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Molecular and Antibacterial Studies of Titanium Dioxide (TiO2) *Aspergillus oryzae* Nanoparticles on Multidrug-Resistance Bacteria

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DEADLY pathogenic multidrug-resistant bacteria (MDR) are becoming more prevalent every day and represent a major danger to human health. This research aimed to isolate and quantify vancomycin resistant MDR (VRMDR) *Enterococcus faecalis* and extended-spectrum β-lactamase MDR (ESBLMDR) *Klebsiella pneumoniae* by detecting specific genes and the use of TiO2 *Aspergillus oryzae* nanoparticles as antibacterial agent against the two strains. One hundred and fifty clinical specimens were collected from Mbarret El-Asafra Hospital, 80 isolates were *E. faecalis* and 70 isolates were *K. pneumoniae*. 21/80 was found to be VRMDR *E. faecalis*, the findings showed that 76.19% of VRMDR *E. faecalis* strains harboured the *VanA* gene and 90.47% harboured the *VanB* gene, while 66.66% of them carried the two resistance genes. On the other hand, 18/70 samples were found to be ESBLMDR *K. pneumoniae*, the findings showed that 72.22% of ESBLMDR *K. pneumoniae* strains harboured the *blaTEM* gene and 61.11% harboured the *blaSHV* gene, while 33.33% of them carried the two resistance genes. 30µg/ml of nano TiO2 *A. oryzae* was found to be the minimal inhibitory concentration (MIC) for the VRMDR *E. faecalis*, while 50µg/ml of nano TiO2 *A. oryzae* was found to be the MIC for the ESBLMDR *K. pneumoniae*. The IC50 of TiO2 *A. oryzae* nanoparticles against human gastric epithelial cell line (GES1) was 563.023±31.7µg/ml compared to chloramphenicol, imipenem drugs and TiO2 nanoparticles showing (563.023±31.7µg/ml, 169.386±9.32µg/ml, 71.692±5.05µg/ml and 30.562±3.22µg/ml) respectively, showing that TiO2 nanoparticles, chloramphenicol and imipenem were more cytotoxic on GES1 normal cells than TiO2 *A. oryzae* nanoparticles.

Keywords: Antibiotics, Vancomycin resistant, Cytotoxicity

Introduction

Enterococcus faecalis is a Gram-positive bacteria cause serious infections, such as meningitis, bacteremia, periodontitis and infections of the gastrointestinal and urinary tracts (Noroozi et al., 2022). Vancomycin-resistant *Eentercoccus* (VRE) can either be inherited or gained by having one of eight different vancomycin resistant genes (*vanA* and *vanB*) for treating systemic enterococcal diseases, the glycopeptide vancomycin is the initial substitute for the penicillin-aminoglycoside variation *Enterococcus* has been found to contain a rarity of vancomycin resistant genes (Foka and Ateba, 2019). A number of enterococcal types have been shown to have *vanA* (top-grade resistant) and *vanB* with moderate to top grades of resistant (Salem-Bekhit et al., 2012). In high of this, the capacity of *enterococcus* to develop resistance to antibiotics by a variety of mechanism (plasmids, transposons and chromosomal exchange or mutation) poses a serious therapeutic obstacle (Simner et al., 2015).

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The pathogen *Klebsiella pneumoniae* is an opportunistic Gram-negative bacterium. Hospital outbreaks and nosocomial infections are caused by *K. pneumoniae* and can occur through complex pathways of cross-transmission from patients to environmental reservoirs or from patients to patients (Gravey et al., 2023). The prevalent cause of antibiotic resistance is the synthesis of extended-spectrum β-lactamases (ESBLs). Enzymes that encode for ESBLs that produce *K. pneumoniae* are mostly found inside plasmids and specifically target the β-lactam ring. Transposons and insertion sequences that promote DNA transfer between bacterial species contain certain ESBL-encoding genes. Mutations in the β-lactamases encoded by *blaSHV* and *blaTEM* are the primary cause of ESBLs. These arise from alterations of amino acids that alter the location of the active enzyme (Al-Sheboul et al., 2023).

Titanium Dioxide (TiO2) nanoparticles are extensively utilized as a photo-catalyst and in cosmetic products. TiO2 is a substance of great technical importance, particularly when used as dielectrics (Rajakumar et al., 2012).

The filamentous fungus *Aspergillus oryzae* is widely regarded as safe. Because *A. oryzae* possesses a comprehensive non-ribosomal peptide synthesis system and an amino acid precursor pool, functional peptides like β-lactam antibiotics have been effectively produced using this fungus. This filamentous fungus like other eukaryotes is the preferred host for enhancing metabolite manufacturing through self-tolerance mechanisms. These methods include the release of metabolites from cells, the alteration of metabolites to produce less toxic versions and the transfer of certain chemicals to storage organelles such vacuoles (Panchanawaporn et al., 2022).

This research sought isolate vancomycin resistant MDR (VRMDR) *E. faecalis* and extended-spectrum β-lactamase MDR (ESBLMDR) *K. pneumoniae* from clinical isolates from Egypt, find particular genes associated with different antibiotic resistance and assess the consequences of different concentrations of Titanium Dioxide (TiO2) *A. oryzae* nanoparticles to prevent and control VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* strains. Moreover, the study has conducted the cytotoxicity effect of TiO2 *A. oryzae* nanoparticles compared with the drug of choice on the human gastric epithelial cell line (GES1).

Material and Methods

Sample collection

One hundred and fifty clinical specimens (Stool, Urine, Sputum and body fluids specimens) were collected during May 2021 to March 2022. These specimens were collected from Mbarret El-Asafra Hospital in Egypt. All of the samples underwent normal processing techniques (Baveja, 2012).

Bacterial isolation and identification

Blood agar and Kenner-Faecal (K.F.) Streptococcal medium agar (selective and differential media for *Enterococcus*) (Oxoid, England) was used to culture the samples and incubated at 37°C for a day. All clinical specimens were identified as *Enterococcus* based on their colonial morphology and ability to grow esculin hydrolysis and tolerant to 6.5% sodium chloride in bile-esculin agar (Jabbari et al., 2017; Rajarajan et al., 2018).

Klebsiella were cultured on MacConkey agar medium (Lab M, United Kingdom) and incubated at 37°C for a day. All clinical isolates underwent biochemical responses and morphological examination to determine colony features on agar medium (Sikarwar and Batra, 2011).

Confirmation tests for *E. faecalis* and *K. pneumoniae* by VITEK 2 system and 16SrRNA test

First, the bacterial confirmation was done by VITEK 2 updating system 9.02 (BioMerieux, USA) (Henning et al., 2015), the bacterium was inoculated into 0.85% NaCl solution then inserted into the optical block of the DensiCheck. The suspension was diluted with 3ml of sterile saline to make 145µl of AST-GN cards. A susceptibility card and this tube were put in the cassette. The VITEK 2 cassette loading station received the tape in less than 10 minutes and the scanned cassette worksheet result was acquired 5 to 8 hours later.

The sequence match was done using Genbank database by 16S rRNA test; primer F: 5'AGAGTTTGATCCTGGCTCAG'3 & R: 5'GGCTACCTTGTTACGACTT'3 with 1500 bp was achieved utilizing the Basic Local Alignment Search Tool (BLAST) software and the sequence match facility in the GenBank database of the National Center for Biotechnology Information (NCBI) (Hall and Beiko, 2018).

Detection of multidrug-resistant isolates by disc-diffusion method

Eleven different antibiotic from various groups were used to test *E. faecalis* and *K. pneumoniae* isolates for their susceptibillity to various antibiotics including penicillins (ampicillin 10µg), carbapenems (imipenem 100µg), macrolides (erythromcyin 5µg), cephalosporins (cephradine 30µg and ceftriaxone 30µg), quinolones (ciprofloxacin 5µg and nalidixic acid 30µg), aminoglycosides (gentamycin 10µg), sulfonamides (trimethoprim/sulphamathoxazole 1.25/23.7µg), glycopeptides (vancomycin 30µg) and phenicols (chloramphenicol 30µg) (Oxoid, England).

Following an overnight development period on Muller Hinton ager plates using 0.5 McFarland inoculum, antibiotic disc first were positioned. After that the plates incubated for a further 24 hours at 37°C. The inhibitory zone's diameter was evaluated and compared to requirements established by the clinical & lab institutional structure (CLSI, 2020).

DNA extraction of E. faecalis and K. pneumoniae

The QIAamp DNAMini Kit (Cat. No. 51304 and 51306, QIAGEN, USA) was utilized to extract the bacterial DNA fragment after *K. pneumoniae* and *E. faecalis* strains were grown separately for a day at 37°C in a 5ml nutrient broth medium (Oates et al., 2012)

Detection of VanA, VanB, blaTEM and blaSHV genes

PCR analysis was used to find the *VanA, VanB, blaTEM,* and *blaSHV* genes. Table 1 shows the regions of genes that were amplified using certain primers (Metabion AG, Germany).

Replication of the PCR was done by a 25µl Dream Taq Green PCR master mix (2X concentration), forward primer (10pmol/l) 2µl, reverse primer (10pmol/l) 2µl and 5µl of DNA extracted in a total volume of 50µl with sterile H_2O Diethyl pyrocarbonate (DEPC) treatment. The cycling circumstances for gene identification were created using the Veriti 96-well Thermal Cycler (Biosystems, USA) (Su et al., 2021).

After that, a 1.5% agarose gel (Vivantis, USA) was used to electrophorese the PCR results (Bio-Rad Laboratories, Hercules, CA, USA) at 100V for approximately 30 minutes in 1X TBE buffer. The gels were stained with 2µl of 10 mg/ml ethidium bromide (Sigma, USA). UVP-gel documentary system (MultiDoc-It™ system) was used to do data analysis (ww.totallab.com, Ver.1.0.1). Purified PCR results were measured spectrophotometrically at 312nm and with micro spin filtering (SYNGENE Model 680XHR, U.K) (Hall and Beiko, 2018).

Preparation of Titanium Dioxide (TiO2) Aspergillus oryzae nanoparticles

A. oryzae was purchased from Microbial Inoculants Center - Faculty of Agriculture - Ain Shams University, Egypt. Stock cultures were kept in a refrigerator at 4°C until they were needed. The starting culture for the production of nanoparticles was created by sub culturing an actively developing stock culture and incubated for 72 hours at 24°C (Raliya and Tarafdar, 2014).

TABLE 1. The particular primer sequences utilized to amplify gene regions.

Genes	Primers (5'-3')		Tm°C	Product size (bp)	Ref.
VanA	Forward	GGGAAAACGACAATTGC	50.0	732	(Depardieu et al., 2004)
	Reverse	GTACAATGCGGCCGTTA	50.0		
VanB	Forward	ACGGAATGGGAAGCCGA	52.0	647	(Depardieu et al., 2004)
	Reverse	TGCACCCGATTTCGTTC	52.0		
blaTEM	Forward	CATCGAGCTGGATCTCAACA	55.0	478	(AbdElMongy et al., 2018)
	Reverse	TTGCCGGGAAGCTAGAGTAA	55.0		
blaSHV	Forward	CTTTCCCATGATGAGCACCT	54.0	606	(AbdElMongy et al., 2018)
	Reverse	GGGGTATCCCGCAGATAAAT	54.0		

A. oryzae ATCC-325 was isolated and injected into broth medium as a single colony. The flasks were incubated at 37°C and 200 rpm in a mechanical shaker. Following five days of incubation, the inoculation medium was centrifuged for ten minutes at 1000 rpm in order to extract the cells. The cells were then twice washed with sterile D.W. before being resuspended in 100ml of D.W., and the combination was then incubated for 24 hours at 35°C. Following the incubation period, the mixture was centrifuged to extract the supernatant or cell-free filtrate, which was then utilized as a biocatalyst to produce Titanium Dioxide (TiO2) nanoparticles by mixing with TiO2 98% while stirring for one hour, resulting in a final concentration of 5mM. One N NaOH was added to the mixture drop by drop until the pH reached eight. The precipitate's transition from light yellow to white color signifies that TiO2 nanoparticles have successfully formed. Following a 24-hour incubation period, the white precipitate was removed, dried in an oven at 200°C for three hours, and then cleaned three times using high purity H_2O (Milli-Q) (Abdel-Maksoud et al., 2023)

Antibacterial effect of A. oryzae, Titanium Dioxide and TiO2 A. oryzae nanoparticles separately on VRMDR E. faecalis and ESBLMDR K. pneumoniae

The filter paper disk diffusion technique has been used to evaluate the antibacterial properties of nano titanium dioxide, nano *A. oryzae* and nano TiO_2 *A. oryzae* separately. Filter paper discs (about 6 mm in diameter) were placed on Muller-Hinton agar surface, each containing 50µl of the nano titanium dioxide, nano *A. oryzae* and nano TiO_2 *A. oryzae* separately. Agar plates are then incubated at 37°C for 24 hours. The inhibitory zone diameter was assessed (Daoud et al., 2019).

Serial dilution of TiO2 *A. oryzae* nanoparticles was done VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* separately was overnight sub cultured. on Mueller-Hinton agar in order to research their impact on it.

The minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC) were detected by using dilutions of TiO2 *A. oryzae* nanoparticles made in D.W. (1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100µg/ml) (Hansen et al., 2018). 95ml of nutrient broth, 100ml of successive TiO2 *A. oryzae* nanoparticle dilutions and 5ml of microbial inoculum were used to fill the tubes.

Negative control tubes contain bacterial suspension without TiO2 *A. oryzae* nanoparticle, whereas positive control tubes only contain TiO2 *A. oryzae* nanoparticle suspension. For 24 hours, every tube was kept in an incubator at 37°C. MIC and MBC values were calculated spectrophotometrically by measuring optical density at 600nm on a spectrophotometer (T80 UV/VIS Spectrometer, UK) (Mutlu-Ingok et al., 2021).

Characterization of TiO2 A. oryzae nanoparticles

Transmission electron microscopy (TEM) at an accelerating voltage of 200.0 kV was used (JEOL JEM-1400 series TEM, Japan) to study the morphological size of TiO2 *A. oryzae* nanoparticles. First, 1mg of TiO2 *A. oryzae* nanoparticles was suspended in 10ml of distilled water and then 2µl drops of nanoparticles were placed onto a parafilm and directly put on electron microscope (E.M.) grids. Finally, the filter paper was used to wick away specimen drop and placed in a petri dish.

Energy dispersive X-ray (EDX) analysis was carried out in high vacuum mode with JCM-6000PLUS apparatus, which has a backscattered electron detector for secondary electrons with a resolution of 126.1 eV (EDAX Inc., USA). TiO2 *A. oryzae* elemental composition could be identified. After being signified, the as-formed powder was suspended in high purity water (Milli-Q) and a few drops were dropped onto the TEM-carbon grid surface. Prior to analysis, the loaded grid is left dry (Correa et al., 2016).

Detection of effects TiO2 A. oryzae nanoparticles on VRMDR E. faecalis and ESBLMDR K. pneumoniae by using TEM

The effect of TiO2 *A. oryzae* nanoparticles on VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* separately were determined by TEM images (JEOL JEM-1400 series TEM, Japan) to study the morphology of *E. faecalis* and *K. pneumoniae* separately as a control sample compared to standard McFarland inoculum of the treated one. First, bacterial suspension was cultured in 2ml nutrient broth and incubated at 37°C for 24 hours then TiO2 *A. oryzae* nanoparticles were added the bacterial suspension then centrifuged and the palette was taken then fixated in glutaraldehyde & osmium tetroxide, and dehydrated by adding alcohol.

The sample was then encased in epoxy resin. A microtome section was obtained with a

thickness of between 500 and 1000m. (Leica Ultracut UCT Ultramicrotome). The camera leica ICC50 HD was used to analyse thin sections that had been dyed with toluidine blue (1X).

Cytotoxicity assay of TiO2 A. oryzae nanoparticles compared with imipenem and chloramphenicol drugs

The American Type Culture Collection provided the human gastric epithelial cell line (GESI), which was cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) (Grand Island, NY, USA), 10ug/ml insulin (Sigma), and 1% penicillin-streptomycin (Sigma). Cell plates ($1x10^3$cells/well) were placed on a 96-well plate with 100ul of the tested chemical in each well and 100µl of full growth media for one day (Chen et al., 2018).

The original enzymatic reduction modification of viability assay was used to calculate cytotoxicity 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to generate blue crystals named formazan (Elshal et al., 2022).

The MTT *in vitro* cytotoxicity measurement technique performed well on multiwell plates. 10% of the growth medium was added to each vial of MTT [M-5655] after it had been reconstituted with 3ml of media. Cultures were placed back in the incubator for 2 hours. Following this, formazan crystals were removed by adding MTT solubilization solution [M-8910] in an amount equal to the volume of the original culture medium. Different concentrations of cells were incubated with TiO2 *A. oryzae* nanoparticles (4.0, 16.0, 63.0, 250.0, 1000.0 µg/l) and imipenem and chloramphenicol drugs (0.4, 1.6, 6.3, 25.0, 100.0 µg/l), dissolved in 10% FBS for 24 hrs. Spectrophotometrically (BioTek Instruments, Inc., Winooski, VT, USA) measured absorbance at wavelength 450nm and then the plates were measured.

Results

One hundred and fifty clinical specimens were collected from Mbarret ElAsafra Hospital during May 2021 to March 2022. Eighty isolates out of 150 (53.33%) were identified as *Enterococcus* while seventy isolates out of 150 (46.67%) were identified as *Klebsiella.* Forty-three isolates out of 80 (53.75%) were identified as *E. faecalis* by the morphology of colonies, gram stain, growth in Azid Maltose agar media, non-fermentation of arabinose and positive results for esculin hydrolysis, while negative results for oxidase and catalase tests. Thirty-five isolates out of 70 (50.0%) were identified as *K. pneumoniae* by morphologically showed lactose fermenting mucoid colonies on MacConkey agar plates and positive results for citrate, urease, voges-proskauer tests and sugar (glucose, sorbitol, mannitol, sucrose and amygdalin) fermentation. All isolates were identified by using VITEK diagnostic systems and confirmed by 16S rRNA test.

The sequences of each isolate were compared to type strains acquired from the Ribosomal Database Project (RDP) using the sequence match tool and BLAST program in the GenBank database at the NCBI, as seen in figure 1.

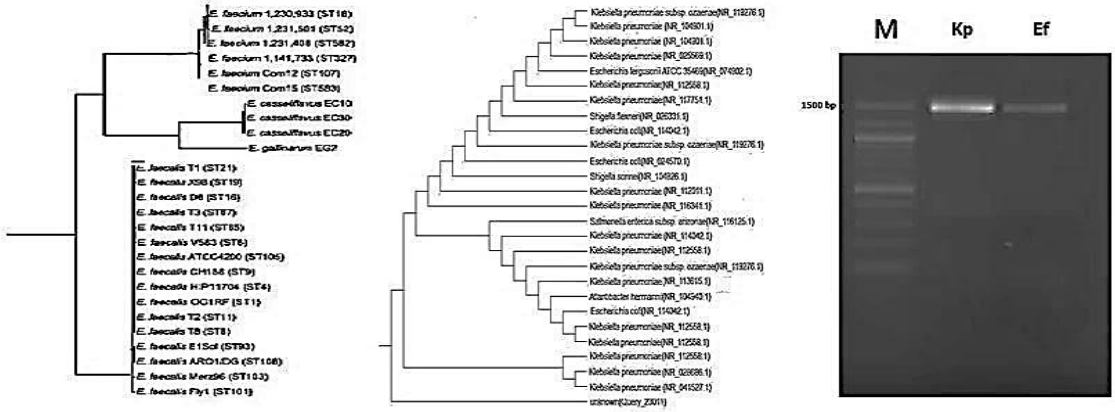

Fig. 1. Phylogenetic tree of the isolated E. faecalis and K. pneumoniae. Sample Kp and Ef was confirmed test for identification of K. pneumoniae and E. faecalis respectively.

The disc diffusion plate technique was utilized to assess the antibiotic susceptibility of forty-three *E. faecalis* and thirty-five *K. pneumoniae* samples. 48.83% (21 out of 43) of them were found to be VRMDR *E. faecalis* strains (Table 2) while 51.42% (18 out of 35) of them were found to be ESBLMDR *K. pneumoniae* strains (Table 3).

VanA (732 bp) and *VanB* (647 bp) genes responsible for vancomycin-resistance in MDR *E. faecalis* strains. Based on the PCR amplification findings, 16 strains out of 21 (76.19%) harbored the *VanA* gene and 19 isolates (90.47%) harbored the *VanB* gene, while fourteen isolates (66.66%) carried the two genes (Figure 2). *blaTEM* (478 bp) and *blaSHV* (606 bp) genes responsible for extended-spectrum β-lactamase in MDR *K. pneumoniae* strains. According to the results of the PCR amplification, 13 strains out of 18 (72.22%) harbored the *blaTEM* gene and 11 isolates (61.11%) harbored the *blaSHV* gene, while six isolates (33.33%) carried the two genes (Figure 3).

Characterization of TiO2 *A. oryzae* nanoparticles was done by electron microscope transmission (TEM) at an accelerating voltage of 200.0 kV to study the morphology of the TiO2 *A. oryzae* nanoparticles (18.2nm). EDX analysis was performed at 15.0 kV to study the morphology of the TiO2 *A. oryzae* nanoparticles (200 µm) (Figure 4)

The minimal inhibitory concentration (MIC) for the VRMDR *E. faecalis* strain harboring the *VanA* and *VanB* genes was 30µg/ml nano TiO2 *A. oryzae*, while the MIC for the ESBLMDR *K. pneumoniae* strain harboring the *blaTEM* and *blaSHV* genes was 50µg/ml nano TiO2 *A. oryzae*.

Nano TiO2 *A. oryzae***,** Nano *A. oryzae* and Nano TiO2 separately were tested for their antibacterial activity on VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* strains separately containing the resistance genes by the filter paper disc method. Figure 5 illustrates that the mean diameter of the growth inhibition zone of Nano TiO2 *A. oryzae* (14mm) and Nano TiO2 (22mm) on VRMDR *E. faecalis,* Nano TiO2 *A. oryzae* (19mm) and Nano TiO_2 (26mm) on ESBLMDR *K. pneumoniae* were larger than that of Nano *A. oryzae* (10mm) on VRMDR *E. faecalis* and Nano *A. oryzae* (13mm) on ESBLMDR *K. pneumoniae.*

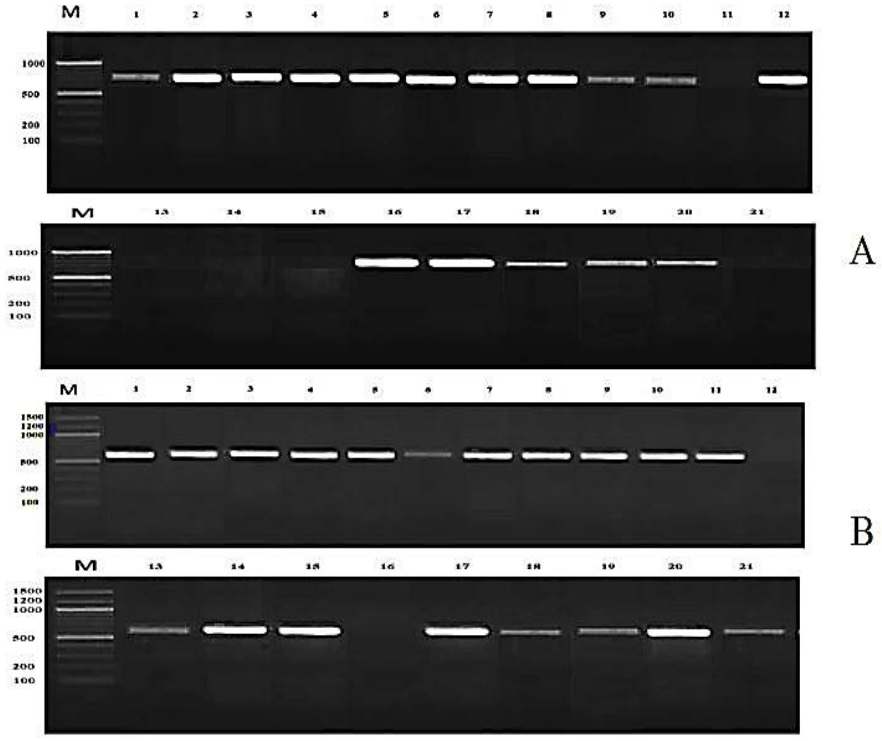

Fig. 2. A. Agarose gel electrophoresis show the amplified 732 bp DNA fragment *VanA* gene of VRMDR *E. faecalis*. B: Agarose gel electrophoresis show the amplified 647 bp DNA fragment *VanB* gene of VRMDR *E. faecalis*. M as Marker & 1, 2, ….to 21 as no. of sample.

TABLE 2. Percentage of susceptible (S), intermediate (I), and resistant (R) VAMDR *E. faecalis:*

Antibiotic susceptibility	Penicillin	Carbapenem	Cephalosporins		Macrolide	Sulfonamide	Glycopeptide	Aminoglyoside	Quinolone		Phenicol
	AM	IPM	CE	CRO	E	SXT	VA	CN	CIP	NA	C
% of susceptibility	24.89	90.12	26.68	35.66	28.00	31.71	00.00	30.90	30.00	28.00	80.12
% of intermediate	39.45	9.88	37.66	40.33	37.66	28.96	00.00	35.66	42.33	45.33	19.88
% of resistance	35.66	00.00	35.66	24.01	34.34	39.33	100.00	33.44	27.67	26.67	00.00

TABLE 3. Percentage of susceptible (S), intermediate (I), and resistant (R) ESBLMDR *K. pneumoniae:*

Antibiotic susceptibility	Penicillin	Carbapenem	Cephalosporins		Macrolide	Sulfonamide	Glycopeptide	Aminoglyoside	Quinolone		Phenicol
	AM	IPM	CE	CRO	E	SXT	VA	CN	CIP	NA	C
% of susceptibility	00.00	70.12	00.00	00.00	30.00	31.71	00.00	30.90	30.00	30.00	94.80
% of intermediate	00.00	29.88	00.00	00.00	35.66	25.96	00.00	35.66	42.33	40.67	5.20
% of resistance	100.0	00.00	100.0	100.0	34.34	42.33	100.00	33.44	27.67	29.33	00.00

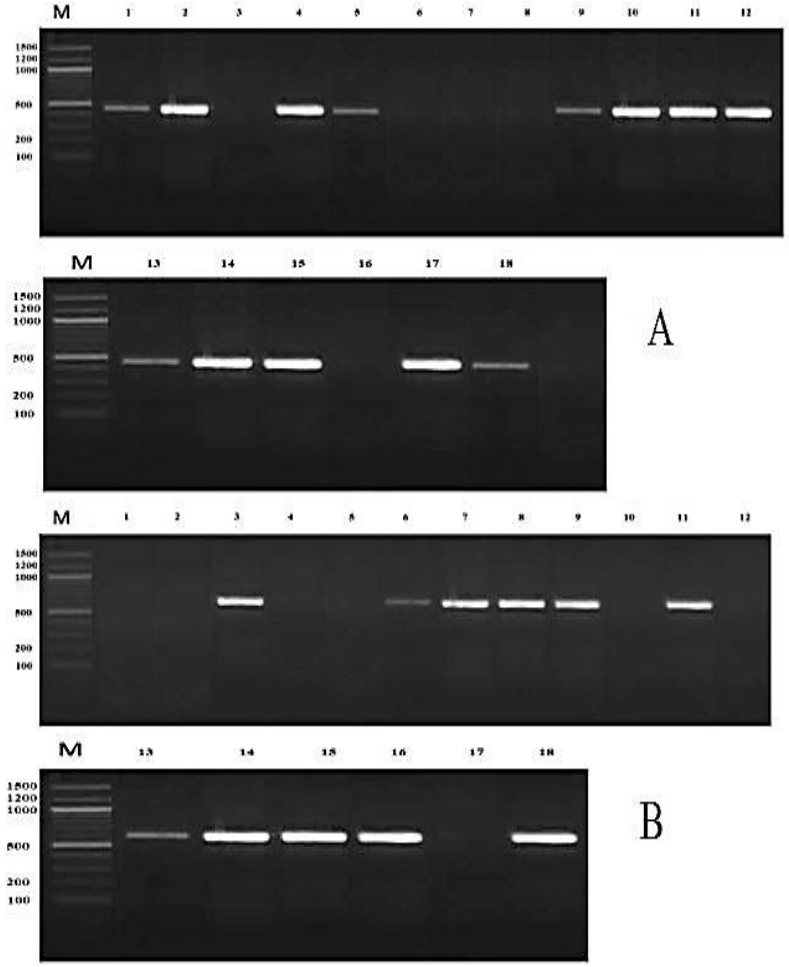

Fig. 3. A. Agarose gel electrophoresis show the amplified 478 bp DNA fragment *blaTEM* gene of ESBLMDR *K. pneumoniae*. B: Agarose gel electrophoresis show the amplified 606 bp DNA fragment *blaSHV* gene of ESBLMDR *K. pneumoniae*. M as Marker & 1, 2,….to 18 as no. of sample.

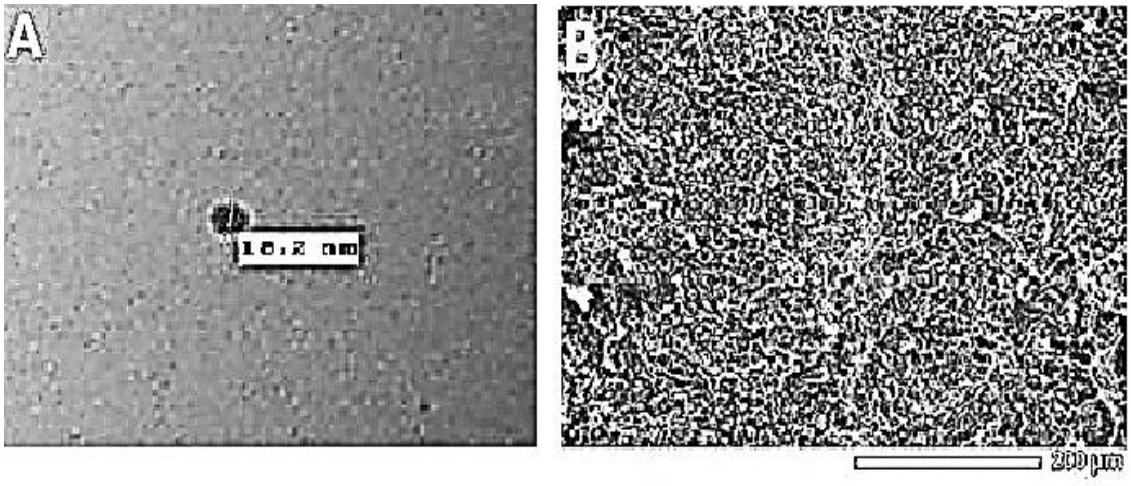

Fig. 4. Characterization of TiO2 *A. oryzae* nanoparticles molecular size using TEM (A: nanoparticle size 18.2 nm) and EDX (B: size 200 µm).

The effect of TiO2 *A. oryzae* nanoparticles on VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* separately strains harboring the two resistance genes were determined by TEM images to study the changes in morphology of bacterial strain compared to the control untreated bacteria.

It was found that TiO2 *A. oryzae* nanoparticles affected by the damaging the cell wall turning it ghost-like as a dead cell (the cell outline remains visible, but its nuclear material and cytoplasmic structures are not stainable formerly) (Figure 6).

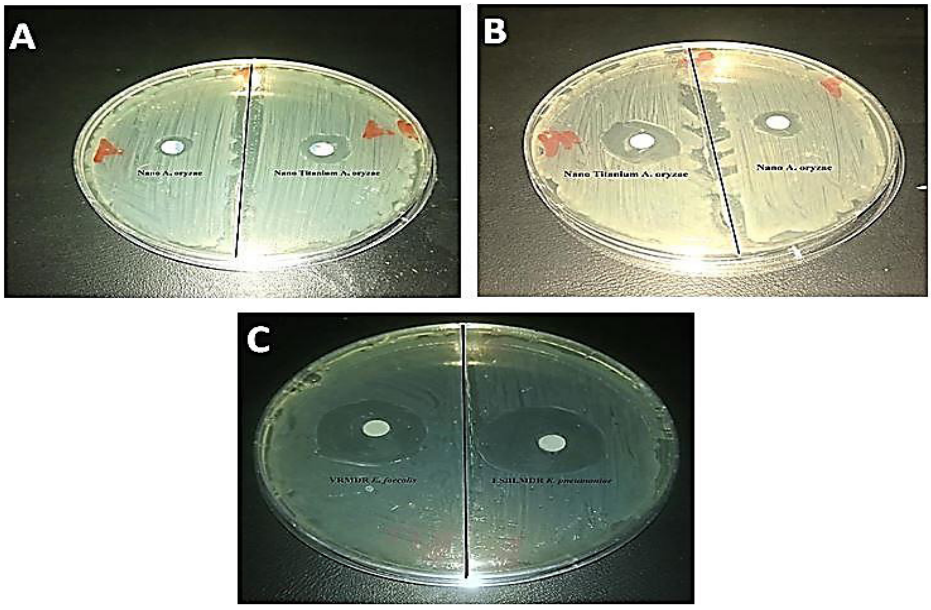

Fig. 5. Bioassay of Nano TiO2 *A. oryzae* and Nano *A. oryzae* separately on VRMDR *E. faecalis* (A), and Nano TiO2 *A. oryzae* and Nano *A. oryzae* separately on ESBLMDR *K. pneumoniae* (B), while Nano TiO2 only on both strains separately (C) by agar disc diffusion method.

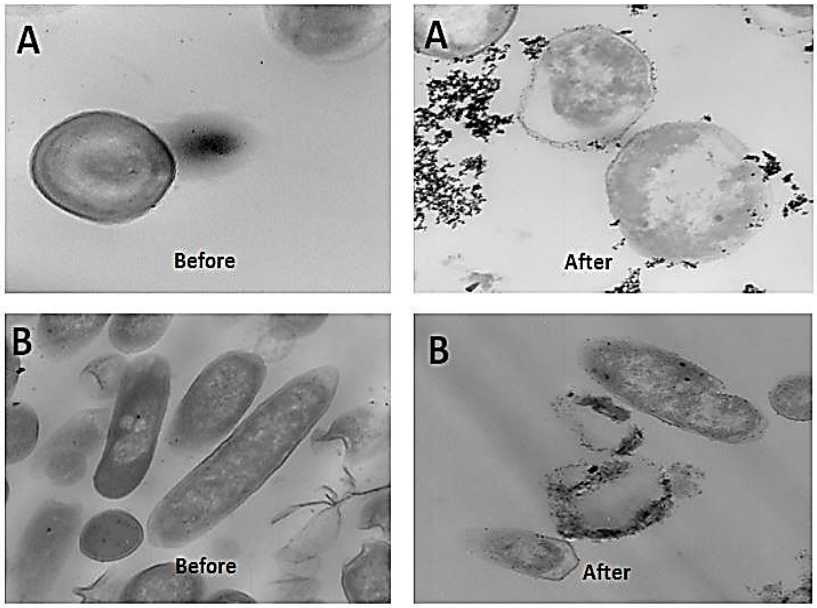

Fig. 6. TEM micrographs of untreated VRMDR *E. faecalis* and after adding TiO2 *A. oryzae* nanoparticles as treated VRMDR *E. faecalis* (A), untreated ESBLMDR *K. pneumoniae* and after adding TiO2 *A. oryzae* nanoparticles as treated ESBLMDR *K. pneumoniae* (B).

TABLE 4. Cytotoxicity test of TiO2 *A. oryzae* nanoparticles, TiO2 nanoparticles, Chloramphenicol and Imipenem drugs on GES1 normal cells:

Sample	Nano TiO2 *A. oryzae* / GES1				Chloramphenicol / GES1				Imipenem / GES1				Nano TiO2 / GES1			
Conc. of dilutions (µg/ml)	1000	250	63	16	4	100	25	6.3	1.6	0.4	100	25	6.3	1.6	0.4	1000	250	63	16	4
Mean value	0.218	0.272	0.312	0.358	0.413	0.26	0.35	0.44	0.46	0.52	0.29	0.35	0.41	0.447	0.466	0.24	0.32	0.41	0.45	0.44
% Percentage	48.42	55.72	62.04	75.61	86.99	41.6	59.1	72.0	77.2	86.9	40.4	56.4	68.7	73.19	77.27	38.2	52.3	60.1	68.5	70.19
Cytotoxicity IC50	563.023 µg/ml					169.386 µg/ml					71.692 µg/ml					30.562 µg/ml				
S.D. (±)	31.7					9.32					5.05					3.22				

The MTT reduction test was used to determine the vitality of the cells. These outcomes demonstrated that imipenem 100µg (the drug of choice to treat VRMDR *E. faecalis*) and TiO2 nanoparticles had a more potent inhibitory activity towards GES1 normal cells than TiO2 *A. oryzae* nanoparticles. On the other hand, chloramphenicol 30µg (the drug of choice to treat ESBLMDR *K. pneumoniae*) and TiO2 nanoparticles had a more potent inhibitory activity towards GES1 normal cells than TiO2 *A. oryzae* nanoparticles.

The inhibitory concentration that is half as large (IC50) was used to measure the cytotoxicity of TiO2 *A. oryzae* nanoparticles compared to chloramphenicol, imipenem drugs and TiO2 nanoparticles showing (563.023±31.7µg/ml, 169.386±9.32µg/ml, 71.692±5.05µg/ml and 30.562±3.22µg/ml) respectively. This indicates that chloramphenicol, imipenem drugs and TiO2 nanoparticles were more cytotoxic on GES1 normal cells than TiO2 *A. oryzae* nanoparticles (Table 4).

Discussion

Antibiotic resistance in microorganisms may increase due to using broad-spectrum antibiotics to treat infections (Ashraf and Iqbal, 2020). The population of *Enterococcus* may become resistant as a result of the use of antibiotics. Horizontal gene transfer is essential to accelerating the spread of resistance (Palmer et al., 2010). For infections brought on by MDR *K. pneumoniae*, treatment choices are frequently restricted. Since the development and widespread usage of new generation extended range antibiotics, the incidence of bacterial species resistant to several drugs has increased dramatically (Odari and Dawadi, 2022).

In the current study, 53.75% (43/80) were identified as *E. faecalis* while 50.0% (35/70) were identified as *K. pneumoniae* from distinct clinical specimens. 48.83% (21 out of 43) of them were found to be VRMDR *E. faecalis* strains while 51.42% (18 out of 35) of them were found to be ESBLMDR *K. pneumoniae* strains. It's possible that Egypt's excessive or incorrect usage of antibiotics is to blame for increasing MDR conditions.

Shridhar and Dhanashree (2019) conducted a similar investigation found that 46.6% were identified as *E. faecalis*. In a study done by Attia et al., (2017) found that VRMDR *E. faecalis* revealed 53.5% of the isolates. In a different

research, individuals in Iraq who had community-acquired pneumonia had a cumulative prevalence of *K. pneumoniae* of 31.9% (Raouf et al., 2022). This result was greater (18.0%) than the earlier research by Temesgen et al. (2019) from Ethiopia and lower (54.0%) than the prior study by Jaaffar et al. (2019) from Iraq. According to a research by Raouf et al. (2022), 65.9% of the isolates have ESBLMDR *K. pneumoniae*. This result was consistent with earlier studies from Brazil (84.0%) (Ferreira et al., 2019).

The current study showed that VRMDR *E. faecalis* cases were highly resistant to the Glycopeptides group (vancomycin 100%), Sulfonamide group (trimethoprim/ sulphamathoxazole 39.33%), Penicillin group (ampicillin 35.66%), and Cephalosporins group (cephradine 35.66% and ceftriaxone 24.01%). In a study done by Ali et al., (2014) who found sixteen (81.2%) MDR isolates were acquired from all of the specimens. Their MDR isolates showed a high incidence of erythromycin and tetracycline resistance (81.2%). In this study showed that ESBLMDR *K. pneumoniae* cases were highly resistant to the Penicillin group (ampicillin 100.0%), Cephalosporins group (cephradine 100.0% and ceftriaxone 100.0%), Glycopeptides group (vancomycin 100%) and Sulfonamide group(trimethoprim/sulphamathoxazole 42.33%). Raouf et al. (2022) conducted a study in Iraq and discovered that *K. pneumoniae*, which is susceptible to imipenem, had high resistance rates to ceftazidime (100.0%), cefotaxime (97.6%), aztreonam (95.1%), ceftriaxone (92.7%), tetracycline (70.7%), and trimethoprim (65.9%). These findings bore a striking resemblance to those reported by Chinese researchers Liu et al. (2019) and French researchers Fils et al. (2021).

The PCR product for VRMDR *E. faecalis* revealed that 76.19% carried the *VanA* gene and 90.47% carried the *VanB* gene, while 66.66% carried the two genes. On the other hand, The PCR product for ESBLMDR *K. pneumoniae* revealed that 72.22% carried the *blaTEM* gene and 61.11% carried the *blaSHV* gene, while 33.33% carried the two genes.

Amplicons are generated by PCR, pooled and subsequently sequenced. Amplicon sequencing can detect variants at very low levels and frequencies. The method allows for multiplexing of samples, where hundreds of PCR fragment sequences can be determined simultaneously. These multiplexing capabilities have made amplicon sequencing efficient at covering large genomic regions. Amplicon sequencing also makes data interpretation during downstream processing more manageable in comparison to data analysis following whole genome sequencing.

Regarding the detection rates of the *VanA* and *VanB* phenotypes, many investigations conducted in various nations produced inconsistent findings. 90.9% of vancomycin resistant *E. faecalis* (VRE) isolates, according to a study by El-Shafei et al., (2008), had the *VanA* genotype. Conversely, in Surendra et al., (2012) analysis, no *VanA* resistance genotype was found, while every isolate tested positive for the *VanB* resistant genotype. Two isolates showed evidence of both the *VanA* and *VanC* genes. El Shenawy et al., (2016), discovered (66.7%) resistance to the *VanA* gene in resistant isolates in a different investigation. They also discovered (21.05%) resistance to the *VanB* gene in the same isolates.

According to Eftekhar et al., (2012) study conducted in Iran, the prevalence of *blaSHV* and *blaTEM* among ESBLMDR *K. pneumoniae* strains was 43.14% and 35.29%, respectively. ESBLMDR *K. pneumoniae* was found to be 30.5% in a recent Iranian research, whereas the rates for *blaSHV* and *blaTEM* were 57% and 30.5%, respectively (Moosavian and Deiham, 2012).

In the current study, the mean diameter of the growth inhibition zone of Nano TiO2 *A. oryzae* (14mm) on VRMDR *E. faecalis* and Nano TiO2 *A. oryzae* (19mm) on ESBLMDR *K. pneumoniae* were larger than that of Nano *A. oryzae* (10mm) on VRMDR *E. faecalis* and Nano *A. oryzae* (13mm) on ESBLMDR *K. pneumoniae.*

The MIC for the VRMDR *E. faecalis* strains harboring the *VanA* and *VanB* genes was 30µg/ ml nano TiO2 *A. oryzae*, while the MIC for the ESBLMDR *K. pneumoniae* strains harboring the *blaTEM* and *blaSHV* genes was 50µg/ml nano TiO2 *A. oryzae*. Consequently, as detected by TEM, TiO2 *A. oryzae* nanoparticles could affect by damaging the cell wall, nuclear material, and cytoplasmic structures.

According to a study by El-Apasery et al. (2022), nano TiO2 have an excellent antibacterial activity. According to a study by Zhang et al. (2015), the two pathogenic bacteria had distorted shapes and their cell membranes had been compromised, allowing a large amount of proteins and nucleic acids to leak out and impeding the bacterial

cells' ability to grow normally. Accordingly, it is hypothesized that the pathogen cell membrane's integrity was compromised by the nano-TiO2 composite membrane solution, which increased the membrane's permeability and caused leakage and the loss of intracellular materials, ultimately resulting in cell death (Xing et al., 2021).

Cytotoxicity was done to demonstrate the cell viability using an MTT reduction test. Results showed that TiO2 *A. oryzae* nanoparticles had more significant cell viability toward GES1 normal cells than TiO2 nanoparticles, chloramphenicol and imipenem drugs. This indicates that TiO2 nanoparticles, chloramphenicol and imipenem drugs were more cytotoxic on GES1 normal cells than TiO2 *A. oryzae* nanoparticles.

Conclusion

The work highlights the significance of TiO2 *A. oryzae* nanoparticles through green chemistry and can be employed as new nano weapons against pathogenic bacteria. TiO2 *A. oryzae* nanoparticles have a higher infectivity against VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* that contain the resistance genes and have lower cytotoxicity on GES1 normal cells than TiO2 nanoparticles, imipenem and chloramphenicol (the most effective drugs of choice on VRMDR *E. faecalis* and ESBLMDR *K. pneumoniae* respectively).

Competing interests:

There are no stated conflicts of interest by the authors.

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