can be by sub-clinically infected hens with salpingitis or peritonitis, or both, continuing to lay and thereby possibly transmitting bacteria to their offspring [7]. Horizontal transmission is by hatchery fluff, contaminated eggs, water, feed, bedding, or a combination of these factors [4, 7].

The breeding system of industrialized poultry production including pedigree lines, and grandparents, at the top of the production pyramid is speculated to be a potential route for the vertical spread of bacteria. If the introduction of pathogenic bacteria occurs at the top of the production pyramid, bacteria might be transmitted to chickens in production via grandparents and parents (vertical transmission) [7, 9]. Accordingly, the current study aimed to survey the antimicrobial resistant *Enterococcus* species in broiler breeder hatcheries in 3 different governorates of Egypt.

## 3. Materials and Methods

### *3.1. Samples*

A total of 1200 yolk sac samples from pipped in shell chicks between 20 and 21 days of incubation according to the hatch window, and 1200 yolk sacs, liver, hearts, joints, bone marrow, and air sac samples from live chicks equally (*n= 200* for each organ) were collected during the period from 2019 to2022 from 3 local hatcheries in Beheira, Sharqeya and Dakahlia.

### *3.2. Isolation and identification of enterococci*

*Enterococcus* species were isolated from different collected samples through primary isolation on tellurite agar (Oxoid, Hampshire UK) and incubated overnight at 37°C for selective and indicative growth of enterococci on Selntz and Bartely medium (Oxoid, Hampshire UK).The colonies of *Enterococcus faecalis* were purified by sub-cultivation on 5% blood agar. Further identification was performed by API-20 Strep and PCR. All isolates were frozen in 15% glycerol at −80°C for further studies [3].

### *3.3. Antimicrobial patterns of E. faecalis isolates*

All *E. faecalis* isolates were tested for antibiotic susceptibility according to CLSI breakpoint guidelines [10] by the disc diffusion technique against 17 different antibiotics arranged as follows: ampicillin 10 µg, amoxicillin 20 µg, penicillin G and V 30 µg, cefotaxime 10 µg, vancomycin, 30 µg, erythromycin 15 µg, oxytetracycline 30 µg, doxycycline 20 µg, amikacin 30 µg, difloxacin 10 µg, florfenicol 10 µg apramycin 10 µg, ceftiofur 10 µg, lincomycin 20 µg,

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spectinomycin 20 µg and gentamicin, 10 µg. While β - lactamase detection in the isolates was carried out using nitrocefine disks (Cefinase®) followed by a combined disc diffusion test.

### *3.4. In-vitro biofilm formation*

*Enterococcus* isolates were incubated in 10 mL of tryptic soy broth (TSB) with 1% glucose for 24 hours at 37°C. Then, 20 µl of each bacterial suspension were transferred to each of three wells of sterile 96-well polystyrene microtiter plates holding 180 µl of TSB with 1% glucose and 200 µl of uninoculated TSB with 1% glucose broth assigned as a negative control. The inoculated microtiter plate was incubated at 37°C for 24 hours. The broth was cautiously withdrawn, and the wells were washed three times with sterile phosphate-buffered saline. Biofilms were then fixed with methanol for 20 min., flicked, and air-dried in a flipped position in a warm room for about 30 min. Biofilms were stained with crystal violet (2%) for 15 min. The wells were washed twice with distilled water and then dried. The dyed adherent cells were resolubilized in 150 µl of acetic acid (33%) for 30 min. without shaking at room temp. Finally, a microtiter plate reader was used to estimate the OD of each well at a wavelength of 570 nm [11].

### *3.5. Virulence and resistance molecular patterns of avian E. faecalis*

The virulence genes; *gelE, cylA* and *asa1*, and the antibiotic resistance genes; *ermB*, *blaZ,* and *vanA* genes were monitored in *E. faecalis* DNA by conventional polymerase chain reactions (cPCR) using primers and PCR settings depicted in {Tables 1 and 2} as described by Vankerckhoven *et al.* [12] and Funda *et al.* [13] , respectively. The DreamTaq Green PCR Master Mix (Thermo Scientific, Sweden) was used in the PCR assays and the PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, Copenhagen, Denmark) in 1x TBE buffer. The *E. faecalis* ATCC 29212 strain was used as a control one.

## 4. Results

The results of isolation and identification of avian *E. faecalis* showed a percentage of 5.4% positive samples from the yolk sacs of pipped in shell chicks. On the other hand, samples collected from live chicks revealed 12.5%, 7.5%, 6%, 5%, 2%, and 1.5% incidences from the liver, heart, joint, yolk sac, bone marrow and, air sacs samples respectively as shown in table 3. The antibiotic susceptibility test of the isolates revealed high sensitivity to penicillin V, amikacin, difloxacin, gentamicin, amoxicillin, and doxycycline with percentages of 85%, 83%, 79%, 63%, 48%, and 45%, respectively. Meanwhile, the resistances to vancomycin, oxytetracycline, erythromycin, cefotaxime, ceftiofur, and penicillin G were expressed by 78%, 64%, 62%, 55%, 40%, and 37% of the tested isolates, respectively. Intermediate sensitivity to spectinomycin, apramycin, florfenicol, ampicillin, and lincomycin was expressed by 50%, 55%,50%, 53%, and 46% of the tested isolates, respectively as shown in Table 4, The result of phenotypic screening by Cefinase® discs was 55% positive, while by combined disc diffusion test was 49% positive as shown in Table 5.

The biofilm formation ability was recorded in 57% of the yolk sac of pipped in shell chicks *E. faecalis* isolates. Concerning samples recovered from the yolk sacs, air sacs, and livers of live chicks the biofilm formation ability was recorded in 50%, 100%, and 67% of the isolates, respectively, while *E. faecalis* isolates from bone marrow, joint, and heart did not form biofilm at all.

PCR investigations on virulence-associated genes revealed that 100% of the yolk sac isolates of pipped in shell chicks and those from live chicks harbored *gelE* and *asa1*genes while no one isolate harbored the *cylA*. Regarding the antibiotic


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resistance genes *ermB, blaZ, vanA* 100%, 57%, and 43%, respectively of the isolates were positive. *asa1* was detected in only 50% of isolates recovered from heart samples. And no one isolates from the organs harbored the *cylA* gene except the yolk sac and heart isolates as 50% were positive. Of the live chick isolates, 100% harbored the *ermB* gene, and all isolates from yolk sac, bone marrow, air sac, and liver samples harbored the *blaZ*. Concerning the *vanA* gene, 67% and 50% of *E. faecalis* from joints and hearts were respectively positive. Meanwhile, 50%, 50% and 33% of isolates from the yolk sac, air sac, liver samples were respectively positive (Tables 6, 7 and Figures 1, 8).

## 5. Discussion

Enterococci are Gram-positive facultative anaerobic bacteria that are part of the normal intestinal microbiota, with densities ranging from $10^5$ to $10^7$ CFU/g of the intestinal content up to $10^{11}$ bacterial cells/gram feces. Enterococci have been proposed as fecal indicator bacteria for microbial source tracking and are often used in tracking trends in resistance to antimicrobials for various resistance surveillance systems. Enterococci are currently ranked among the most prevalent multidrug resistant hospital pathogens worldwide. They are the third most commonly isolated healthcare pathogen and are capable of causing a variety of infections including endocarditis, sepsis, surgical wound infections, and urinary tract infections. The genus *Enterococcus* consists of over 40 ecologically diverse species, yet more than 90 percent of enterococcal infections are caused by two species: *E. faecalis* and *E. faecium*[14].

In the United States, enterococci are common nosocomial pathogens, accounting for 10.0% of hospital-acquired infections. This genus ranks second or third among the most common bacterial agents of urinary tract infections, wound infections, and bacteremia in hospitals. Enterococci are responsible for about 16.0% of nosocomial urinary tract infections, bacteremia caused by *E. faecalis* has significant relevance for public health since it is linked with an increase of a subsequent endocarditis, one of the most severe enterococcal infectious diseases [15]. Genetic relatedness was found between *E. faecalis* isolates from urinary tract infection cases and those from poultry, reinforcing the zoonotic potential of this species and suggesting a possible role of poultry in its spread to humans [16].

In poultry, enterococci have been associated with septicemia, endocarditis, and other diseases. The safety issue regarding enterococci has not been recognized in poultry meat; however, concerns about the transmission of antimicrobial resistant enterococci to humans have been reported [17].

Recently, the isolation of AMR *E. faecalis* strains from broilers with vertebral osteomyelitis disease has been reported [16]. Antibiotic resistant enterococci have been reported in poultry retail meats. The ability of enterococci to acquire AMR through the transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge to infection control. Mobile genetic elements, including transposons, play an important role in the dissemination of AMR through horizontal gene transfer in bacteria including enterococci [16].

So, in the present study it was assumed that enterococci occurrence in hatcheries represents a marker for hygienic conditions, as it is present at a very low rate in pipped chicks (5.4%) and (5.8%) in live chicks from different organs. The uniformity of the *E. faecalis* population under confined conditions can be explained by a very strict breeding program.

For many years, the use of antibiotics in food-producing animals has been considered the main driver of multidrug


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resistant (MDR) bacteria selection, with chicken-meat or sub products potential vehicles for the transmission of such bacteria or their mobile genetic elements to humans and the environment [18].

Enterococci has a role in the movement of antibiotic resistance from farm to table to clinic, a study was conducted by characterizing over 300 isolates of *Enterococcus* cultured. *Enterococcus faecalis* and *Enterococcus faecium* were the predominant species found, and antimicrobial susceptibility testing uncovered striking levels of resistance to medically important FDA approved antibiotic classes. The ability of enterococci to persist in the food system positions them as vehicles to move resistance genes from the industrial farm ecosystem into more human-proximal ecologies [19].

High level of resistance to antibiotics used in animal production was observed in isolates from chicken meat. The vast majority of the observed resistance in the current study was to drugs used in animal production such as lincomycin (23% of isolates), tetracycline (61%), and erythromycin (53%). It was similar to results of previous study obtained by Manson et al. [19].

A study conducted to evaluate the susceptibility of *Enterococcus* spp. from chicken meat collected 20 years apart to antibiotics showed that a high rate of recent chicken meat in Portugal are vehicles of MDR *Enterococcus* spp. and the decrease of antibiotic use at farm level in Europe during the last decade was not enough to reduce antibiotic resistance rates associated with samples recovered >20 years apart [20].

In the current study, the recovered isolates showed a relatively high resistance patterns to vancomycin and oxytetracycline at the rate of 78.4% and 64%, respectively. It may be attributed to the misuses of such medications in the field as it was explained by Hog et al. in their study in Denmark [21]. On the other hand, such isolates showed a relatively higher sensitivity patterns for amikacin and gentamicin (83% and 63%, respectively) which may be owed to a similar reason, but on the contrary that those antimicrobials are uncommonly used in the field.

Glycopeptide and macrolide growth-promoting agents are banned in Denmark but macrolides are used for treatment. The high number of resistant strains of vancomycin and erythromycin found in this study could be related to co-selection for other antimicrobial agents. The resistance genes for macrolides and glycopeptides can be found on the same mobile genetic elements [22–24].

In the current study, the phenotypic screening for β-lactamases of *E. faecalis* showed that combined disc diffusion is more sensitive than chromogenic Cefinase® technique as the recorded results revealed that there were 74/134 positive isolates by Cefinase®, while 65/134 were positive isolates by combined disc diffusion test.

Numerous genetic determinants confer antibiotic resistance across the genus *Enterococcus*. Co-occurrence of resistance genes and virulence factors is of particular concern from a clinical standpoint, *vanA* gene clusters are the most common in clinical isolates and frequently integrated into a wide range of plasmids producing clinical levels of resistance to vancomycin. Several PCR assays have been developed for the detection of virulence factors common to *Enterococcus*, including: cytolysin (*cyl*) and gelatinase secretion proteins (*gelE*) that are predominantly found in endocarditis isolates [25].

Pathogenesis of enterococci is attributed to an array of virulence factors. Aggregation substance (*as*), gelatinase


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(*gel*), cytolysin (*cyl*), enterococcal surface protein (*esp*) and hyaluronidase (*hyl*). Among the 5 virulence determinants screened, *asa1* was significantly more common in *E. faecalis* followed by *gelE,*and *cylA* (86.51%, 85.39%, 59.55%) respectively [26].

The nature of biofilm structure confers an inherent resistance to antimicrobial agents. Mechanisms responsible for resistance may be delayed penetration of the antimicrobial agent through the biofilm matrix, altered growth rate of biofilm microorganisms, and other physiological changes due to the biofilm mode of growth [15].

In the present study we agree that *gelE* and *asa1* are the most common virulence genes as the percentage of detection of both of them are above 95% of the tested samples. However, there was a tendency for a lower prevalence of *cylA* in the current study as it was totally negative in pipped in shell samples and with 14% positive samples only from live chicks samples, *vanA* detected with 43% in pipped in shell samples and as low as 21% in live chicks samples. Also the prevalence of biofilm formation ability in current study was 57% of pipped in shell isolates.

Several investigations have been made on transmission routes, antimicrobial resistance, ESβLs ratio, and genetic diversity of *E. faecalis* in industrialized broiler production systems. As knowledge on the prevalence and diversity in broiler breeder farms and hatcheries is very limited, the aim of this investigation was to establish knowledge on the intestinal *E. faecalis* populations occurrence and antimicrobial resistance patterns in broiler breeders' farms and hatcheries. Such knowledge might be used to set up control strategies to reduce *E. faecalis* that are most frequently associated with disease and antibiotic resistance.

## 6. Conclusion

In conclusion, the prevalence of *E. faecalis* in the hatcheries of broiler breeder flocks was very low, but the isolated strains were virulent and resistant so it is considered as a source of multidrug resistance reservoir. Further studies should investigate the potential of *E. faecalis* to be considered as pathogenic and cause disease under certain conditions, and how to limit strains with higher pathogenicity and antibiotic resistance.

*Conflict of interest* Nothing to declare

## 7. References

1. Devriese, L., Baele, M., &Butaye, P. The Prokaryotes book. Third edition. Springer US, 2006; (4): 163-174.
2. Chadfield, M. S., Christensen, J. P., Christensen, & H., Bisgaard, M. Characterization of streptococci and enterococci associated with septicaemia in broiler parents with a high prevalence of endocarditis, Avian Pathology, 2004; 33 (6): 610–617.
3. Gregersen, R. H., Petersen, A., Christensen, H., & Bisgaard, M. Multilocus sequence typing of *Enterococcus faecalis* isolates demonstrating different lesion types in broiler breeders, Avian Pathology, 2010; 39 (6): 435–440.
4. Fertner, M. E., Olsen, R. H., Bisgaard, M., & Christensen, H. Transmission and genetic diversity of *Enterococcus faecalis* among layer chickens during hatch, Acta Veterinaria Scandinavica, 2011; 53-56.
5. Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health, Journal of Antimicrobial

Chemotherapy, 2003; 52 (2): 159–61.
6. Coburn, P. S., Baghdayan, A. S., Dolan, G. T., & Shankar, N. Horizontal transfer of virulence genes encoded on the *Enterococcus faecalis* pathogenicity island, Molecular Microbiology, 2007; 63 (2): 530–544.
7. Olsen, R. H., Schønheyder, H. C., Christensen, H., & Bisgaard, M. *Enterococcus faecalis* of Human and Poultry Origin Share Virulence Genes Supporting the Zoonotic Potential of *E. faecalis*, Zoonoses Public Health, 2012; 59 (4): 256–263.
8. Rogers, L. A., Strong, K., Cork, S. C., McAllister, T. A., Liljebjelke, K., Zaheer, R., et al. The Role of Whole Genome Sequencing in the Surveillance of Antimicrobial Resistant *Enterococcus* spp.: A Scoping Revie, Front. Public Health, 2021; 9: 599285.
9. Cox, J. M., & Pavic, A. Advances in enteropathogen control in poultry production, Journal of Applied Microbiology, 2010; 108: 745–755.
10. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 31st ed. CLSI supplement M100. USA, 2021.
11. Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Ćirković, I., et al. Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci, APMIS, 2007; 115 (8): 891–899.
12. Vankerckhoven, V., *Van A*utgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., et al. Development of a multiplex PCR for the detection of *asa1, gelE, cylA, esp*, and *hyl* genes in enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*, J ClinMicrobiol, 2004; 42 (10): 4473 – 4479.
13. Funda, B. A., Ikiz, S., Ak, S., &Yakut, Ö. N. Isolation of Vancomycin Resistant Enterococci from Animal Faeces, Detection of Antimicrobial Resistance Profiles and Vancomycin Resistant Genes, KafkasUniv Vet FakDerg, 2015; 21 (1): 87–94.
14. Van Tyne D, Martin, M. J., & Gilmore, M.S. Structure, function, and biology of the *Enterococcus faecalis* cytolysin, Toxins, 2013; 5 (5): 895-911.
15. Ceci, M., Delpech, G., Sparo, M., Mezzina, V., Bruni, S.S.,& Baldaccini, B. Clinical and microbiological features of bacteremia caused by *Enterococcus faecalis*, The Journal of Infection in Developing Countries, 2015; 9 (11): 1195-1203.
16. Rehman, M.A., Yin, X., Zaheer, R., Goji, N., Amoako, K. K., & McAllister, T., et al. Genotypes and phenotypes of enterococci isolated from broiler chickens, Frontiers in Sustainable Food Systems, 2018; 2: 83.
17. Souillard, R., Laurentie, J., Kempf, I., Le Caër, V., Le Bouquin, S.,& Serror, P., et al. Increasing incidence of *Enterococcus*-associated diseases in poultry in France over the past 15 years, Vet. Microbiol, 2022; 269: 426.
18. Fatoba, D.O., Amoako, D.G., Akebe, A.L., Ismail, A., & Essack, S.Y. Genomic analysis of antibiotic-resistant *Enterococcus* spp. reveals novel enterococci strains and the spread of plasmid-borne Tet (M), Tet (L) and Erm (B) genes from chicken litter to agricultural soil in South Africa, Journal of Environmental Management, 2022; 302: 114101.


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19. Manson, A.L., Van Tyne, D., Straub, T.J., Clock, S., Crupain, M. & Rangan, U., et al. Chicken meat-associated enterococci: influence of agricultural antibiotic use and connection to the clinic, Applied and environmental microbiology, 2019; 85 (22): e01559.
20. Rebelo, A., Duarte, B., Ferreira, C., Mourão, J., Ribeiro, S., & Freitas, A.R., et al. *Enterococcus* spp. from chicken meat collected 20 years apart overcome multiple stresses occurring in the poultry production chain: Antibiotics, copper and acids, International journal of food microbiology, 2023; 384:109981.
21. Høg, B., Bisgaard, H., Sönksen, W., Bogø, L., Nygaard, A., Knegt, de., et al. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, Danmap, DTU Library 2015; 65-70.
22. Simjee, S., Jensn, L. B., Donabedian, S. M. and Zervos, M. J. *Enterococcus* In: Aarestrup, F. M. Antimicrobial resistance in bacteria of animal origin, ASM Press, 2006 ; Jul;12(7) 315- 328.
23. Hasman, H., Aarestrup, F. M., Dalsgaard, A., & Guardabassi, L. Heterologous expression of glycopeptide resistance vanHAX gene clusters from soil bacteria in *Enterococcus faecalis*, Journal of Antimicrobial Chemotherapy, 2006; 57 (4): 648–653.
24. Hasman, H., Kempf, I., Chidaine, B., Cariolet, R., Ersbøll, A. K., Houe, H., et al. Copper resistance in *Enterococcus faecium*, mediated by the tcrB gene, is selected by supplementation of pig feed with copper sulfate, Appl Environ Microbiol, 2006 ;72 (9): 5784–5789.
25. Davis, B.C., Keenum, I., Calarco, J., Liguori, K., Milligan, E., & Pruden, A., et al. Towards the standardization of *Enterococcus* culture methods for waterborne antibiotic resistance monitoring: A critical review of trends across studies. Water Research X, 2022; 17: 161.
26. Kiruthiga, A., Padmavathy, K., Shabana, P., Naveenkumar, V., Gnanadesikan, S., & Malaiyan, J. Improved detection of *esp, hyl, asa1, gelE, cylA* virulence genes among clinical isolates of enterococci, BMC Research Notes, 2020; 13: 1-7.
27. Matsuda, K., Tsuji, H., Asahara, T., Matsumoto, K., Takada, T&Nomoto, K. Establishment of an Analytical System for the Human Fecal Microbiota, Based on Reverse Transcription-Quantitative PCR Targeting of Multicopy rRNA Molecules, Appl. Environ. Microbiol., 2009; 75 (7): 1961.
28. Homan, W. L., Ttibe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E.,etal.Multilocus sequence typing scheme for *Enterococcus faecium*, J. Clin. Microbiol., 2002; 40: 1963–1971.
29. Zoletti, G. O., Siqueira, J. F. & Santos, K. R. N. Identification of *Enterococcus faecalis* in Root-filled Teeth With or Without Periradicular Lesions by Culture-dependent and-Independent Approaches, JOE, 2006; 32: 722–726.
30. Schlegelova, J., Vlkova, H., Babak, V., Holasova, M., Jaglic, Z., Stosova, T., etal.Resistance to erythromycin of *Staphylococcus* spp. isolates from the food chain,VeterinarniMedicina, 2008; 53 (6): 307-314.
31. Patel, R., Uhl, J. R., Kohner, P., Hopkins, M. K. & Cockerill Iii, F. R. Multiplex PCR Detection of *vanA, vanB, vanC-1*, and *vanC*-2/3 Genes in Enterococci, Journal of clinical Microbiology, 1997; 35: 703-707


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**Table (1): Oligonucleotide primers' sequences***

| Gene | Primer Sequence 5'-3' | Amplified product | Reference |
|---|---|---|---|
| ***Enterococcus 16S rRNA*** | ATCAGAGGGGGATAACACTT | 337 bp | [27] |
| | ACTCTCATCCTTGTTCTTCTC | | |
| ***E. faecium atpA*** | CGG TTC ATA CGGAAT GGC ACA | 556 bp | [28] |
| | AAG TTC ACG ATA AGC CAC GG | | |
| ***E. faecalis 16S rRNA*** | GTT TAT GCC GCA TGG CAT AAGAG | 310 bp | [29] |
| | CCG TCA GGG GAC GTT CAG | | |
| ***gelE*** | TATGACAATGCTTTTTGGGAT | 213 bp | [12] |
| | AGATGCACCCGAAATAATATA | | |
| ***asa1*** | GCACGCTATTACGAACTATGA | 375 bp | |
| | TAAGAAAGAACATCACCACGA | | |
| ***cylA*** | ACTCGGGGATTGATAGGC | 688 bp | |
| | GCTGCTAAAGCTGCGCTT | | |
| ***ermB*** | CATTTAACGACGAAACTGGC | 425 bp | [30] |
| | GGAACATCTGTGGTATGGCG | | |
| ***blaZ*** | TACAACTGTAATATCGGAGGG | 833 bp | [13] |
| | CATTACACTCTTGGCGGTTTC | | |
| ***vanA*** | CATGACGTATCGGTAAAATC | 885 bp | [31] |
| | ACCGGGCAGRGTATTGAC | | |

* Source: Midland Certified Reagent Company oilgos (USA).

**Table (2): Cycling conditions of the different primers during cPCR**

| Gene | Primary denaturation | Secondary denaturation | Annealing | Extension | No. of cycles | Final extension |
|---|---|---|---|---|---|---|
| ***Enterococcus 16S rRNA*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 40 sec. | 35 | 72˚C 10 min. |
| ***E. faecium atpA*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| ***E. faecalis 16S rRNA*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 40 sec. | 35 | 72˚C 10 min. |
| ***gelE*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 10 min. |
| ***asa1*** | 94˚C 5 min. | 94˚C 30 sec. | 53˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 10 min. |
| ***cylA*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| ***ermB*** | 94˚C 5 min. | 94˚C 30 sec. | 51˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| ***blaZ*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 50 sec | 35 | 72˚C 10 min. |
| ***vanA*** | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 50 sec. | 35 | 72˚C 10 min. |

**Table (3): Incidence of *E. faecalis* in broiler breeder hatcheries**

| **Samples** | **Pipped chicks (*n=1200*)** | **Live chicks (*n=1200*)** | | | | | | **Total** |
|---|---|---|---|---|---|---|---|---|
| **Type** | **Yolk sac** | **Yolk sac** | **Bone marrow** | **Joint** | **Air sac** | **Heart** | **Liver** | |
| **Total no. of samples** | 1200 | 200 | 200 | 200 | 200 | 200 | 200 | 2400 |
| ***E. faecalis* no.** | 65 | 10 | 4 | 12 | 3 | 15 | 25 | 134 |
| **%** | 5.4 | 5 | 2 | 6 | 1.5 | 7.5 | 12.5 | 5.6 |

**Table (4): Resistance pattern of avian *E. faecalis* (*n=134* ) to different antibiotics**

| Antimicrobial agents | Resistance patterns | | | | | |
|---|---|---|---|---|---|---|
| | R | | I | | S | |
| | No. | % | No. | % | No. | % |
| Penicillin V, 30 µg | 15 | 11 | 5 | 4 | 114 | 85 |
| Penicillin G, 30 µg | 50 | 37 | 44 | 33 | 40 | 30 |
| Amoxicillin, 20 µg | 31 | 23 | 39 | 29 | 64 | 48 |
| Ampicillin, 10 µg | 55 | 41 | 71 | 53 | 8 | 6 |
| Cefotaxime, 10 µg | 74 | 55 | 52 | 39 | 8 | 6 |
| Ceftiofur, 10 µg | 54 | 40 | 33 | 25 | 47 | 35 |
| Difloxacin ,10µg | 9 | 7 | 19 | 14 | 106 | 79 |
| Vancomycin, 30 µg | 105 | 78.4 | 14 | 10.4 | 15 | 11.2 |
| Erythromycin, 15 µg | 83 | 62 | 40 | 30 | 11 | 8 |
| Oxytetracycline, 30 µg | 86 | 64 | 37 | 28 | 11 | 8 |
| Doxycycline, 20 µg | 43 | 32 | 31 | 23 | 60 | 45 |
| Amikacin, 30 µg | 5 | 4 | 18 | 13 | 111 | 83 |
| Gentamicin, 10 µg | 19 | 14 | 31 | 23 | 84 | 63 |
| Florfenicol, 10 µg | 50 | 37 | 67 | 50 | 17 | 13 |
| Apramycin,10 µg | 55 | 41 | 74 | 55 | 5 | 4 |
| Lincomycin, 20 µg | 43 | 32 | 62 | 46 | 29 | 22 |
| Spectinomycin, 20 µg | 31 | 23 | 67 | 50 | 36 | 27 |


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**Table (5): Result of Cefinase® and combined disc diffusion test of *E. faecalis isolates (n=134)***

| Result | Cefinase® | Combined Disc diffusion test |
|---|---|---|
| **Positive isolates** | 74/134 (55%) | 65/134 (49%) |
| **Negative isolates** | 60/134 (45%) | 69/134 (51%) |

**Table (6): Biofilm formation ability in comparison with occurrence of virulence and resistance genes in *E. faecalis* isolates (*n=21*).**

| Isolate number | Source of isolates | *asa1* | *cylA* | *gelE* | *ermB* | *blaZ* | *vanA* | Biofilm |
|---|---|---|---|---|---|---|---|---|
| 1 | *E. faecalis* from pipped yolk sac | + | - | + | + | - | - | - |
| 2 | *E. faecalis* from piped yolk sac | + | - | + | + | - | - | - |
| 3 | *E. faecalis* from piped yolk sac | + | - | + | + | - | + | + |
| 4 | *E. faecalis* from piped yolk sac | + | - | + | + | + | + | + |
| 5 | *E. faecalis* from piped yolk sac | + | - | + | + | + | - | - |
| 6 | *E. faecalis* from piped yolk sac | + | - | + | + | + | + | + |
| 7 | *E. faecalis* from piped yolk sac | + | - | + | + | + | - | + |
| 8 | *E. faecalis* from chicks bone marrow | + | - | + | + | + | - | - |
| 9 | *E. faecalis* from chicks air sacs | + | - | + | + | + | - | + |
| 10 | *E. faecalis* from chicks air sacs | + | - | + | + | + | + | + |
| 11 | *E. faecalis* from chicks yolk sac | + | - | + | + | + | + | + |
| 12 | *E. faecalis* from chicks yolk sac | + | + | + | + | + | - | - |
| 13 | *E. faecalis* from chicks heart | - | + | + | + | - | - | - |
| 14 | *E. faecalis* from chicks heart | + | - | + | + | + | - | - |
| 15 | *E. faecalis* from chicks liver | + | - | + | + | + | - | + |
| 16 | *E. faecalis* from chicks liver | + | - | + | + | + | + | + |
| 17 | *E. faecalis* from chicks liver | + | - | + | + | + | - | - |
| 18 | *E. faecalis* from chicks joint | + | - | + | + | + | - | - |
| 19 | *E. faecalis* from chicks joint | + | - | + | + | + | - | - |
| 20 | *E. faecalis* from chicks joint | + | - | + | + | + | - | - |
| 21 | *E. faecalis* from chicks joint | + | - | + | + | - | - | - |
| **Total** | | **20** | **2** | **21** | **21** | **16** | **6** | **9** |

**Table (7): Collective data for virulence and resistance genes patterns in Avian *E. faecalis* isolates (*n=21*).**

| Origin | | Pipped chicks | Live chicks | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| Type | | Yolk sac | Yolk sac | Bone marrow | Joint | Air sac | Heart | Liver | |
| No. of *E.faecalis* isolates | | 7/1200 | 2/200 | 1/200 | 4/200 | 2/200 | 2/200 | 3/200 | 21/2400 |
| Biofilm formation ability | | 4/7 | 1/2 | 0/1 | 0/4 | 2/2 | 0/2 | 2/3 | 9/2400 |
| Virulence genes | *gelE* | 7/7 | 2/ 2 | 1/1 | 4/4 | 2/2 | 2/2 | 3/3 | 21/2400 |
| | *asa1* | 7/7 | 2/ 2 | 1/1 | 4/4 | 2/2 | 1/2 | 3/3 | 20/2400 |
| | *cylA* | 0/7 | 1/2 | 0/1 | 0/4 | 0/2 | 1/2 | 0/3 | 2/2400 |
| Antibiotic resistance genes | *ermB* | 7/7 | 2/ 2 | 1/1 | 4/4 | 2/2 | 2/2 | 3/3 | 21/2400 |
| | *blaZ* | 4/7 | 2/ 2 | 1/1 | 3/4 | 2/2 | 1/2 | 3/3 | 16/2400 |
| | *vanA* | 3/7 | 1/2 | 0/1 | 0/4 | 1/2 | 0/2 | 1/3 | 6/2400 |

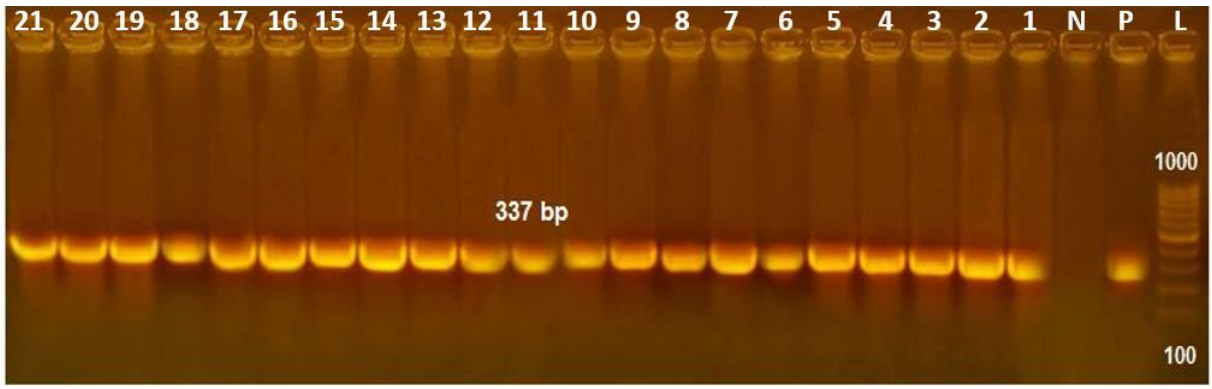


**Fig (1):** Agarose gel electrophoresis showing positive amplification of 16s *rRNA* for genus *Enterococcus* at 337 bp fragm*ent*.
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

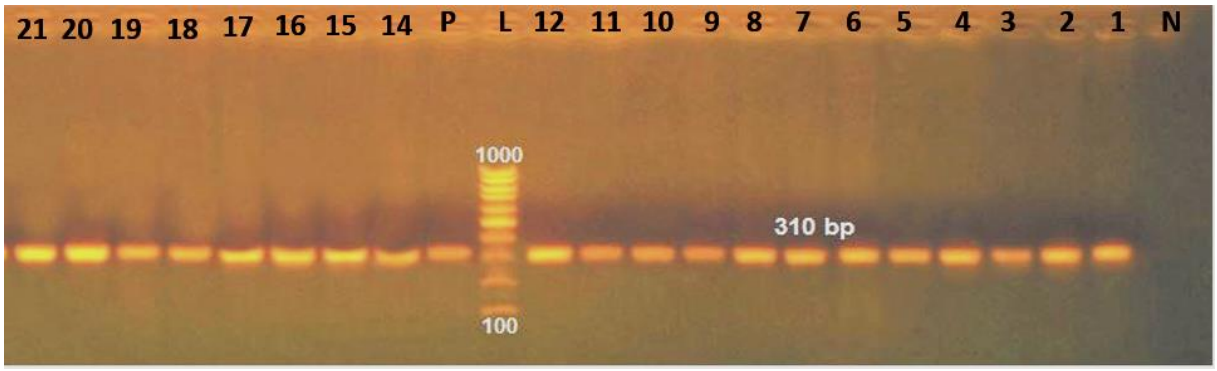


**Fig (2):** Agarose gel electrophoresis showing positive amplification of 16s rRNA for *E. faecalis* at 310 bp fragment
Lane L:DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

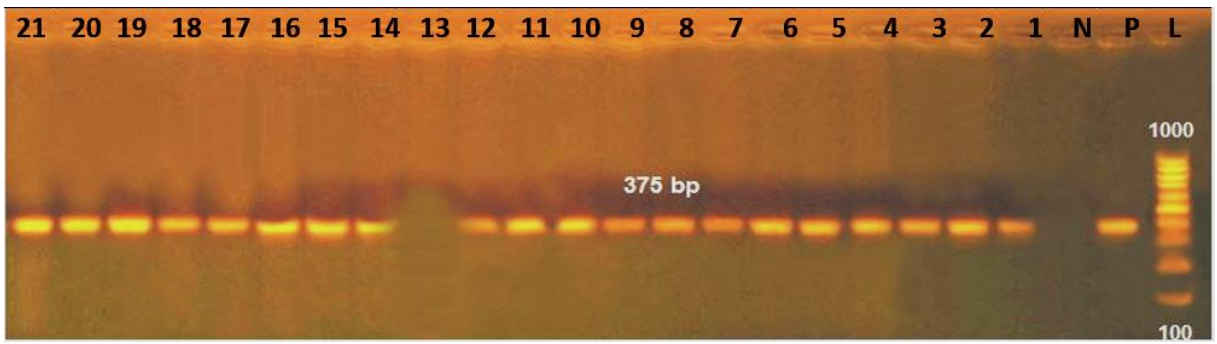


**Fig (3):** Agarose gel electrophoresis showing positive amplification of *asa1* gene of *E. faecalis* at 375 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

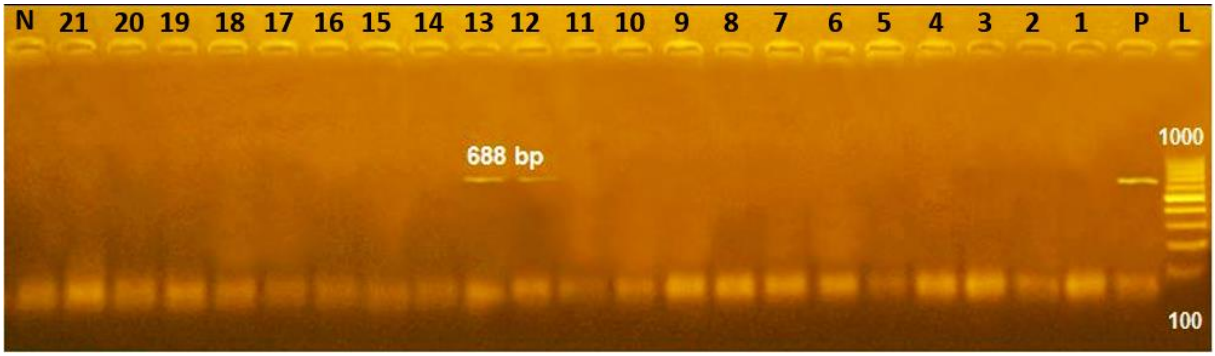


**Fig (4):** Agarose gel electrophoresis showing positive amplification of *cylA* gene of *E. faecalis* at 688 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

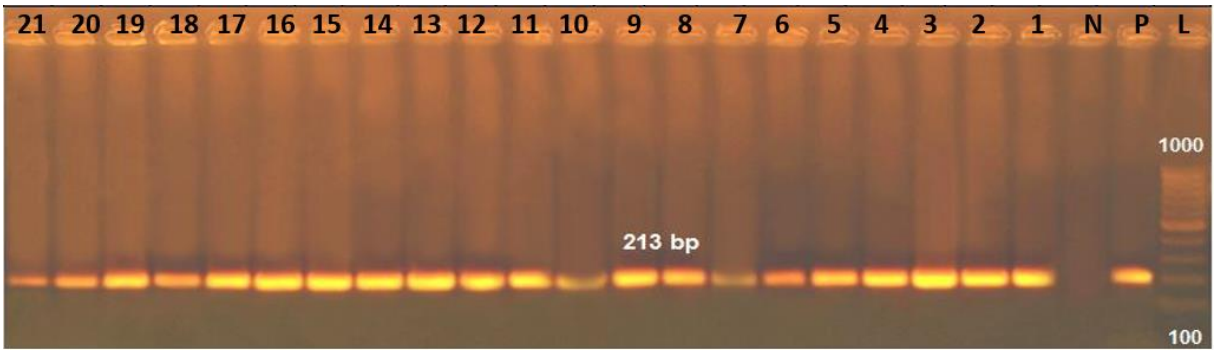


**Fig (5):** Agarose gel electrophoresis showing positive amplification of *gel*E gene of *E. faecalis* at 213 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.


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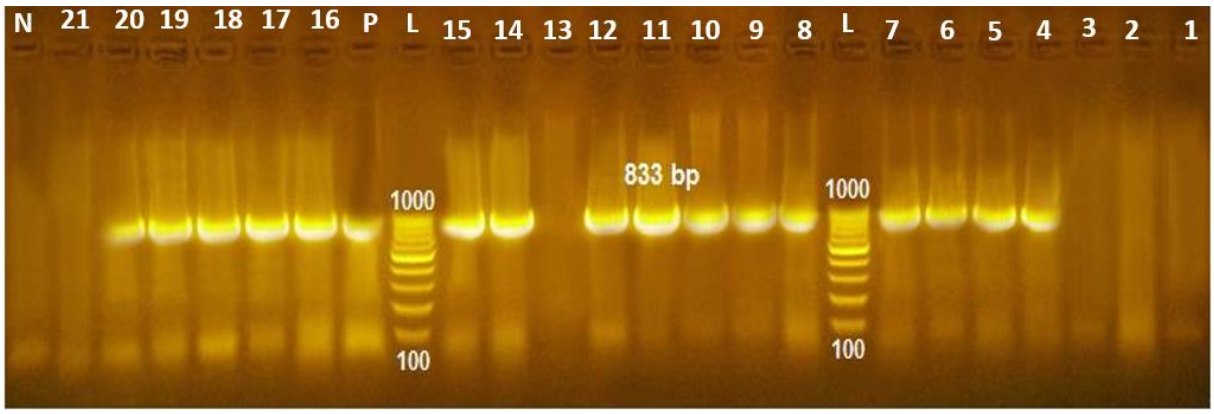


**Fig (6):** Agarose gel electrophoresis showing positive amplification of *bla*Z gene of *E. faecalis* at 833 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

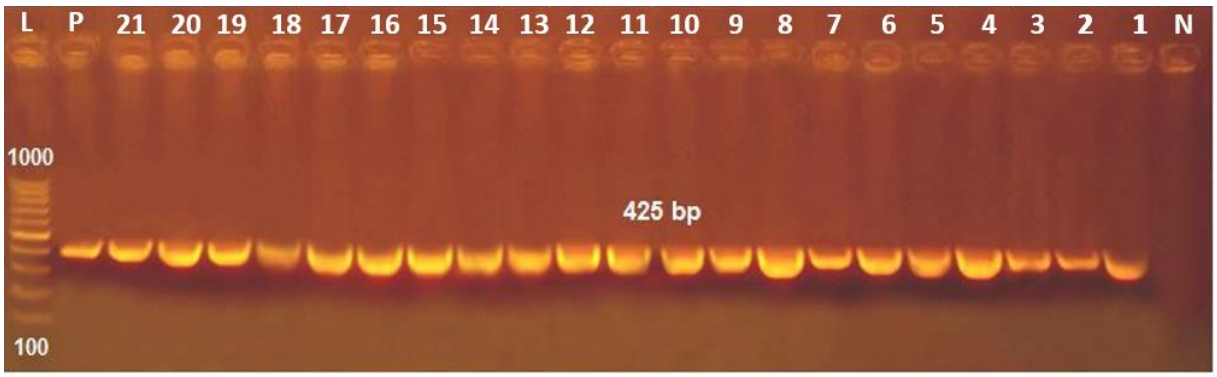


**Fig (7):** Agarose gel electrophoresis showing positive amplification of *erm*B gene of *E. faecalis* at 425 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

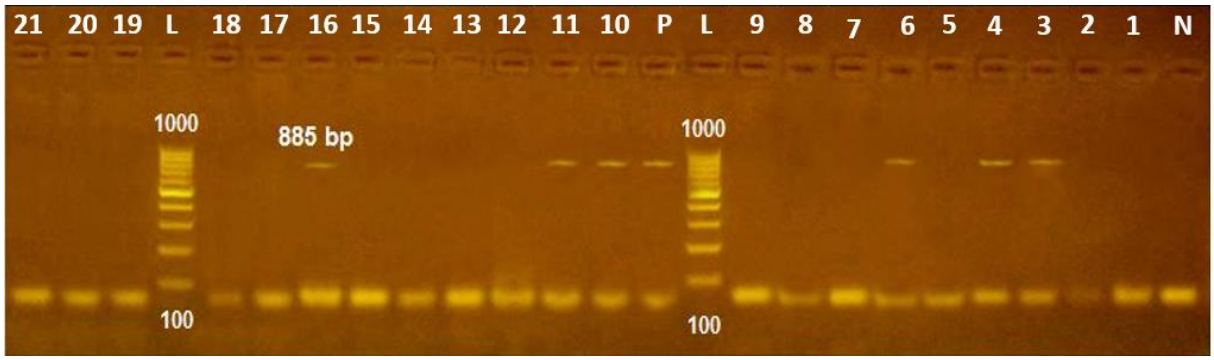


**Fig (8):** Agarose gel electrophoresis showing positive amplification of *van*A gene of *E. faecalis* at 885 bp fragment
Lane L: DNA marker (cat. no. SM0243) from Fermentas (100-1000 bp), lane P: positive control, lane N: negative control.

| | | | |
|---|---|---|---|
| ***L*** | ***100-1000 bp. ladder*** | **10** | ***E.faecalis* from chicks air sacs** |
| **P** | **Positive control** | **11** | ***E.faecalis* from chicks yolk sac** |
| **N** | **Negative control** | **12** | ***E.faecalis from* chicks yolk *sac*** |
| **1** | ***E.faecalis* from pipped yolk sac** | **13** | ***E.faecalis* from chicks heart** |
| **2** | ***E.faecalis* from piped yolk sac** | **14** | ***E.faecalis* from chicks heart** |
| **3** | ***E.faecalis* from pipped yolk sac** | **15** | ***E.faecalis* from chicks liver** |
| **4** | ***E.faecalis* from pipped yolk sac** | **16** | ***E.faecalis* from chicks liver** |
| **5** | ***E.faecalis* from pipped yolk sac** | **17** | ***E.faecalis* from chicks liver** |
| **6** | ***E.faecalis* from pipped yolk sac** | **18** | ***E.faecalis* from chicks joint** |
| **7** | ***E.faecalis* from pipped yolk sac** | **19** | ***E.faecalis* from chicks joint** |
| **8** | ***E.faecalis* from chicks bone marrow** | **20** | ***E.faecalis* from chicks joint** |
| **9** | ***E.faecalis* from chicks air sacs** | **21** | ***E.faecalis* from chicks joint** |

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