Pseudomonas aeruginosa outer membrane proteins: Profile analysis and immunogenicity in relation to the antibiotic resistance

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To analyze the major outer membrane proteins (OMPs) of the sensitive or resistant *Pseudomonas aeruginosa* strains, the OMPs were separated from the cellular elements by sarcosyl extraction method. OMPs profiles were conducted by SDS-polyacrylamide gel electrophoresis. Amoxicillin clavulanic acid (AMC) sensitive *P. aeruginosa* serotype K showed four protein bands; 35.713, 31.159, 26.107 and 22.869 KD. While AMC sensitive *P. aeruginosa* serotype H showed three bands of 35.713, 27.164 and 23.174 KD. Whereas AMC resistant *P. aeruginosa* serotype G, that was positive for the *bla*$_{TEM}$ gene by the PCR, modified its protein pattern. It has five protein bands of 52.142, 38.525, 30.690, 27.164 and 22.569 KD. These findings suggested that *bla*$_{TEM}$ gene and the outer membrane protein barrier are contributed to the resistance to amoxicillin clavulanic acid in *P. aeruginosa*. To determine a possible relationship between the resistance of *P. aeruginosa* and the production of antibodies against its outer membrane protein, antibodies against OMPs of AMC sensitive and resistant *P. aeruginosa* strains were prepared in mice and evaluated by ELISA. Our results showed that there was no association between immunogenicity of the outer membrane proteins and resistance of *P. aeruginosa* to antibiotics.

Pseudomonas aeruginosa is a clinically significant pathogen that exhibits intrinsic resistance to various antimicrobial agents (Quinn, 2002). The B-lactamase inhibitor; amoxycillin clavulanic acid (trade name; co-amoxyclav or synulox or augmentin) is indicated for treatment of both Gram positive and Gram negative bacterial infection in both human and veterinary practice (Ball *et al.,* 1980). Little is recorded about amoxycillin clavulanic acid susceptibility of *P. aeruginosa in* animals.

Many reports have demonstrated that the intrinsic resistance of *P. aeruginosa* to beta-lactams is due to the interplay among multiple resistance mechanisms (Li *et al.,* 2000 and Putman *et al.,* 2000). Among which; plasmid-encoded TEM-type β-lactamase (*bla*$_{TEM}$ gene) (Kaye *et al.,* 2004) and the inhibition of drug entry into the cell (Putman *et al.,* 2000).

Attention has been addressed to the outer membrane proteins of *P. aeruginosa* and their consideration as a penetration barrier responsible for some antibiotic resistance *P. aeruginosa* (Cullmann *et al.,* 1986). The first aim of this study was to analyze the major OMPs of the sensitive or resistant *P. aeruginosa* strains for a comparison between them by SDS-polyacrylamide gel electrophoresis.

P. aeruginosa OMPs are valuable immunogens (Gilleland *et al.,* 1984). Previous studies have been performed to evaluate immunogenicity of the outer membrane proteins of *P. aeruginosa* in human and animals (Alexander and Fisher, 1974; Gilleland *et al.,* and Jang *et al.,* 1999).

A trial was performed to determine a possible relationship between the resistance of *P. aeruginosa* and the production of antibodies against its outer membrane protein.

Material and Methods

Organisms. Six isolates of *P. aeruginosa* from samples of chicken origin were included in this work. The six strains belong to serotype F, L G, K, H and F as shown in (Table 1).

Antibiotic susceptibility. The antibiotic susceptibilities of the *P. aeruginosa* isolates were analyzed by the disk diffusion method using Mueller-Hinton broth and agar (Oxoid). Antibiotic discs (Oxoid, Ltd., London, England) were used and interpretation was done as described by the National committee for clinical laboratory standards (1993). The following antibiotics were tested: amoxicillin-clavulanic acid (AMC, 30μg), Ampicillin (Amp, 10μg), ceftriaxone (CRO, 30μg), neomycin (N, 30ug),

streptomycin (S, 10ug) and norfloxacillin (NOR, 10μg).

Detection of *bla*$_{TEM}$ gene: PCR amplification of the *bla*$_{TEM}$, gene was carried out for screening purposes, as described (Belaaouaj *et al.,* 1994). *P. aeruginosa* cells were lysed by heating and cellular debris was removed by centrifugation. The supernatant was used as the templates for the following PCR. Primers MATEMF (5'-ATGA GTATTCAACATT TTCGTGC-3') and MATEMR (5'CCAATGCTTAATCAGTGAG-GCAC C-3') were used. The PCR program was as follows: 95°C for 5 min and then 35 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s followed by 72°C for 10 mm. The amplified product was visualized by standard gel electrophoresis of 10 μl of the PCR mixture on 1.5 % agarose gel stained with 0.5μg/ml ethidium bromide. The amplicon size was evaluated by comparison band with the sizes of the fragments of Hae III molecular weight marker (Invitrogen, USA).

Outer membrane proteins preparation. The outer membrane proteins of AMC resistant and sensitive *P. aeruginosa* strains were prepared as described by (Caballero *et al.,* 2001). Basically, bacteria were grown at 37°C in nutrient agar (Oxoid); the cells were harvested and broken by sonication in an ice bath (20 kHz for 2 minutes, 5 cycles). Whole cells were eliminated by centrifugation at 5000 *g* for 20 minutes. The outer membrane proteins were separated from the other cellular components by sarcosyl extraction (N-lauroyl-sarcosine Na 2% concentration) at room temperature for 30 minutes followed by centrifugation (38000 *g* for 1 hour at 4°C). The pellets with outer membranes were resuspended in 50 mM Tris-HCL (PH 6.8) and frozen at -70°C until used. The total protein concentration of the preparation was calculated by the method of (Lowry *et al.,* 1951).

OMPs analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The OMPs were analyzed using vertical electrophoresis in 10% (wt/vol) SDS-polyacrylamide gel as described by (Laemmli, 1970) with a Protean II slab electrophoresis system (Bio-Rad Laboratories, Richmond, Calif. USA). Protein fractions were solubilized in sample buffer, heated at 95°C for 5 min. The OMPs were loaded in amounts of approximately 35 mg and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% (wt/vol) acrylamide and 0.1% (wt/vol) piperazine diacrylyl in the running gel at a constant current of 20 Am. The gel was stained with Coomassie brilliant blue R-250 (Bio-Rad). Quantitation of the stained OMPs bands was done by using scanning spectrophotometry at absorbance of 605 nm(Hames and Rickwood,1987).

Production of antibodies against OMPs in mice. Antibodies against OMPs of sensitive and resistant strains of *P. aeruginosa* strains were prepared as described by (Muthukumar and Muthukkaruppan, 1993). Four groups of 10 mice, were immunized with the OMPs antigens of *P. arugenosa*. Each mouse was injected with 50 ug/ 0.2 ml of the indicated antigen subcutaneously (S/C) twice at 15 day intervals. The first, the second and the third groups were immunized with OMPs antigen of *P. aruginosa* AMC sensitive serotype K, AMC sensitive serotype H and AMC resistant serotype G, respectively. The fourth group served as control non-injected group. Fifteen days after the last injection, sera were collected from each group of mice and stored at -20 till used.

Detection of the antibody level (Leitner *et al.,* 1990). The humoral immune response was assessed, 15d after the 2nd immunization. The indicated OMPs antigens (20 μg/ ml) in carbonate buffer was used to coat 96-well microtiter plates (Nunc Immunoplates), which were then incubated at 4°C overnight. Following antigen coating, ELISA plates were washed and blocked with 3% bovine serum albumin in PBS for 1 h prior to addition of the serum. Serum was diluted in PBS containing 0.3% bovine serum albumin and 0.05% Tween 20. Following serial dilution, diluted sera were incubated in the wells for 2 h at 37°C. Wells were washed with PBS containing 0.05% Tween 20, 100 μl of the appropriate goat anti-mice IgG conjugate was added for 2 h at 37°C. Wells were then washed, and bound conjugate was detected by using ABTS peroxidase substrate. Reactions were stopped with 20 μl of 2.5 M H_2SO_4 and optical densities (ODs) for the tested and control sera were read at 405 nm.

Results

Characterization of clinical strains. It was illustrated in (Table 1), by disk diffusion method, that three strains (serotypes; F, L and G) were resistant to amoxicillin clavulanic acid (zone diameter ≤ 13 mm) and other antibiotics; ampicillin, ceftriaxone, neomycin and strept-omycin. Three strains (serotypes; K, H and F) were sensitive to amoxicillin clavulanic acid (zone diameter ≥17 mm**).** The *bla*$_{TEM}$ gene was

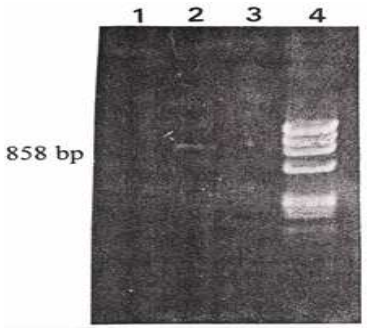

Fig. (1): The amplification of 858 bp fragment of *P. aeruginosa bla*$_{TEM}$ gene positive, Lane (2). *P. aeruginosa* negative for *bla*$_{TEM}$ gene Lane (1 and 3). Hae III molecular weight marker (Lane).

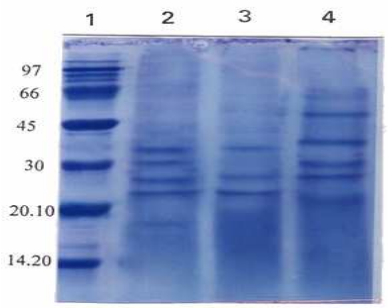

Fig. (2): Outer membrane proteins profile analysis for sensitive and resistance strains of *P. aeruginosa that* was separated by SDS-PAGE electrophoresis. Lane (1) is the molecular weight marker in KD**. Lane (2) is the AMC sensitive *P. aeruginosa* serotype K. Lane (3) is the AMC sensitive *P. aeruginosa* serotype H. Lane (4) is the AMC resistant *P. aeruginosa* serotype G and positive for *bla*$_{TEM}$ gene. Arrows indicate Outer membrane protein bands, which were subjected to scanning spectrophotometric analyses.**

recovered from only one of three *P. aeruginosa* isolates (serotype G). PCR amplification product of 858 bp fragment of *bla*$_{TEM}$ gene was shown in Fig. (1).

The SDS-PAGE profile of the outer membrane proteins of AMC sensitive and resistant strains of *P. aeruginosa*. As shown in (Fig 2), not all the visible Coomssie blue stained bands were quantitated by the scanning spectrophotometer at absorbance of 605 nm. AMC sensitive *P. aeruginosa* serotype K induced four bands; 35.713, 31.159, 26.107 and 22.869 KD. While AMC sensitive *P. aeruginosa* serotype H induced three bands; 35.713, 27.164 and 23.174 KD**.** Whereas AMC resistant *P. aeruginosa* serotype G *and* positive for *bla*$_{TEM}$ gene modified the protein pattern of its OMPs. It has five bands of 52.142, 38.525, 30.690, 27.164 and 22.569 KD (Table 2).

The immunigenic differences afforded by OMPs of AMC sensitive or resistant *bla*$_{TEM}$ gene positive *P. aeruginosa* strains. It was clear from Table (3) that there was a significant increase in antibody levels against OMPs of sensitive and resistant *P. aeruginosa* but without differences in the OD values within mice groups injected with the OMPs antigens, in comparison with the control group. It is interesting to note that there was no relation between numbers of protein bands of the OMPs of *P. aeruginosa* detected by SDS–PAGE and the levels of anti-outer membrane protein antibodies detected by ELISA.

Discussion

Pseudomonas aeruginosa is known for its ability to develop resistance to many antibiotics. The relations among OMPs quantity, permeability, and antibiotics resistance are much better established for *E. coli* than for *P. aeruginosa*, and the possible existence of additional barrier layers besides the outer membrane in the latter species cannot be excluded **(**Livermore, 1988).

In the present study, a new technique was adopted to selectively solubilize the cytoplasmic membrane with sodium lauroyl sarcosinate for isolation of the outer membrane proteins of *P.aeruginosa* strains. This method was described by (Zuo *et al.,* 1999) as a relative simple method.

The outer membrane proteins (OMPs) profile analysis of AMC sensitive and resistant strains of *P. aeruginosa* were detected by SDS-PAGE. AMC sensitive *P. aeruginosa* serotype K and H induced three common protein bands equal or about (35.713, 26-27 and 23 KD). AMC sensitive *P. aeruginosa* serotype K additionally induced a protein band of 31.159 KD. Whereas AMC resistant *P. aeruginosa* serotype G modified its outer membrane protein patterns. It has two more protein bands of 52.142 and

Table (1): The results of antibiotic susceptibility test and the presence of *bla*$_{TEM}$ gene in the clinical *P. aeruginosa* strains of chicken origin.

Antibiotic	*P. aeruginosa* serotypes					
	F	**L**	**G**	**K**	**H**	**F**
Amoxicillin-clavulanic acid (AMC, 30μg)	R	R	R	S	S	S
Ampicillin (Amp, 10μg)	R	R	R	R	S	S
Ceftriaxone (CRO, 30μg)	I	I	I	S	S	S
Neomycin (N, 30ug)	I	I	R	R	R	S
Streptomycin (S, 10ug)	I	S	S	S	S	S
Norfloxacillin (NOR, 10μg)	S	S	S	S	S	S
***Bla*$_{TEM}$ gene detected by the PCR**	-	-	+			

R: resistant, I: intermediate, S: sensitive

Table (2): Characterization of AMC sensitive and resistant *P. aeruginosa* outer membrane protein by SDS- PAGE technique.

Marker			The OMPS of *P. aeruginosa* serotype					
			*K		*H		*G	
Band	**Mol. W.**	**%**	**Mol. W.**	**%**	**Mol. W.**	**%**	**Mol. W.**	**%**
1	97	18.2	35.713	20.9	35.713	4.91	52.142	15.4
2	66	15.3	31.159	12.2	27.164	6.20	38.525	9.78
3	45	17.1	26.107	16.2	23.174	88.9	30.690	7.12
4	30	13.2	22.869	50.7			27.164	5.58
5	20.1	23.3					22.569	62.1
6	14.2	13.0						

The molecular weight is in KD and the amount of the OMPs band is in percentage. The OMPs were loaded in of approximately 35 mg *K is the AMC sensitive *P. aeruginosa* serotype K. *H is the AMC sensitive *P. a* serotype H. *G is the AMC resistant *P. aeruginosa* serotype G and positive for *bla*$_{TEM}$ gene.

Table (3): The relationship between the level of the anti-outer membrane protein antibodies detected by ELISA, the OMPs bands detected by SDS-PAG and the AMC susceptibility of *P. aeruginosa*.

	OMPs of *P. aeruginosa* serotype		
	K	**H**	**G**
The mean OD (405 nm) of sera of the injected mice grou	0.627	0.562	0.542
The mean OD (405 nm) of the control group	0.174	0.169	0.158
The AMC sensitivity	S	S	R
The number of bands detected by SDS -PAGE	4	3	5

38.525 KD than the AMC sensitive *P. aeruginosa.* Three shared protein bands were shown between AMC sensitive and AMC resistant *P. aeruginosa* about (31, 27 and 23 KD). Hostacka and Karelova, (1997) revealed protein bands of 45, 38, 35 and 23 KD from control *P. aeruginosa* strains. While Zhang *et al.* (2000) stated that many of the MDR strains of *P. aeruginosa* expressed either moderate or high levels of a novel outer membrane protein band of about 50 kD molecular mass. It can therefore be safely stated that sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMPs from AMCr strains of *P. aeruginosa* (resistant to AMC) revealed a unique OMP of 52.142 KD when compared with AMC-susceptible strains of *P. aeruginosa*. This unique OMP of 52.142 KD is involved in AMC resistance specifically. These suggestions are similar to those previously reported by (Livermore, 1988) who supported that the outer membrane, composed of porin proteins, which is the major individual barrier. He added that the outer membrane proteins of *P. aeruginosa* play important though not exclusive role in enhancing strain resistance against the AMC. Furthermore, Okamoto *et al.,* (2001**)** confirmed that, resistance mechanism in *P. aeruginosa* was not strong after loss of either the outer membrane barrier.

It is interesting to note from our results that the OMPs overproduction of AMC resistant *P. aeruginosa* is associated with cross resistance to other antibiotic such as ampicillin, ceftriaxone and neomycin. The finding agree with that of (Masuda *et al.,* 1995) who reported that outer membrane proteins responsible for multiple drug resistance in *P. aeruginosa* induced a band of approximately 50 KD.

Although *P. aeruginosa* has an outer membrane with low permeability (Yoshimura and Nikaido**,** 1982) that can enhance strain resistance against some antibiotics (Cipriani *et al.,* 1995). This alone does not adequately explain *P. aeruginosa* intrinsic resistance, as an additional mechanism interfering with the access of the agents to their targets in this bacterium (Nikaido**,** 1985). It was clear from the obtained results in this work that the expression of bla_{TEM} gene in the AMC resistant *P. aeruginosa* strains indicated that the outer membrane proteins acting in synergy with beta-lactamase encoded by bla_{TEM} gene. This synergistic effect probably responsible for AMC resistance in *P. aruginosa*. The same was obtained by Briñas *et al.* (2002) who added that susceptibility to β-lactamase inhibitors could be affected by different mechanisms. The most frequent one is the hyperproduction of classical β-lactamases or the synthesis of inhibitor-resistant TEM (IRT) β-lactamases by amino acid substitutions in TEM-1 or TEM-2 that encoded by bla_{TEM} gene or by other possible changes in membrane quantity and permeability.

The outer membrane proteins can influence the virulence and immunological properties of bacteria (Hale *et al.,* 1983**).** Attention has been addressed to the role of the outer membrane proteins of *P. aeruginosa* in the induction of specific immunity (Grundmann *et al.,* 1991 and Jang *et al.,* 1999). Our research has been focused on the antibodies against the outer membrane proteins from AMC susceptible and resistance strains**.** Alexander and Fisher (1974) and Gilleland *et al.,* (1984) successfully used purified outer membrane proteins of *Pseudomonas aeruginosa* to induce OMPs-specific IgG antibodies in sera of mice.

In the present study specific antibody against two doses of 50 µg of OMPs of sensitive or bla_{TEM} positive AMC resistant *P. aeruginosa* strains were determined by ELISA. Analysis of OD values of the pooled sera of mice, two weeks after last S/C injection indicated no immunogenic differences between antibodies afforded by OMPs of AMC susceptible strains of *P. aeruginosa* or resistant with bla_{TEM} gene.

It is important to note that there was no relation between numbers of bands detected by SDS-PAGE and the levels of anti-outer membrane protein antibodies detected by ELISA. These findings suggested that the additional outer membrane protein band of 52.142 kD does not affect the immubogenic responce. Norris and Sciortino (1988) produced monoclonal antibodies against an OMP band specific for the resistant strains of *P. aeruginosa*. Furthermore, Caballero *et al.* (2001) found a correlation between the levels of antibodies and number of bands of the OMPs of *P. aeruginosa* strains of human origin.

In conclusion, resistance to amoxicillin clavulanic acid in *P. aeruginosa* strains could depend not only, the present of bla_{TEM} gene but also on outer membrane protein alterations. There was no association between immunogenicity of the outer membrane proteins and resistance of *P. aeruginosa*.

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