BACTERIOLOGICAL AND MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM B-LACTAMASES IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM KURDISTAN REGION, IRAQ.

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ABSTRACT

A total of 275 clinical isolates of *Klebsiella pneumoniae* were collected from three general hospitals in Duhok, Erbil, and Sulymania, during the period September 2010 to June 2011. The Minimum Inhibitory Concentration (MIC) of these isolates was measured using the Gram-negative susceptibility card (GNC) of Phoenix system. Only 187 ESBL producing *K. pneumoniae* isolates were detected by this system. These isolates were confirmed as 100% ESBLs producers by the Double Disk Synergy Test (DDST). All 187 *K. pneumoniae* isolates were 100% resistant to ampicillin, cefazolin, cefepime, ceftriaxone, cefotaxime, cefuroxime, ceftazidime, and aztreonam.

These isolates showed different percentages of resistance 81.8%, 68.5%, 65.8%, 52.4%, 50.3%, 34.2%, 25.2%, and 12.3% towards, ampicillin/sulbactam, gentamicin, trimethoprime-sulfamethoxazole, ciprofloxacin, piperacillin-tazobactam, amikacin, amoxicillin-clavulanate, and levofloxacin respectively. Molecular characterization by PCR was employed using specific primers for three different ESBLs (TEM, SHV, and CTX-M). Results obtained revealed that SHV-type ESBLs were the most common ESBL occurring in 87% of the isolates with phenotypic evidence of ESBLs production. While those for TEM-type and CTX-M-type were 60% and 58% respectively.

***Key word*: *Klebsiella pneumoniae, ESBLs, SHV, TEM, CTX-M*.**

INTRODUCTION

Since the first report in Germany in 1983 (Knothe *et al.*, 1983), the emergence of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* and *Escherichia coli* has become a serious problem in hospitalized patients worldwide (Hawser *et al*., 2009; Livermore *et al*., 2007; Paterson & Bonomo, 2005).

According to Ambler's classification, ESBLs belong to the class A β-lactamases, which possess an active-site serine residue essential for the inactivation of β-lactam antimicrobial drugs (Ambler, 1980). The term 'extended spectrum β-lactamase' was originally applied to describe the TEM (came from the patient's name, Temoniera) and SHV (sulphydryl variable) variants that can hydrolyse oxyiminocephalosporins (Paterson & Bonomo, 2005). The 'extended spectrum' activity is defined in terms of hydrolyzing oxyimino-cephalosporins or aztreonam at more than 10% of the activity of hydrolysing benzylpenicillin. Generally, ESBLs confer resistance to all penicillins, first-to-fourth generation cephalosporins and monobactams, but not to cephamycins or carbapenems (Bush and Jacoby 1995, Rupp 2003). However, like many other class A β-lactamases, ESBLs are inactivated by the β-lactamase inhibitors, such as clavulanate (Bradford, 2001).

Most of the genes encoding ESBLs are plasmid-borne and are often located on the transposons and integrons, facilitating their mobilization with other resistance determinants. Thus the genes encoding ESBLs may be easily transferred between bacteria (Eckert *et al*., 2006).

The most prevalent ESBLs are included in three groups: TEM, SHV and CTX-M (cefotaximase). The TEM enzyme was first discovered in *E. coli* in Greece (Jacoby 1997). The SHV enzymes were named after the thiol variable active site and are commonly associated with *K. pneumoniae* (Livermore 1995). A CTX-M-type ESBL is a plamid encoded ESBL related to the chromosomal β-lactamase of *Kluyvera ascorbata* (Humeniuk *et al*., 2002). This enzyme was characterized by a better hydrolyzation of cefuroxime, cefotaxime and cefepime than that of ceftazidime (Bernard *et al.*, 1992). CTX-M-type of ESBLs has spread rapidly and is now regarded as the most dominant types of ESBLs

in many countries (Livermore *et al.*, 2007; Lee *et al.*, 2009).

Materials and Methods

Bacterial Isolation

Two hundred and seventy five clinical isolates of *K. pneumoniae* were collected from various clinical specimens (urine, blood, sputum, and wound) from September 2010 to June 2011 from three general hospitals (Duhok, Erbil, Sulymania) Kurdistan region/Iraq. All these isolates were characterized using different conventional bacteriological and biochemical methods (Bagley *et al*., 1981; Monnet and Freney, 1994).

Antimicrobial Susceptibility and ESBLs Test Using the Phoenix System

The antimicrobial susceptibility and the ability to produce ESBLs of all 275 isolates were tested by the Phoenix system (Beckton Dickinson Diagnostic Systems, USA) using Gram-negative susceptibility card (GNS).

Double Disk Synergy Test (DDST)

All ESBL producing isolates were tested by Double Disk Synergy Test (DDST) with disks containing cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg) were placed 25 mm (centre to centre) from an Amoxicillin-clavulanic acid disk (30 and 10 µg, respectively), incubated at 35°C over night.

Detection of ESBL Genes by Polymerase Chain Reaction

Genomic DNA was extracted from 100 *K. pneumoniae* ESBLs-positive isolates using DNA extraction kit (Genaid, Korea). The ESBLs genes TEM, SHV, CTX-M were detected using specific pair of primers for each gene (EUROFINS, mwg, operon, Germany). Polymerase chain reactions **(**PCR) were employed as indicated Table.3, and the resulting PCR products were electrophoresed using 1.5% agarose gel.

Results and Discussion

Antimicrobial Susceptibility Test

Results from Table (1) showed that all 187 *K. pneumoniae* isolates were 100% resistant to ampicillin, cefazolin, cefepime, ceftriaxone, cefotaxime, cefuroxime, ceftazidime, and aztreonam. These isolates showed different percentages of resistance 81.8%, 68.5%, 65.8%, 52.4%, 50.3%, 34.2%, 25.2%, and 12.3% towards, ampicillin/sulbactam, gentamicin, trimethoprime-sulfamethoxazole, ciprofloxacin, piperacillin-tazobactam, amikacin, amoxicillin-clavulanate, and levofloxacin respectively. *K. pneumoniae* isolates harboring ESBLs are significantly more frequently found to be resistant to antibiotic than non-producing isolates (Mohammad *et al*., 2009; Dechen *et al*., 2009).

Table 1. Antibiotic susceptibility of ESBL-producing *K. pneumoniae* clinical isolates using Phoenix system.

Antibiotic	Resistant Isolates		Sensitive Isolates	
	No.	Percentage%	No.	Percentage%
Amikacin	64	34.22	123	65.8
Gentamicin	128	68.5	59	31.5
Ertapenem			187	100
Imipenem			187	100
Meropenem			187	100
Cefazolin	187	100		
Cefuroxime	187	100		
Cefoxitin	27	14.4	160	85.6
Ceftazidime	187	100		
Ceftriaxone	187	100		
Cefepime	187	100		
Cefotaxime	187	100		
Aztreonam	187	100		
Amoxycilin/clavulanic	47	25.2	140	74.8
Ampicillin-Sulbactam	153	81.8	34	18.2
Piperacillin-Tazobactam	94	50.3	93	49.7
Trimethoprim-Sulfamethoxazole	64	65.8	36	34.2
Ampicillin	187	100		
Ciprofloxacin	98	52.4	89	47.6
Levofloxacin	23	12.3	164	87.3

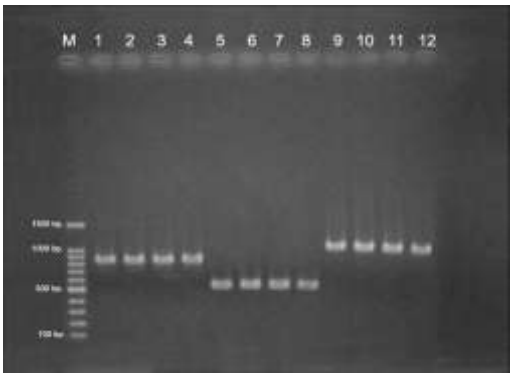
Double Disk Synergy Test (DDST)

All 175 isolates showed positive results by the DDST (Fig.1) which indicates that all these isolates were putative ESBLs producers. These results were 100% the same as that obtained by Phoenix System (Lee *et al.,* 2008). The disk synergy test is regarded as the most effective for detecting ESBL-producing strains (Dechen *et al*., 2009).

Figure(1). Double Disk Synergy Test (DDST)
Center: Clavulanic acid disk; around: 3rd generation cephalosporins (cefotaxime, ceftazidime and ceftriaxone).

Detection of *bla*TEM, *bla*SHV, and *bla*CTX-M Genes by PCR

Only 100 isolates identified as ESBL-producers which were tested for the presence of genes coding for the TEM, SHV, and CTX-M, using PCR employing primers that are specific for each gene. Results shown in Table (2) and Fig (2) revealed that 60 isolates harbored TEM-type enzymes, 87 isolates harbored SHV-type enzymes and 58 isolates harbored CTX-M type enzymes.



Figure(2). Detection of PCR amplified products of *bla*TEM, *bla*SHV and *bla*CTX-M genes using 1.5% agarose gel electrophoresis.
Lane 1-4 *bla*SHV
lane 5-8 *bla*CTX-M,
lane 9-12 *bla*TEM
M: marker

Table(2). Detection of ESBL genes in *Klebsiella pneumoniae* clinical isolates by PCR using specific primers.

ESBLs gene type	No. of isolates
TEM only	2
SHV only	26
CTX-M only	4
TEM+SHV	14
TEM+CTX-M	7
SHV+CTX-M	10
TEM+SHV+CTX-M	37
Total	**100**

Table(3). Primers used for detection of ESBLs.

Primer	Primer sequences	PCR Cycles
TEM/F TEM/R	5′- ATAAAATTCTTGAAGAAGACGAAA -3′ 5′- GACAGTTACCAATGCTTAATC -3′	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 1 min 30 sec at 45°C, 1 min at 72°C); cycle of 10 min at 72°C
SHV/F SHV/R	5′- TCGTTATGCGTTATATTCGCC -3′ 5′- GGTTAGCGTTGCCAGTGCT -3′	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 30 sec at 48°C, 1 min at 72°C); cycle of 10 min at 72°C
CTX- M/F CTX-M/R	5′- CGCTTTGCGATGTGCAG -3′ 5′- ACCGCGATATCGTTGGT 3′	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 1 min at 58°C, 1 min at 72°C); cycle of 10 min at 72°C

To our knowledge this study is the first to be conducted not only in Kurdistan region but in all Iraq to give a snapshot on the molecular characterization of ESBLs. A major finding was that SHV-Type ESBLs were by far the most dominant (Table.2). Recent study in Korean hospitals has also confirmed the persistence of isolates producing SHV-type (Kim *et al.*, 2006).

Recent European studies on Enterobacteriaceae have also confirmed the persistence of strains producing TEM and SHV, and the increasing prevalence of strains producing CTX-M (Rupp *et al.*, 2003). The prevalence of ESBL productions revealed a significant geographical differences, ranging from 0% (Iceland) to less than 1% (Estonia) to 41% for *E. coli* (Romania) and 91% (Romania) for *K. pneumonia* (Coque *et al.*, 2008). The ESBL production is much less frequent in Europe than in Latin America and Asia, and they are even less frequent in the Pacific than in North America (Coque *et al*., 2008).

The CTX-M gene predominates in Europe, while in other countries, the ESBL genes are more diverse (Livermore *et al.*, 2007). In the United Kingdom, a recent dramatic increase of the ESBL producing strains was observed both in hospitals and in the community, and this increase is attributed to CTX-M (Coque *et al.*, 2008). In Norway and Portugal, the CTX-M is the ESBL enzyme most frequently found in *E. coli* (Tofteland *et al*., 2007; Machado *et al*., 2007). In Italy, the prevalence of *E. coli* producers of ESBL has also increased with a predominance of TEM , SHV and the emergence of CTX-M (Carattoli *et al.*, 2008). Other studies also reported the type of ESBL produced by these strains and some showed the presence of TEM, SHV and the dramatic emergence of CTX-M (Daoud *et al*., 2003; Matar *et al*., 2007and Kanj *et al*., 2008).

REFERENCES

Ambler, R. P. (1980). The structure of beta-lactamases. Philos Trans. R. Soc. Lond. B. Biol. Sci., 289: 321–331.

Bagley, S. T., Seidler R. J., and Brenner, D. J. (1981). *Klebsiella planticola* sp. nov.: a new species of *Enterobacteriaceae* found primarily in non-clinical environments. Curr. Microbiol., 6:105-109.

Bernard, H., Tancrede, C., Livrelli, V., Morand, A., Barthelemy, M. & Labia, R. (1992). A novel plasmid-mediated extended-spectrum betalactamase not derived from TEM- or SHV-type enzymes. J Antimicrob Chemother., 29: 590–592.

Bradford, P. A. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev., 14: 933–951.

Bush, K., Jacoby, G.A., Medeiros, A.A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother., 39: 1211-33

Carattoli, A., Garcia-Fernandez, A., Varesi, P., Fortini, D., Gerardi, S. and Penni, A. (2008). Molecular epidemiology of *Escherichia coli* producing extended-spectrum ß-lactamases isolated in Rome, Italy. J. Clin. Microbiol. 46:103-108.

Coque, T.M., Baquero, F. and Canton, R. (2008). Increasing prevalence of ESBL producing Enterobacteriaceae in Europe. Eurosurveillance. 13: 47.

Daoud, Z. and Hakime, N.(2003). Prevalence and susceptibility patterns of extended-spectrum ß-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in a general university hospital in Beirut, Lebanon. Revista Espanola de Quimioterapia. 16:233-238

Dechen, C., Tsering, S., Das, Luna, A., Ranabir, P., and Takhellambam, S. (2009). Extended Spectrum Beta-lactamase Detection in Gram-negative Bacilli of Nosocomial Origin. J. Glob. Infect. Dis., 2:87–92.

Eckert, C., Gautier, V. & Arlet, G. (2006). DNA sequence analysis of the genetic environment of various *bla*CTX-M genes. J. Antimicrob. Chemother., 57:14–23.

Hawser, S. P., Bouchillon, S. K., Hoban, D. J., Badal, R. E., Hsueh, P. R. & Paterson, D. (2009). Emergence of high levels of extendedspectrum β-lactamase-producing Gram-negative bacilli in Asia/ Pacific. Antimicrob. Agents Chemother., 53: 3280–3284.

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R. & Philippon, A. (2002). Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother., 46: 3045–3049.

Jacoby, G. (1997). Extended-spectrum β-lactamases and other enzymes providing resistance to oxyimino-β-lactams. Infect. Dis. Clin. North Am., 11: 875- 887.

Kanj, S.S., Corkill, J.E., Kanafani, Z.A., Araj, G.F., Hart, C.A., Jaafar, R. and Matar, G.M. (2008) Molecular characterisation of extended spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella* spp. isolates at a tertiary-care centre in Lebanon. Eur. J. Clin. Microbiol. Infect. Dis., 14: 501-504

Kim, K.Y., Kim, T.U., and Baik, H. S. (2006). Characterization of Extended Spectrum β-Lactamase Genotype TEM, SHV, and CTX-M producing *Klebsiella pneumoniae* Isolated from Clinical Specimens in Korea.J. Microbiol., 16:889-895.

Knothe, H., Shah, P., Krcmery, V., Antal, M. & Mitsuhashi, S. (1983).Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection, 11: 315–317.

Lee, K.K., Kim, S.T., Hong, K.S., Huh, H.J., and Chae, S.L.(2008). Evaluation of the Phoenix automated microbiology system for detecting extended-spectrum beta-lactamase in *Escherichia coli*, *Klebsiella* species and *Proteus mirabilis*. Korean J. Lab. Med., 3:185-90

Lee, S. G., Jeong, S. H., Lee, H., Kim, C. K., Lee, Y., Koh, E., Chong, Y. & Lee, K. (2009). Spread of CTX-M-type extended-spectrum betalactamases among bloodstream isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a Korean hospital. Diagn. Microbiol. Infect Dis. 63: 76–80.

Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G., Ayala, J., Coque, T. M., Kern-Zdanowicz, I. (2007). CTX-M: changing the face of ESBLs in Europe. J. Antimicrob. Chemother.,59: 165–174.

Livermore, DM. (1995). β-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev., 8: 557-84.

Machado, E., Coque, T.M., Canton, R., Novais, A., Sousa, J.C., Baquero, F. (2007) High diversity of extended-spectrum ß-lactamases among clinical isolates of Enterobacteriaceae from Portugal. J Antimicrob Chemother., 60:1370-1374.

Matar, G.M, Jaafar, R., Sabra, A., Hart, C.A., Corkill, J.E., Dbaibo, G.S., Araj, G.F (2007). First detection and sequence analysis of the *bla*CTX-M-15 gene in Lebanese isolates of extended-spectrum ß-lactamase producing *Shigella sonnei*. Ann. Trop. Med. Para.,101:511- 517.

Mohammad, H.M., Al-Agamy, Atef, M. S., and Abdelkader, F.T. (2009). Prevalence and molecular characterization of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in Riyadh, Saudi Arabia. Ann. Saudi Med., 29: 253–257.

Monnet, D., and J. Freney. (1994). Method for differentiating *Klebsiella planticola* and *Klebsiella terrigena* from other *Klebsiella* species. J. Clin. Microbiol., 32:1121-1122.

Paterson, D. L. & Bonomo, R. A. (2005). Extended-spectrum beta lactamases:a clinical update. Clin Microbiol. Rev., 18: 657–686.

Rupp, M. and Fey, P. (2003). Extended Spectrum β-Lactamase (ESBL)-Producing Enterobacteriaceae. Adis. International Limited. 63: 353-365.

Tofteland, S., Haldorsen, B., Dahl, K.H., Simonsen, G.S., Steinbakk, M., and Walsh, T.R. (2007). Effects of phenotype and genotype on methods for detection of extended-spectrum ß-lactamase producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. J. Clin. Microbiol., 45:199-205.

التوصيف البكتيريولوجي والجزيئي لانزيمات البيتالاكتيميز الواسعة الطيف في العزلات السريرية لبكتريا *Klebsiella pneumoniae* المعزولة من أقليم كردستان العراق

الخلاصة

جمعت 275 عزلة سريرية لبكتريا *Klebsiella pneumoniae* من ثلاث مستشفيات عامة في محافظات اقليم كردستان: دهوك و أربيل والسليمانية خلال الفترة مابين شهر ايلول عام 2010حتى شهر حزيران عام 2011. تم قياس التركيز المثبط الادنىMIC لتلك العزلات بأستخدام جهاز Phoenixوالذي يعتمد على كارت حساسية البكتريا السالبة لصبغة كرام، حيث كانت 187 عزلة فقط منتجة لانزيمات البيتالاكتيميز الواسعة الطيف. تم تأكيد هذة العزلات بانها 100% منتجة لانزيمات البيتالاكتميز الواسعة الطيف بواسطة اختبارالقرص الثنائي المتساند(DDST).

كانت جميع هذة العزلات مقاومة 100%لكل من المضادات الحيوية ampicillin, cefazolin, cefepime, ceftriaxone, cefotaxime, cefuroxime, ceftazidime, aztreonam و. كما اظهرت نسب مقاومة متباينة 81.8%, 68.5%, 65.8%, 52.4%, 50.3%, 34.2%, 25.2% و 12.3% تجاه كل من ampicillin/sulbactam , gentamicin , trimethoprime-sulfamethoxazole, ciprofloxacin و amoxicillin-clavulanate,amikacin, piperacillin-tazobactam, levofloxacin على التوالي.

استخدمت تقنية تفاعل السلسلة المتضاعفة لغرض التوصيف الجزيئي لهذه الانزيمات وذلك باستخدام البوادىء المتخصصة لثلاثة انواع من انزيمات البيتالاكتيميز الواسعة الطيف (TEM, SHV,CTX-M). حيث اظهرت النتائج بان النوع SHVمن انزيمات البيتا لاكتميز الواسعة الطيف كان الاكثرتواجدا 87%من تلك العزلات و كانت نسبة العزلات المنتجة لكل من النوع TEMو CTX-Mهي 60% و58% على التوالي.

سیفەتە بەکترلوجییەکان و گەردیلیەکانی ئەنزیمی بێتا لاکتیمەیزی شەبەنگە فراوەکانی بەکتریای *Klebsiella pneumoniae* جیاکەرەوە لە نەخوشخانەکانی هەرێمی کوردستانی عیراق .

پوختە

توانیم(275)**بەکتریایی جوری** (*Klebsiella pneumoniae*) **لە نەخوشخانە سەرەکییەکانی هەر سێ پارێزگایی هەرێمی کوردستان ,دهوك ،هەولێر ,سلێمانی لەماوەی نیوان مانگی ئەیلولی** 2011 **تا حوزەیرانی** 2012 **جیابکەمەوە . رێژەی چری نزمترین راگر**(MIC) **مان خوێندەوە بو ئەم بەکتریا جیاکەرەوانە بەبەکارهینانی ئامێری** (Phoenix) **کەبە هوی کارتی هەستیاری بو بەکتریا گرام نەگەتیفەکان** (GNC) **کار دەکات ، لەهەموو بەکتریا جیاکەرەوان تەنها**(187)**بەکتریا بەرهەم هێنەری ئەنزیمی بێتا لاکتیمەیزی شەبەنگە فراوان** (Broad spectrum) **بوون ،** (100 ٪) **لەم ئەنجامە دلنیا بوون بەهووی تاقیکردنەوەی پەپکە دووانە پێشگیرەکان** Double Disk Synergy Test **، هەموو ئەو**(187)**بەکتیریا (** *Klebsiella pneumoniae*) **جیاکەرەوە لە** (100 ٪)**رزستن بوون بۆ ئەم ئەنتی بایوتیکانە :**
(ampicillin ,cefazolin ,cefepime ,ceftriaxone, aztreonam ,cefotaxime ,cefuroxime , ceftazidime)

بەلام رێژەی رزستنی جیاواز یان هەبوو :

بەرامبەر 12.3% **و** 25.2%, 34.2%, 50.3%, 52.4%, 65.8%, 68.5%, 81.8%, ampicillin/sulbactam , gentamicin , trimethoprime-sulfamethoxazole, amoxicillin-clavulanate,amikacin, piperacillin-tazobactam, ciprofloxacin **و** levofloxacin

یەك بەدووایەك .

توانیمان سیفەتە گەردیلەکانیان ئەم بەکتریا جیاکەرەوانە بخوێنینەوە بەبەکارهێنانی ئامێری کارلێکردنی زنجیرە چەند چارەکان (PCR) **بشت بەستن بە دستپێکە تایبەتییەکانی** specific primers**جۆرە جیاوازەکانی ئەنزیمی بێتا لاکتیمەیزی شەبەنگە فراوەکانی** (CTX-M , SHV , TEM)

لە کوتاییا بۆ مان دەرکەوت کە زۆربەی ئەنزیمی بێتا لاکتیمەیزی شەبەنگە فراوەکان و بەرێژەی (87٪) **لە جۆری** (SHV) **ئەمەش بەهوی سیفەتە دایکینییەکان** phenotypic evidence **بەلام جۆری** (TEM) **لە**(60٪) **و جۆری** (CTX-M) **لە** (58٪) **بوون .**