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# Regulation of pertussis toxin and lipopolysaccharide levels of *Bordetella pertussis* 134 in response to modulators

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**ABSTRACT**

Whooping cough (pertussis) is a highly contagious disease of the human respiratory tract, which is caused by *Bordetella pertussis*. Reemerge of pertussis in some highly immunized populations and divergency in gene order among several *B. pertussis* strains promoted this research to study the change of pertussis toxin (PT) and lipopolysacharide levels in response to the different environments. This study conducted an extensive investigation of antigenic modulation of *B. pertussis* strain 134 in the presence of different chemicals. One of the findings of this research, for the first time, was that barium ion has growth inhibitory role on *B. pertussis* 134, when added in high concentration to CBA plates. Nicotinic acid, magnesium sulfate, and magnesium chloride have shown the significant modulating effect on the basis of reduction of PT levels. Our data demonstrated quantitatively that the modulation of *B. pertussis* yields high levels of LPS. Our results have showed the strong modulatory effects of $FeCl_3$ on reduction of PT levels. In general, this study provided collective data which are strongly applicable to explain modulation of *B. pertussis*, and also introduces new modulators which promote more study on gene order of *B. pertussis*.

***Keywords:*** Pertussis, Modulation, Pertussis Toxin, Lipopolysaccharide

## INTRODUCTION

Pertussis, or whooping cough, is an acute infectious disease caused by the *Bordetella pertussis*, isolated by Bordet and Gengou (1906). This disease occurs worldwide and is a highly contagious disease of the human respiratory tract, which is particularly severe in infants. Despite the availability of vaccines, pertussis has remained an endemic disease, epidemics occurring every 3 to 5 years (Mooi *et al* 2000). In fact, pertussis has been classified by the CDC as one of the re-emerging diseases (CDC 1998). Several explanations have been put forward for the resurgence of pertussis in vaccinated populations. Improved surveillance, changes in case definitions and diagnostic techniques may result in an apparent increase in the incidence of pertussis. However, it seems that the mentioned factors could not explain all situations in which a dramatic rise in pertussis has been observed (Baron *et al* 1998, de Melker *et al* 1997). Other factors that may result in a true increase in the incidence of pertussis include demographic changes,

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waning vaccine-induced immunity, changes in vaccine effectiveness, changes in vaccine coverage and adaptation of the *Bordetella pertussis* population to vaccine-induced immunity. To gain insight into the reasons for the re-emergence of pertussis, study of regulation of virulence factors in response to the different environments, which is called modulation, is necessary. *Bordetella pertussis* expresses its pathogenic potential through the action of an array of virulence factors. These virulence factors have traditionally been grouped into toxins and adhesions and mutagenesis studies have shown many of these toxins and adhesions are required for in vivo persistence in animal models (Gross *et al* 1992, Mooi *et al* 1992, Weiss *et al* 1984, Weiss *et al* 1989). Phenotypic modulation and phase variation depend on a two-component system encoded by the bvg locus (Stibitz *et al* 1989). The expression of *B. pertussis* virulence factors is almost controlled by the Bvg A/S system (Weiss *et al* 1983, Arico *et al* 1989, Stibitz *et al* 1989, Uhl & Miller 1994). BvgA and BvgS are members of a superfamily of signal transducing proteins designated as two-component regulatory systems. BvgS is a transmembrane sensor and BvgA is a response regulator. Mutants lacking BvgA/S are avirulent in murine and cellular models (Cotter & Miller 2001). In the absence of modulators, BvgS undergoes autophosphorylation. After several intramolecular phosphorylation steps, the phosphate group is finally transferred to the amino-terminal domain of the second component, BvgA, a transcriptional activator. Phosphorylation activates BvgA and increases its affinity for the promoter regions of *B. pertussis* virulence activated genes (vag) (Boucher *et al* 1994). It was shown that at low temperatures or in the presence of nicotinic acid or sulfate ions, the phosphorylation is interrupted and the vag genes are silent and the bvg–repressed genes are expressed. This is called phenotypic modulation. Essentially irreversible repression of the vag genes can also occur by mutations in the bvg A/S operon during phase variation (Stibitz *et al* 1989). The data of monitoring in vivo gene expression in *B pertussis* using the recombinase-based in vivo technology (RIVET) system revealed that differential regulation of virulence factors similar to that seen in vitro also occurs during infection (Veal-Carr & Stibitz 2005). Then, in this study we focused on pertussis toxin (PT) and lipopolysaccharide (LPS) as two important virulence factors of *B. pertussis* to explain the response of these bacteria to different modulators. Pertussis toxin (PT), the main toxin of the pertussis organism, is responsible for different biological activities and, accordingly, is called lymphocyte promoting factor (LPF), histamine sensitizing factor (HSF), and islet activating protein (IAP) (Munoz *et al* 1985). PT has been postulated to be the toxin responsible for most of the systemic symptoms of disease in patients with pertussis (Pittman 1979). Lipopolysaccharide (LPS) is another virulence factor of *B. pertussis* which is smaller than many other bacterial LPS structures and is therefore often referred to as a lipooligosaccharide (LOS). The structure of *B. pertussis* LPS differs in that it lacks the O antigen (Kerr & Matthews 2000). Biological activities exhibited by *B. pertussis* LPS include antigenic and immunomodulating properties (Amano *et al* 1990, Watanabe *et al* 1990). The amount of LPS in whole cell pertussis vaccine has been shown to have a statistically significant association with the frequency of fever after vaccination (Baraff *et al* 1989). Modulation is defined as responses of bacteria to different environmental conditions. Metabolism of amino acids may be varied during these responses. Thus, ammonium, as a by-product of metabolism of amino acids was another factor which was examined in our study.

## MATERIALS AND METHODS

***B. pertussis* strain.** *Bordetella pertussis* strain 134 was obtained from Razi vaccine and serum research institute (RVSRI, Iran), which has been

used routinely for production of whole cell pertussis vaccine.

**Charcoal blood agar plates.** Charcoal blood agar (CBA) plates were made by dissolving 10.0 g beef extract, 10.0 g casein peptone, 10.0 g starch, 4.0 g charcoal, 5.0 g NaCl, 1.0 mg nicotinic acid, and 12.0 g agar in 1 liter of distilled water, pH 7.4. The mixture was autoclave sterilized at 121 °C for 15 min and 200 ml of pre-warmed defibrinated sheep blood were added aseptically. To make modulating CBA plates, the 10X solution of each chemical (Table 1), was added to the mixture by filter sterilizing (0.2 µm, Schleicher and Schuell, Germany), just before adding the defibrinated blood. These were subsequently dispensed into sterile petri dishes and incubated overnight at 37 °C for a preliminary sterility check.

**CBA plates culture conditions.** Lyophilized *Bordetella pertussis* 134 suspended in autoclave sterilized casamino acids solution (1 g casamino acids, 0.5 g NaCl, 0.01 g $MgCl_2.6H_2O$, and 0.0016g $CaCl_2.2H_2O$ in 100 ml of distilled water) and directly cultured on triplets of either CBA or modulating CBA plates at 35 °C. In another treatment cultivation was performed on triple alone CBA at 22 °C. The plates stored in humidified container and checked daily for growth and purity. The grown cells on each plate were washed using 5 ml of sterile phosphate buffer saline (PBS), centrifuged at 10,000 g for 20 min, and then re-suspended in 2 ml of sterile PBS. Cell lysate was prepared by sonication using 5x60 s bursts at 60 amplitude each separated by 10 s cooling on ice. The lysate was centrifuged (10,000 rpm, 20 min) and supernatant was collected for further studies.

**Chinese hamster ovary cell clustering assay of PT.** Quantification of pertussis toxin was performed using CHO cell clustering assay according to Gillenius (1985). Cell line of CHO cells (Razi Cell Bank, RVSRI, Iran) were cultured in the Ham F-12 medium (Sigma) supplemented with 0.118% (w/v) sodium bicarbonate (Merck) and 10% (v/v) fetal calf serum (Gibco) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. Single cell suspensions were obtained from confluent monolayers of CHO cells by treatment with a solution of 0.1% (w/v) trypsin and 0.02% (w/v) EDTA in PBS, pH 7.2. The released cells were adjusted to a final concentration of $3.0x10^5$ cells per milliliter in Ham F-12.

**CHO cell assay.** A 200 µl portion of complete Ham F-12 medium was placed in each flat-bottom well of first column of 96-well microplates, and a 100 µl in other columns. 40 µl of each cell lysate, distilled water, and PT standard were added to the wells of first column of a microplate and serially twofold diluted to last wells. Cell suspension, with the concentration of $3.0x10^5$ cells per ml, added to each well in a volume of 100 µl. After incubating for 24 h, the clustering effect of test samples on the cells was observed using an inverted microscope. The highest dilution of a test sample showing the clustering effect was considered as titer, and the concentration of PT determined using standard of PT. Standard PT showed clustering effect in concentration above of 0.1 ng per ml.

**Quantification of endotoxin.** The quantitative chromogenic Limulus Amebocyte Lysate (LAL) method (Charles River Endosafe, USA) was used to measure lipopolysaccharide (LPS) of samples. 50 µl of each cell lysate in tenfold serial dilution, and endotoxin standards (1.2, 0.6, 0.3, and 0.15 EU/ml) were added to glass tubes and were incubated at 37 °C for 5 min. Then, 50 µl of LAL solution was added to each tube. After approximately 7 min incubation at 37 °C, pre-warmed (37 °C) substrate solution with the volume of 100 µl added and mixed. After incubation at 37 °C for approximately 5 min, 100 µl of 20% acetic acid was added to each tube and mixed immediately to stop the reaction. Absorbance measured at 405 nm, and LPS content (EU/ml) of test samples determined using standard curve of absorbance versus endotoxin unit (EU)/ml.

**Protein determination.** Protein concentration was determined by Biuret method in microplate format using bovine serum albumin (BSA) as standard. 250 µl of Biuret reagent (2.25 g sodium potassium tartrate, 0.75 g $CuSO_45H_2O$, 1.25 g potassium iodide, 25 ml of 6.0 M NaOH in 250 ml of distilled water) was added to each flat-bottom wells of microplate. Then, 50 µl of each BSA standards up to 2 mg/ml, unknown samples, and distilled water were added and mixed. The mixtures were incubated for 30 min at RT. The absorbance was measured at 540 nm using a microplate reader. Protein concentration of unknown samples was determined using standard curve of absorbance versus mg/ml protein.

**Ammonium assay.** The Nessler's reaction used to determine the ammonium levels in test samples. The Nessler's reagent was prepared by dissolving of 1 g mercuric iodide, and 0.7 g potassium iodide in a small amount of water. This mixture was added to a cooled solution of 1.6 g of NaOH in 5 ml of water, and diluted to 10 ml. The supernatant of centrifugation, 5000 g for 15 min, was used as working reagent. 125 µl of each sample, standards, and distilled water were placed into the wells of a flat-bottom microplate. Then 10 µl of Nessler's working reagent was added to each well and mixed. The mixtures were incubated for 15 min at room temperature. Subsequently absorbance was measured by microplate reader at 450 nm. Concentrations of NH3-N (µg/ml) were determined using standard curve of absorbance versus µg/ml of NH3-N.

## RESULTS

**Cultivation of *B. pertussis*.** Various chemicals (Table 1), each in two different concentrations, were added to the triplets CBA plates and *B. pertussis* strain 134 was cultivated on them at 35 °C. Another treatment was cultivation of bacteria on alone CBA plates at 22 °C and all were checked daily. Colonies on all plates were appeared after four days, except on CBA plates containing 140 mM $BaCl_2$, which no bacteria were grown even after 10 days. All efforts to cultivation of *B. pertussis* 134 on CBA plates containing 140 mM $BaCl_2.H_2O$ were unsuccessful. Our results showed that *B. pertussis* strain 134 is not able to grow on CBA plates containing 140 mM $BaCl_2.H_2O$.

**Table1.** Chemicals and concentrations used to induce modulation on CBA plates.

| Chemicals | Concentration (mM) | |
|---|---|---|
| | Level 1 | Level 2 |
| $MgSO_4.7H_2O$ | 40 | 140 |
| $MgCl_2.6H_2O$ | 40 | 140 |
| $BaCl_2.2H_2O$ | 40 | 140 |
| NaCl | 80 | 280 |
| Nicotinic acid | 8 | 28 |
| $FeCl_3$ | 2 | 7 |

Gram-staining was applied for grown bacteria of each plate and bacterial morphology was studied. Morphologies of bacteria were not significantly different between various treatments and bacteria appeared as single or paired minute coccobacillus (Figure 1).

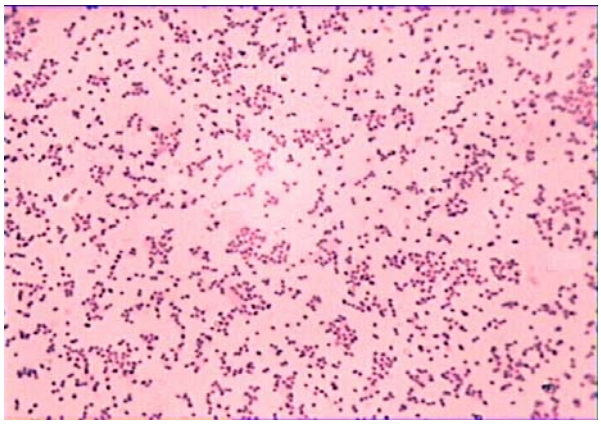

**Figure 1.** Microscopic view of *B. pertussis* strain 134. Gram-staining, 1000X.

**Cell lysate.** The cells were harvested from plates and lysates were prepared by sonication. Amounts of specific levels of pertussis toxin (µg PT/mg

protein), lipopolysaccharide (EU/mg protein), and ammonium (µg N /mg protein) of each lysate were determined and presented in Table 2.

**Table 2.** Amounts of specific levels of pertussis toxin (PT), lipopolysaccharide (LPS), and ammonium of modulation of *B. pertussis* 134.

| Chemicals | Conc. (mM) | PT (µg/mg prot.) | LPS (EU/mg prot.) | Ammonium (µg N /mg prot.) |
|---|---|---|---|---|
| $MgSO_4.7H_2O$ | 40 | 0.349 | 155.55 | 6.39 |
| | 140 | 0.026 | 4.73 | 20.67 |
| $MgCl_2.6H_2O$ | 40 | 0.265 | 32.76 | 6.58 |
| | 140 | 0.045 | 68.75 | 3.39 |
| $BaCl_2.2H_2O$ | 40 | 0.343 | 103.32 | 2.68 |
| | 140 | - | - | - |
| NaCl | 80 | 0.233 | 11.25 | 4.67 |
| | 280 | 0.218 | 119.01 | 7.87 |
| Nicotinic acid | 8 | 0.148 | 12.66 | 2.71 |
| | 28 | 0.055 | 32.07 | 2.91 |
| $FeCl_3$ | 2 | 0.277 | 0.91 | 3.21 |
| | 7 | 0.075 | 2.65 | 3.71 |
| Tem. 22 °C | - | 0.26 | 62.72 | 6.02 |
| CBA alone | - | 0.543 | 0.83 | 3.41 |

**Levels of pertussis toxin.** Comparisons of the levels of PT revealed that all chemicals reduced the pertussis toxin production and caused modulation in *B. pertussis* 134 (Figure 2A). Results showed that five most effective modulators for *B. pertussis* 134, which reduced the PT level, were 140 mM $MgSO_4$, 140 mM $MgCl_2$, 28 mM nicotinic acid, 7 mM $FeCl_3$, and 8 mM nicotinic acid. The lowest effects belonged to 40mM $MgSO_4$ and 40 mM $BaCl_2$. Comparative data of PT production for all modulators are presented in Figure 2B. These results showed that modulation was more intensive when higher concentration of each chemical was applied.

**Levels of lipopolysaccharide.** The modulation effects of each chemical on lipopolysaccharide (LPS) of *B. pertussis* 134 are presented in Figure 3 (A). In general, results showed that modulation led to increase of LPS levels. Results showed that highest effects of increasing of LPS level belonged to 40 mM $MgSO_4$, 280 mM NaCl, 40 mM $BaCl_2$, 140 mM $MgCl_2$, and temperature 22 °C with impact factors of 187, 143, 124, 83, and 75, respectively. The $FeCl_3$ showed the lowest effect in this regard. The comparative data of LPS production under each concentration of modulators are represented in Figure 3 (B).

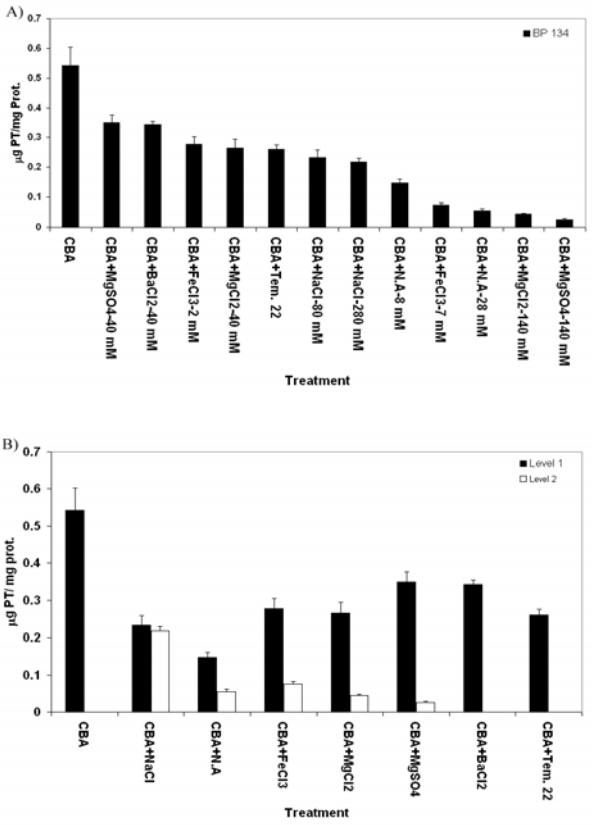


**Figure 2.** Effect of modulators on the levels of pertussis toxin of *B. pertussis* 134. A) Comparison the effects of all chemicals. B) The paired comparisons of each chemical. (■): Low concentration. (□): High concentration.

For all chemicals except $MgSO_4$, increase of LPS level was concentration dependent. In the case of $MgSO_4$, even though concentrations of 40 and 140 mM caused increase of LPS, the effect of low concentration was 32 folds more than high concentration.

**Levels of ammonium**. Results showed that some modulators increase and some ones decrease the

ammonium levels (Figure 4A). CBA plates containing 140 mM $MgSO_4$ had most increasing effect and CBA plates containing 8 mM nicotinic acid showed the most decreasing effect on ammonium level with impact factors of 6 and 1.25, respectively.

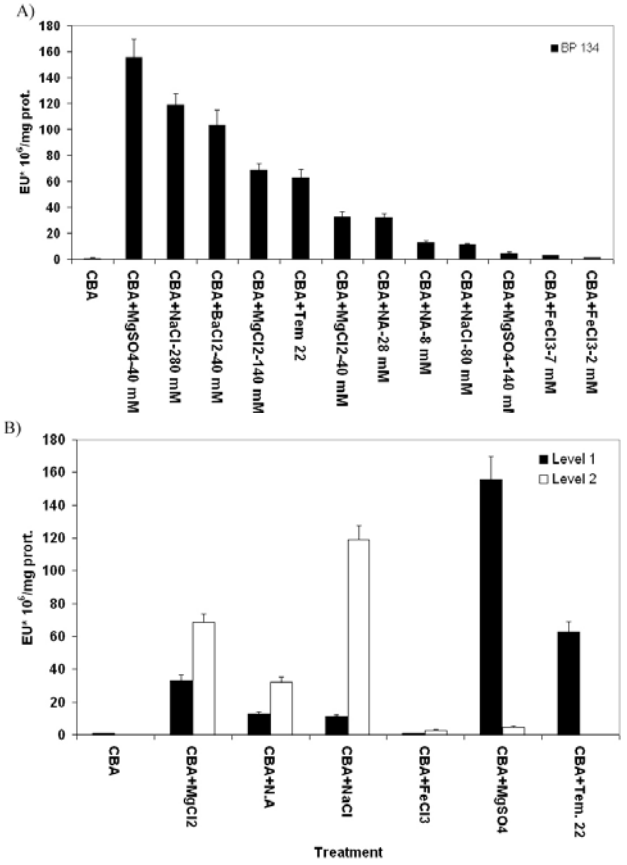


**Figure 3.** Effect of modulators on the levels of lipopolysaccharide of *B. pertussis* 134. A) Comparison the effects of all chemicals. B) The paired comparisons of each chemical. (■): Low concentration. (□): High concentration.

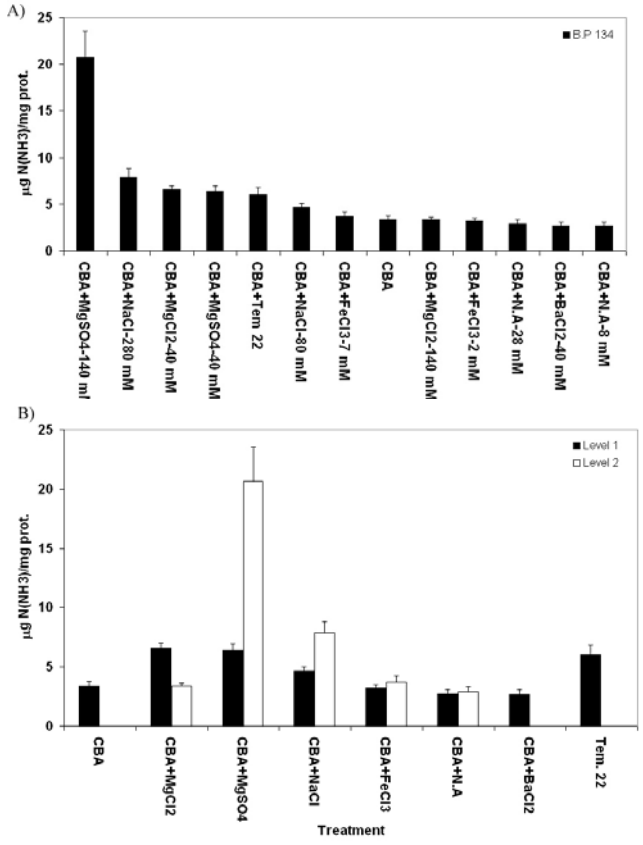


**Figure 4.** Ammonium Effect of modulators on the levels of ammonium of *B. pertussis* 134. A) Comparison the effects of all chemicals. B) The paired comparisons of each chemical. (■): Low concentration. (□): High concentration.

Comparative data (Figure 4B) showed that with exception of $MgCl_2$, increase in chemical concentration led to increase of ammonium level.

## DISCUSSION

Whooping cough is a severe and highly contagious respiratory disease caused by *Bordetella pertussis*. Prior to immunization, pertussis was widespread and responsible to high morbidity and mortality in infants and children (Cherry 1984). The whole cell pertussis vaccine was available individually in the early 20th century. 1999).

The combination vaccine (DTP) was developed in 1943 and was licensed in 1948 (Plotkin and Orenstein The acellular pertussis vaccine, then replaced the whole cell pertussis vaccine in some countries. Despite widespread use of pertussis vaccine, the pertussis is remerged in some highly

vaccinated populations and during outbreak in Netherland, United States, and France different clinical strains of pertussis were isolated (Baron *et al* 1998, deMelker *et al* 1997, Bass *et al* 1994). Previous studies by Stibitz and Yang (1997) indicated divergency in gene order among various strains of *B. pertussis*. Antigenic drift may be contributing factor to the persistence of pertussis. Antigenic drift often is established antigenic modulation which takes place under different conditions of bacterial cultivation. In this study, extensive experiments were conducted to explain the modulation effects of different chemicals on *B. pertussis* strains 134. Modulating effects of different chemicals consisting of $MgSO_4$, $MgCl_2$, $BaCl_2$, nicotinic acid, and $FeCl_3$ were investigated as additive into charcoal blood agar (CBA) plates. As another modulating condition, bacteria were grown on alone CBA plate at 22 $^oC$. Each chemical was used in two different concentrations, both higher than the minimum requirement for inducing modulation. $MgSO_4$ and $MgCl_2$ were used as a final concentration of 40 and 140 mM, where previously reported modulating amounts of them were 20 mM (Beattie *et al* 1990). $BaCl_2$, which is used for the first time in this study, was used in equivalent normality of $MgSO_4$ and $MgCl_2$. NaCl was applied as final concentrations of 80 and 280 mM to produce equivalent normality of $MgSO_4$ and $MgCl_2$. Nicotinic acid (N.A), with modulating effect at 5 mM (McPheat *et al* 1983), was used as final concentrations of 8 and 28 mM. $FeCl_3$ was used as a final concentration of 2 and 7 mM. *B. pertussis* strain 134 was cultivated on alone CBA and CBA plates containing each chemical at 35 $^oC$. As another modulating condition, bacteria were grown on alone CBA plate at 22 $^oC$. Microscopic studies, using Gram-staining, have performed for bacteria collected from all plates. Also, bacteria were scraped from the plates and disrupted by ultrasonication. The amounts of pertussis toxin (PT), lipopolysaccharide (LPS), and ammonium, normalized to protein content for each treatment.

*B. pertussis* 134 was able to grow on all CBA treatment plates, except CBA plates containing 140 mm of $BaCl_2$. Microscopic studies showed that bacteria cultivated on all CBA plates were appeared as single or paired minute coccobacillus. The inhibitory effect of high concentration of $BaCl_2$ on growth of *B. pertussis* is reported for the first time in this study. Another interesting subject was observation of few mutants of *B. pertussis* 134 on CBA plates containing 140mM $BaCl_2$. In this mutant, morphology of bacteria has been changed to chains of long bacillus instead of single or paired minute coccobacillus.

Our study, for the first time, showed the modulation effect of $BaCl_2$ at concentration of 40 mM at CBA plates; however Bogdan *et al* (2001) indicated that $BaCl_2$ induce higher level of PT at concentration at 20 mM in liquid medium. Hot *et a*l (2003) applied microarray to study the effects of different conditions on antigenic modulation of *B. pertussis*. With comparing the DNA microarray of genes, which are involved in modulation in the present of $BaCl_2$, we could reveal the genes involved in persistence of the mutants of *B. pertussis* 134 on 140 mM $BaCl_2$ CBA plates.

Considerable evidence indicated that pertussis toxin (PT) is a major virulence factor of *B. pertussis* and is important for immunity to disease (Smith *et al* 2001). It has been shown that the expression of pertussis toxin (PT) is under the control of Bvg A/S system, and it was reduced under modulating conditions. Measurement of pertussis toxin indicated the modulating effects of used chemicals and also growing bacteria at low temperature as well. The five most effective modulators for *B. pertussis* 134 were 140 mM $MgSO_4$, 140 mM $MgCl_2$, 28 mM nicotinic acid, 7 mM $FeCl_3$, and 8 mM nicotinic acid, which have reduced the PT levels by 21, 12, 10, 7, and 4 folds, respectively. These findings were in agreements with studies

which explained the modulating effect of $MgSO_4$, $MgCl_2$ and nicotinic acid (Beattie *et al* 1990, McPheat *et al* 1983). The lowest modulating effect of used chemicals on PT of *B. pertussis* 134 belonged to 40 mM $MgSO_4$ and 40 mM $BaCl_2$. Melton and Weiss (1989) explained modulating effect of $SO_4$ salts, while no modulating effect of $MgCl_2$, which is in contrast with (Beattie *et al* 1990, McPheat *et al* 1983) and also our findings. These differences may be attributed to the differences in *B. pertussis* strains used in these studies.

Lipopolysaccharide (LPS) was another virulence factor which was examinined in this study. The levels of LPS determined by quantitative chromogenic Limulus Amebocyte lysate (LAL) method. As a general finding, our data indicated the increase of LPS levels under modulating conditions of *B. pertussis* strain 134.

The results showed that highest effects belonged to 40 mM $MgSO_4$, 280 mM NaCl, 40 mM $BaCl_2$, 140 mM $MgCl_2$, and growth at 22 $^oC$, where increased the LPS levels by 187, 143, 124, 83, and 75 folds, respectively. $FeCl_3$ had the lowest effects on LPS levels of *B. pertussis* 134. A controversial result was the different effects of 40 and 140 mM of $MgSO_4$ on PT and LPS levels of *B. pertussis* 134. While 40 mM $MgSO_4$ was the strongest effector on LPS levels, it was a weak effector on the PT levels.

Altogether, these data indicated that production of lipopolysaccharide (LPS) is not under the control of BvgA/S regulatory system, which controls the pertussis toxin expression. This finding was in agreement to that reported by van den Akker (1998), which indicating increased levels of LPS of bacteria grown at 25 $^oC$, by the use of electrophoretic analysis. The quantitative data of our study confirm that finding and indeed, our results quantitavely describe the increasing effects of $MgSO_4$ and also other used chemicals on LPS levels. Ammonium, as a by-product of amino acids metabolism was another factor examined in our modulation studies. Modulation is a response of bacteria to the different environmental conditions. Metabolism of amino acids may be varying during these responses. The amounts of ammonium were determined by the quantitative Nessler's reaction and normalized to the total protein. The ammonium levels of *B. pertussis* 134 at the presence of various modulators were compared to the ones grown on alone CBA plates. Results indicated that some treatments increased and the other ones decreased the levels of ammonium. These results were not in accord with the trend of PT and LPS variations. It seems at this level, we are not able to find any relation between modulation and ammonium levels. In conclusion, this study for the first time introduced new modulators of *B. pertussis* consisting of $BaCl_2$ and $FeCl_3$. It was shown before (Veal-Carr & Stibitz 2005) that in vitro modulation of *B. pertussis* is in accord to modulation which takes place during infection. Thus with using these modulators we can gain new insights on pertussis infection. This study also confirmed strongly that production of lipopolysaccharide, in contrast to pertussis toxin, is not under the control of Bvg A/S system.

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