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Metal Stress and Antibiotic Susceptibility Profile of Some Bacterial and Fungal Strains

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In this study the effect of metal stress and antibiotics was evaluated on pathogenic strains of bacteria and fungi, due to an alarming rate of diseases caused by emergence of multi drug resistant pathogenic microorganisms and patients, untreated due to lack of appropriate drugs. The study was performed by standard Kirby Bauer Disk diffusion method approved by National Committee on Clinical Laboratory Standards (NCCLS) and broth dilution method. The inhibitory effect was analyzed by calculating Zone of inhibition (ZOI), minimum inhibitory concentration (MIC) and minimum bactericidal/ fungicidal concentrations (MBC/MFC) values. Antibiotic ceftriaxone (ZOI=20±2mm, MIC=250-315μg/ml and MBC≤2.5μg/ml) at concentration of ≥1000μg/ml, gentamycin (ZOI=19±2mm, and MBC=2.5μg/ml) and tetracycline (ZOI=19±2mm, MIC=250-315μg/ml and MBC≤1.5±0.5 μg/ml) at ≥500μg/ml were most effective against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Citrobacter freundii, Micrococcus luteus, Staphylococcus aureus, Escherichia coli* and *Rhizopus stolonifer, Microsporum gyseum*. While gentamycin (MIC=10mg/ml and MFC=10mg/ml) and tetracycline (MIC=20mg/ml) were also found to be most effective against fungus *R. stolonifer, M. gyseum* and *P. crysogenum*. The metal stress also significantly contributed in the inhibition of growth of bacterial strains as compared to the fungal strains. Cr^{2+} and Cu^{2+} at concentration of ≥40mg/ml gave highest antibacterial activity than other metal ions. The Cu^{2+} was observed to be toxic at concentration ≥80mg/ml for bacteria. While Mn^{2+} found resistant against bacterial strains but it found to be most effective against all selected fungal strains at concentration of ≥60mg/ml with ZOI>20mm. Zn^{2+} was also effective against fungal growth at concentration of ≥80mg/ml but fails in the inhibition of growth of bacterial strains. The most prompting candidate order to inhibit the growth of fungal strains investigated in this study is as follows: $Mn2^{+} > Zn^{2+} > Cu^{2+} > Cr^{2+} > Fe^{2+}$. This study suggests that metal ions may be used as antimicrobial/antifungal agents along with the antibiotics such as tetracycline, ceftriaxone and gentamicin and may be good drug of choice against bacterial and fungal pathogenesis in future.

Key words: Antibacterial, Metal Stress, ZOI, MIC, MBC and MFC.

The increased numbers of resistant organisms have made susceptibility testing a crucial aspect of the treatment of serious bacterial/ fungal illness. The World Organization for Animal Health (OIE), the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) reports resistant pathogenic bacteria as a serious global human and animal

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health problem. The wide spread use of antibiotics plays a significant role in the emergence of resistant bacterial and fungal species. The bacterial growth is inhibited by antibiotics means the multiplication of the cells is decreased significantly. This may result from an effect on the cell growth rate due to cell lysis. In the case of the penicillins and cephalosporins the target site is usually be the cell wall (Linnet P. E. *et al.,* 1973; Tomasz, 1979). However, it has not been established whether or not the rate of cell growth prior to rapid bacteriolysis is influenced by the antibiotic. Determination of the effect of antibiotics on bacterial and fungal growth by means of viable counts or by turbidimetric methods does not establish the rate of cell growth inhibition. The spectrophotometric measurements indicate the growth of the population of cells as a whole but not the rate of growth of the individual, because as time proceeds the proportion of the population capable of further growth may diminish as a result of the action of an antibiotic.

Apart from the antibiotics the metal stress affects the growth, morphology, and biochemical activities that ultimately results in decreased population biomass of microbial and fungal cells (T.M. Roane, I.L. Pepper, 2000). Heavy metals displaces essential metals from their native binding sites and hence results in damage of the cell membranes, alter enzymatic properties, and disrupt cellular functions. The metal stress can result alterations in the conformational structure of nucleic acids, proteins and can cause changes in biochemical functions like oxidative phosphorylation and osmotic balance (R.K. Poole *et al.,* 1989 and M.R. Bruins *et al.,* 2000). Metal ion binds through the interactions of the ions with the sulfhydryl groups of cysteine residues (Erbe *et al.,* 1995). Heavy metals-induced delay in the increased metabolic activity in response to substrate arrival, as well as oxygen mass transfer limitation during active aeration. This has been incorporated into microbial kinetics models by Sengor *et al.,* in 2009.

The present study was performed on various pathogenic bacterial and fungal strains to check the efficacy and killing potency of metal stress and antibiotics with an aim to find the antibacterial/antifungal effect by agar well diffusion method, broth dilution method. The MBC and MFC were also determined. In this study tetracycline, ceftriaxone and gentamicin proved to be drug of choice against bacterial strains. The study also suggests that antibiotics along with metal ions may be used as antimicrobial/antifungal agents to treat the diseases caused by pathogenic organisms.

MATERIALS AND METHODS

Collection of antibiotics and metal salts

Pure antibiotics from Ranbaxy Pvt. Ltd were purchased at Gwalior (M.P.) India in powder form. The Muller Hinton's Agar/Broth medium (MHA/MHB), and metal ions were taken in the form of metal sulphates ($ZnSO_4$, $CuSO_4$, $Cr_2(SO_4)_3 \cdot 12(H_2O)$, $MnSO_4(H_2O)$, and $FeSO_4$), were purchased from Hi-media Pvt. Ltd.

Preparation of antibiotic and metal ion stock solutions

The powders were accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware. Stock solutions were frozen at -20ºC for future experimental work. Stock solutions were prepared by using the formula:

$$\frac{1000}{P} \times V \times C = W$$

where

P=Potency given by the manufacturer in relation to the base

V= Volume in ml required

C=Final concentration of solution (multiples of 1000)

W= Weight of the antimicrobial to be dissolved in the volume V

Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), was used. The standard tubes were prepared by mixing varying amount of 1% $BaCl_2$ and 1% H_2SO_4 in air tight tubes as Table 6. The turbidity of overnight broth culture of test organism was compared with McFarland Standard scale tubes against white background and concentration was approximated.

Inoculum Preparation

The bacterial strains *viz., Citrobacter freundii, Escherichia coli, Klebsiella pneumoniae,*

Micrococcus luteus, Pseudomonus aeruginosa, Staphylococcus aureus, and the fungal strains *viz., Rhizopus stolonifer, Microsporum gyseum,* and *Penicillium crysogenum* were collected from Department of Biotechnology, Jiwaji University Gwalior - 474002, India. The growth method was used for inoculum preparation. Three to five well-isolated colonies of the same morphological type were selected from MHA plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4 to 5 ml of MHB. The broth culture was incubated at 37°C until it achieves optimum turbidity. The turbidity of the actively growing broth culture was adjusted with broth to obtain turbidity optically comparable to that of the 0.5 McFarland standard. This resulted in a suspension containing approximately1-2x10^7CFU/ml for bacteria and 1-2x10^6 CFU/ml for fungus. Potato Dextrose Agar (PDA) and Potato Dextrose broth (PDB) were used for sub-culturing of fungal stains at 28°C for 48 hrs in the same manner as for bacterial strains.

Disc diffusion method

The Disc diffusion method also known as Kirby-Bauer method was used for antimicrobial susceptibility testing as being recommended by the NCCLS. NCCLS is approved by FDA-USA and recommended by WHO. MHA was used for disk diffusion susceptibility testing, according to NCCLS and international guidelines. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The dried surface of a MHA plates were inoculated by streaking the swab over the entire sterile agar surface. The lid was left ajar for 5 minutes to allow for any excess surface moisture to be absorbed before impregnate well in agar. The wells of 6mm in diameter were impregnated using Eppendrof tip and were filled with 20µl of each antimicrobial agent.

Reading plates and interpretation of results

After 24 and 48 hrs of incubation each plate was examined for bacterial and fungal growth, respectively. The diameter of the zones of complete inhibition was measured, including the diameter of the disc. The zones were measured to the nearest whole millimeter using a ruler (Table 1, 4 & 5).

Determination of Minimum Inhibitory Concentration (MIC)

Broth dilution susceptibility testing method was used to determine the minimal concentration of antimicrobials to inhibit or kill the bacterial and fungal test strains. This method has an advantage that the same tubes were taken for MBC tests. 1mg/ml antibiotic was diluted serially in MHB and PDB test tubes. Fresh 20µl inoculum (for bacteria 1×10^7 CFU/ml and 1×10^6 CFU/ml for fungus) equivalent 0.5 McFarland Standard was added to each test tube and incubated for 24hr (37°C) and 48hr (28°C) for bacteria and fungus, respectively. Each extract was assayed in duplicate and each time 10 sets of test tubes were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in test tubes. MIC values were taken as the lowest dilution/ concentration of antimicrobials, which inhibit growth in the test tube observed by lack of turbidity after incubation spectrophotometrically (Table 2 & 3) (Chandrabhan *et al*., 2011 & Singh *et al*., 2009).

Determination of Minimum bactericidal/ fungicidal concentration (MBC/MFC)

The test tubes showing no visible sign of growth or turbidity in MIC were assessed for minimum bactericidal/fungicidal concentration. The control tube containing no antibiotic was subcultured (Before incubation) by spreading a loopful culture evenly, over a quarter of MHA and PDA medium plates, suitable for the growth. The plates were incubated overnight at 37°C and 48 hrs at 28°C for bacterial and fungal strains growth, respectively. The MIC of the control was read to check that the antibiotic concentrations were correct. Similarly, all test tubes not showing visible growth were processed in the same manner as the control. The least concentration of antibiotic showing no visible growth on subculturing was taken as MBC/MFC (Table 3).

RESULTS AND DISCUSSION

The specific antibacterial and antifungal susceptibility test were used on bacterial and fungal pathogenic strains; the test may influenced by variety of factors such as inoculum preparation, concentration, temperature, pH and chemical properties of agents (Pfaller, 1990). The MIC, MBC/ MFC against selected antibiotics was also performed by broth dilution method. With the fact

that there is presence of large number of bacteria and fungi in infection cycle; we evaluated antibiotics and metal ions as antibacterial and antifungal agents.

In case of ceftriaxone: all bacterial strains fall in category of intermediate (ZOI=13-17mm) at drug concentration of ≤500µg/ml whereas at higher concentration ≥1000µg/ml all strains are highly susceptible. The *P. aeruginosa* and *E. coli* were most susceptible with ZOI=25mm each. MIC=250µg/ml and MBC=2.0mg/ml was observed for *P. aeruginosa* while MIC=500µg/ml and MBC=2.5mg/ml was observed for *E. coli*. All other strains have ZOI=20mm at drug concentration of 2000µg/ml with MIC=250µg/ml and MBC=2.5mg/ml.

Tetracycline was found effective against all bacterial strains at concentration of ≥500 µg/ml. *P. aeruginosa* was most susceptible strain (ZOI=23mm) at all concentration with MIC=250-315µg/ml and MBC≤1.56mg/ml while *K. pneumoniae* showed resistance against tetracycline at ≤1000µg/ml but was susceptible at higher concentration (2000µg/ml) (ZOI=20mm) with MIC=315 µg/ml and MBC ≤1.56mg/ml.

The ampicillin did not inhibit growth of test strains at any concentration hence, all strains found resistant (Table 1). In accordance with ampicillin streptomycin was also found less effective against all test strains. But streptomycin was found most effective against *K. pneumoniae* at all concentrations ZOI ≥19mm with MIC=250µg/

Table 1. Effect of antibiotics on bacterial strains by agar well diffusion method (well diameter in mm)

Antibiotics	Test Organism	250(µg/ml)	500(µg/ml)	1000(µg/ml)	2000(µg/ml)
	Pseudomonas aeruginosa	17	17	20	25
	Micrococcus luteus	15	15	20	20
Ceftriaxone	*Citrobacter freundii*	14	15	18	20
	Staphylococcus aureus	15	15	18	20
	Escherichia coli	17	17	20	25
	Klebsiella pneumoniae	12	12	18	20
	Pseudomonas aeruginosa	23	23	25	27
	Micrococcus luteus	17	20	20	25
Tetracycline	*Citrobacter freundii*	15	19	19	20
	Staphylococcus aureus	15	15	17	20
	Escherichia coli	17	19	20	25
	Klebsiella pneumoniae	R	R	20	20
	Pseudomonas aeruginosa	R	09	10	12
	Micrococcus luteus	R	R	R	10
Ampicillin	*Citrobacter freundii*	R	09	10	10
	Staphylococcus aureus	R	09	12	12
	Escherichia coli	R	R	R	10
	Klebsiella pneumoniae	R	R	10	11
	Pseudomonas aeruginosa	15	15	20	23
	Micrococcus luteus	15	14	15	20
Streptomycin	*Citrobacter freundii*	R	R	08	10
	Staphylococcus aureus	R	R	10	10
	Escherichia coli	15	14	15	20
	Klebsiella pneumoniae	16	18	20	25
	Pseudomonas aeruginosa	22	23	25	25
	Micrococcus luteus	17	20	20	22
Gentamicin	*Citrobacter freundii*	19	19	23	25
	Staphylococcus aureus	15	17	19	20
	Escherichia coli	19	20	25	25
	Klebsiella pneumoniae	16	17	19	20

Well Diameter (mm) ≤12 Resistant, 13-17 Intermediate, ≥18 Susceptible

ml and MBC=2.5µg/ml. *C. freundii* and *S. aureus* were found resistant to streptomycin at concentration ≤1000µg/ml.

The gentamicin at a concentration of ≥250 µg/ml was found most effective against *P. aeruginosa* (ZOI=22mm, MIC=500µg/ml and MBC=2.0mg/ml), *C. freundii* (ZOI=19mm, MIC=500µg/ml and MBC=2.5mg/ml) and *E. coli* (ZOI=19mm, MIC=250µg/ml and MBC=2.5mg/ml) and were characterize as susceptible. All other strains came under category of intermediate until the concentration of drug was increased to 1000 µg/ml which rendered all strains to be susceptible. The growth of *S. aureus* was least affected by gentamicin but at highest concentration of drug ≥2000µg/ml *S. aureus* found susceptible (ZOI=20mm, MIC=1000 µg/ml and MBC=5mg/ml).

It was observed that gentamicin and tetracycline were effective against fungus *R. stolonifer, M. gyseum* and *P. crysogenum* with MIC=10mg/ml and MIC=20mg/ml, for gentamicin and tetracycline respectively. In this study tetracycline, ceftriaxone and gentamicin proved to be good drug of choice against bacterial and fungal pathogenesis. Other antibiotics were found resistant against all fungal strains.

In addition to antibiotics the study was also performed to evaluate the effect of metal ion stress on selected bacterial and fungal strains. Cu^{2+} is an important cofactor for many enzymes, including those essential for catabolic pathways and energy metabolism; however, high levels of copper are toxic (Rensing & Grass 2003). Cu^{2+} ions inhibited bacterial growth and was found effective at concentrations ≥40mg/ml where all strains were categorized as intermediate tends to be susceptible

Table 2. MIC/MBC of antibiotics against bacterial strains by broth dilution method

Antibiotics	Test Organism	MIC (µg/ml)	MBC(mg/ml)
	Pseudomonas aeruginosa	250	2.0
	Micrococcus luteus	500	2.5
Ceftriaxone	*Citrobacter freundii*	250	2.25
	Staphylococcus aureus	250	2.5
	Escherichia coli	500	2.5
	Klebsiella pneumoniae	250	2.5
	Pseudomonas aeruginosa	250	<1.56
	Micrococcus luteus	315	1.56
Tetracycline	*Citrobacter freundii*	250	1.25
	Staphylococcus aureus	250	2.5
	Escherichia coli	315	<=1.56
	Klebsiella pneumoniae	315	<1.56
	Pseudomonas aeruginosa	1000	5
	Micrococcus luteus	2500	>5
Ampicillin	*Citrobacter freundii*	1000	5
	Staphylococcus aureus	1000	5
	Escherichia coli	2500	>5
	Klebsiella pneumoniae	1000	5
	Pseudomonas aeruginosa	250	2.5
	Micrococcus luteus	500	5.0
Streptomycin	*Citrobacter freundii*	>5mg	-
	Staphylococcus aureus	5mg	5.0
	Escherichia coli	500	5.0
	Klebsiella pneumoniae	250	2.5
	Pseudomonas aeruginosa	500	2.0
	Micrococcus luteus	250	2.5
Gentamycin	*Citrobacter freundii*	500	2.5
	Staphylococcus aureus	1000	5.0
	Escherichia coli	250	2.5
	Klebsiella pneumoniae	500	2.5

Table 3. MIC/MFC of antibiotics against fungal strains by broth dilution method

Antibiotics	Test Organism	MIC (mg/ml)	MFC (mg/ml)
	Rhizopus stolonifer	50	-
Ampicillin	*Microsporum gyseum*	50	-
	Penicillium crysogenum	50	-
	Rhizopus stolonifer	25	-
Streptomycin	*Microsporum gyseum*	50	-
	Penicillium crysogenum	25	50
	Rhizopus stolonifer	10	-
Gentamicin	*Microsporum gyseum*	10	10
	Penicillium crysogenum	10	-
	Rhizopus stolonifer	20	-
Tetracycline	*Microsporum gyseum*	20	-
	Penicillium crysogenum	20	-

Table 4. Effect of metal stress on growth of bacterial strains by Agar Well Diffusion method (diameter in mm)

Metal ions	Test organism	20mg/ml	40 mg/ml	60 mg/ml	80 mg/ml
	Pseudomonas aeruginosa	R	15	17	20
	Micrococcus luteus	12	14	16	16
Zn^{2+}	*Citrobacter freundii*	10	12	18	18
	Staphylococcus aureus	12	16	18	18
	Escherichia coli	09	12	15	18
	Klebsiella pneumoniae	12	18	20	22
	Pseudomonas aeruginosa	11	13	20	23
	Micrococcus luteus	12	17	19	23
Cu^{2+}	*Citrobacter freundii*	R	12	22	24
	Staphylococcus aureus	R	16	19	21
	Escherichia coli	12	19	20	25
	Klebsiella pneumoniae	12	16	20	25
	Pseudomonas aeruginosa	14	17	19	19
	Micrococcus luteus	12	16	17	20
Cr^{2+}	*Citrobacter freundii*	13	14	17	17
	Staphylococcus aureus	14	16	20	20
	Escherichia coli	14	14	21	22
	Klebsiella pneumoniae	14	15	20	20
	Pseudomonas aeruginosa	R	R	11	20
	Micrococcus luteus	R	10	10	16
Mn^{2+}	*Citrobacter freundii*	R	12	15	20
	Staphylococcus aureus	R	15	16	18
	Escherichia coli	R	R	R	11
	Klebsiella pneumoniae	R	09	19	20
	Pseudomonas aeruginosa	11	12	15	19
	Micrococcus luteus	10	12	14	19
Fe^{2+}	*Citrobacter freundii*	10	12	14	15
	Staphylococcus aureus	10	12	13	16
	Escherichia coli	10	12	13	16
	Klebsiella pneumoniae	10	12	13	14

Well Diameter (mm) ≤12 Resistant, 13-17 Intermediate, ≥18 Susceptible

at higher concentrations ≥60mg/ml. At higher concentration ZOI ≥20mm was found for *P. aeruginosa, E. coli, K. pneumoniae* and *C. freundii,* ZOI=22mm and ZOI=19mm for *S. aureus* and *M. luteus,* respectively. The Cu^{2+} was highly toxic at concentration of ≥80mg/ml for *P. aeruginosa, S. aureus, E. coli, M. luteus* and *K. pneumoniae* with ZOI ≥20mm and falls under susceptible category (Table 4).

All bacterial strains were found resistant against Mn^{2+} ions but it found significantly most effective against all selected fungal strains at concentration of ≥60mg/ml with ZOI>20mm. Zn^{2+} was also found effective against fungal strains at concentration of ≥80mg/ml but fails to inhibit the growth of bacterial strains. The high levels of Zn^{2+} reduced the bacterial diversity, with only a very limited number of resistant bacteria survivals (Kelly *et al.,* 2003). Bacterial strains remain intermediate towards Fe^{2+} and Zn^{2+} at 80mg/ml and 60mg/ml, respectively. Fe^{2+} is directly limit the growth of bacteria and high concentrations of Fe^{2+} causes oxidative damage, due to the ability of Fe^{2+} to reduce H_2O_2 (Touati, 2000).

Cu^{2+} has less activity against fungal strains at concentration ≥60mg/ml and were categorized as resistant but at concentrations ≥60mg/ml was found most effective against *R. stolonifer* (ZOI=19mm), *M. gyseum* (ZOI=21mm) and *P. chrysogenum* (ZOI= 25mm) and categorized as susceptible. Zn^{2+} was found effective against all fungal strains at concentration ≥60mg/ml while resistant at ≥60mg/ml. There were no significant inhibition was observed against Fe^{2+} and Cr^{2+} against all fungus strains even at high concentration of e"100mg/ml and were categorized as resistant (Table 5).

Table 5. Effect of metal stress on growth of fungal strains by Agar Well Diffusion method (diameter in mm)

Metals	Test organism	20 mg/ml	40 mg/ml	60mg/ml	80mg/ml	100mg/ml
	Rhizopus stolonifer	13	16	14	20	17
Zn^{2+}	*Microsporum gyseum*	12	17	21	16	25
	Penicillium crysogenum	14	14	18	24	24
	Rhizopus stolonifer	08	11	14	20	19
Cu^{2+}	*Microsporum gyseum*	08	08	14	15	21
	Penicillium crysogenum	08	08	20	17	25
	Rhizopus stolonifer	08	16	17	17	22
Cr^{2+}	*Microsporum gyseum*	08	15	15	16	20
	Penicillium crysogenum	08	16	16	17	21
	Rhizopus stolonifer	12	15	22	24	27
Mn^{2+}	*Microsporum gyseum*	12	20	24	24	24
	Penicillium crysogenum	12	18	27	30	32
	Rhizopus stolonifer	08	09	11	12	11
Fe^{2+}	*Microsporum gyseum*	08	11	12	14	11
	Penicillium crysogenum	08	09	11	14	13

Well Diameter (mm) ≤12 Resistant, 13-17 Intermediate, ≥18 Susceptible

Table 6. McFarland Standard scale

McFarland standard Scale	1% $BaCl_2$ (ml)	1% H_2SO^4 (ml)	Approx. bacterial density value X 10^7 (cells/ml)
1	0.1	9.9	300
2	0.2	9.8	600
3	0.3	9.7	900
4	0.4	9.6	1200
5	0.5	9.5	1500
6	0.6	9.4	1800
7	0.7	9.3	2100

CONCLUSION

The metal ions stress significantly inhibited the growth of bacterial strains as compared to the fungal strains. The effects of metal ion stress on microbial cells suggest that individual strains adapt to elevated metal ion concentrations (Giller *et al.,* 1998). The metal resistance in bacteria has been associated with single or multiple drug resistance. Fortunately, microorganisms can affect the reactivity and mobility of metals. Toxic metals exert toxicity in a number of ways including the displacement of essential metals from their normal binding sites on biological molecules, inhibition of enzymatic functioning and disruption of nucleic acid structure. Khan & Scullion in 2002 investigated that the fungal strains were less sensitive to heavy metals than bacteria. In agreement to their study, we also observed that fungal strains were more resistant at low metal ions concentration ≤ 60mg/ml but found susceptible at higher concentration ≥ 60mg/ml**.** The most prompting candidate order to inhibit the growth of fungal strains investigated in this study is as follows: $Mn2^{+} > Zn^{2+} > Cu^{2+} > Cr^{2+} > Fe^{2+}$. This study suggests that metal ions may be used as antimicrobial/antifungal agents along with the antibiotics such as tetracycline, ceftriaxone and gentamicin and may be a good drug of choice against bacterial and fungal pathogenesis in future.

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