ORIGINAL ARTICLE

Monospecific antibody against *Bordetella pertussis* Adenylate Cyclase protects from Pertussis

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ABSTRACT

Objectives: Acellular pertussis vaccines has been largely accepted world-wide however, there are reports about limited antibody response against these vaccines suggesting that multiple antigens should be included in acellular vaccines to attain full protection. The aim of present study was to evaluate the role of *Bordetella pertussis* adenylate cyclase as a protective antigen.

Materials and methods: Highly mono-specific antibody against adenylate cyclase (AC) was raised in rabbits using nitrocellulose bound adenylate cyclase and the specificity was assessed by immuoblotting. *B.pertussis* 18-323, was incubated with the mono-specific serum and without serum as a control. Mice were challenged intra-nasally and patho-physiolgical responses were recorded.

Results: The production of *B.pertussis* adenylate cyclase monospecific antibody that successfully recognized on immunoblot and gave protection against fatality ($p < 0.01$) and lung consolidation ($p < 0.01$). Mouse weight gain showed significant difference ($p < 0.05$).

Conclusion: These preliminary results highlight the role of the *B.pertussis* adenylate cyclase as a potential pertussis vaccine candidate. *B.pertussis* AC exhibited significant protection against pertussis in murine model. *J Microbiol Infect Dis 2012; 2(2): 36-43*

Key words: Pertussis; monospecific; antibody; passive-protection

Bordetella pertussis'e karşı gelişen monospesifik antikor Adenilat Siklaz Boğmaca'ya karşı koruyucudur

ÖZET

Amaç: Asellüler boğmaca aşısı dünya çapında geniş kabul görmekle beraber bu aşılara karşı gelişen antikor cevabının sınırlı olduğunu ve tam koruma elde etmek için çoklu antijenlerin asellüler aşılara dahil edilmesi gerektiğini bildiren raporlar bulunmaktadır. Bu çalışmanın amacı *Bordetella pertussis* adenilat siklazın koruyucu bir antijen olarak rolünü değerlendirmek idi.

Gereç ve yöntem: Tavşanlarda nitroselüloza bağlı adenilat siklaz kullanılarak adenilat siklazına karşı yüksek derecede monospesifik antikorlar elde edildi ve spesifitesi immuoblotting metodu kullanılarak ile değerlendirildi. *B.pertussis* 18-323, monospesifik serumla ve kontrol için serum olmadan inkübe edildi. Farelere intra-nazal uygulandı ve patofizyolojik cevaplar kaydedildi.

Bulgular: *B.pertussis* adenilat siklaz monospesifik antikor yapımı immunoblotla başarıyla gösterildi ve ölüme ($p < 0.01$) ve akciğer konsolidasyonuna ($p < 0.01$) karşı koruyuculuğu gösterildi. Farelerin kilo kazanımı anlamlı farklılık gösterdi ($p < 0.05$).

Sonuç: Bu ön çalışmanın sonuçları potansiyel bir boğmaca aşısı adayı olarak *B.pertussis* adenilat siklazın rolüne işaret etmektedir. *B.pertussis* AC fare modelinde boğmacaya karşı kayda değer bir koruma gösterdi.

Anahtar kelimeler: Boğmaca, monospesifik, antikor, pasif-koruma

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INTRODUCTION

Pertussis or whooping cough caused by Bordatella pertussis is a non-invasive, highly communicable acute infection of ciliated cells of upper respiratory tract, which mainly affects infants, young children and adults[1,2] with little effect of therapeutic intervention after the onset of the disease.[3]

The global burden of pertussis having highest incidence rate in developing countries is approximately 91,689 reported cases and 195,000 deaths per year with 86% DTP3 coverage.[4,5] Transmission is usually due to droplet infection from infected carriers especially within a closed environment.[6,7] Protection is achieved by whole-cell pertussis vaccine (DTwP) and acellular pertussis vaccine (DTaP), composed of few purified antigens (PT, FHA, PRN and Fim).[8,9]

Data of re-emergence of pertussis in Netherlands, Finland, Italy and USA has presented a new challenge to pertussis vaccine program.[10-13] In a recent study about DtaP vaccine response, a high antibody titer was found against the antigens that were present in the DTaP vaccine as compared to the antigens that were not present in that vaccine suggesting that DtaP vaccine formulation should contain additional antigens to confer full protection against pertussis.[14] Adenylate cyclase (AC) is one of the important virulence factors of *B.pertussis* that is produced during pertussis infection in humans and it induces production of high titers of anti-AC antibody that may persist into adulthood. These antibodies are also produced after vaccination.[14] Active immunization with purified AC has been shown to protect mice against *B.pertussis* intra-nasal infection and shorten the colonization period.[16] The aim of present study was to investigate the role of AC as protective antigen against the pathophysiological responses of *B.pertussis* infection through passive protection test in murine model using mono-specific antibodies.

MATERIALS AND METHODS

Preparation of AC

Crude AC was prepared as urea extract from *B.pertussis* strain BP348 (pRMB1)[17] and AC-deficient mutant strain BP348;[18] was resolved on SDS-PAGE (7.5% acrylamide gel) using the method of Laemmli.[19] The resolved 210 kDa AC was transferred to nitrocellulose membrane (Schlerche and Schell) overnight at 30 V at 15°C in Bio-Rad transblot apparatus.

Preparation of nitrocellulose- bound immunogen was performed using method of Knudsen et.al.20; with few modifications. Protein bands were visualized by staining with ponceau S (0.5% ponceau S in 0.1% acetic acid). The membrane was rinsed in sterile deionized distilled water for 3 to 4 times after which the bands at 210 kDa were carefully cut out. Precautions were taken to handle membrane carefully with gloved hands and alcohol-sterilized scalpel and forceps. Bands were de-stained in sterile PBS (Dulbecco A, pH 7.3, Oxoid) and stored frozen at - 20°C. When material from 20 bands was collected (representing 60 μg protein), the frozen bands were thawed and further chopped into 2-3 mm3 pieces. The material was suspended in sterile PBS (500 μl/band) and sonicated at maximal output with[20] seconds cooling intervals in an ice bath until the nitrocellulose was reduced to fine suspension and the particle size was small enough to pass through an 18-gauge hypodermic needle.

The suspension of AC- bearing nitrocellulose in PBS was emulsified in Freund's incomplete adjuvant (Sigma) in 1:1 ratio.

A female New Zealand Albino rabbit was injected with 1.5 ml of this suspension, sub-cutaniously at two sites. Before injection, the rabbit was bled for pre-immune serum. The first injection was followed by two boosters at 15 day intervals. The rabbit was bled on day 40 and serum was collected and designated as mono-specific1 (Ms1).

Immunoblot assessment of Ms1

The method used for immunoblotting was essentially described by Towbin et al.[21] Crude AC was prepared as urea extract from wild type *B.pertussis* Tohama, recombinant *B.pertussis* BP (pRMB1)16, delta AC *B.pertussis* strain BP 34818 and *E.coli* H1469 (pRMB6)17 harboring AC gene and subjected to 7.5% acrylamide resolving gel, and transferred to nitrocellulose. Nonspecific binding sites were blocked with 3% non-fat dry milk (Marvel) in PBS pH 7.3 and probed with pre-immune serum, Ms1 (1:1000 in PBS Dulbecco A, pH 7.3, Oxoid), adsorbed Ms1 (adsorbed with urea extract of BP 348 to neutralize cross reacting epitopes; 1:1000 in PBS Dul-

becco A, pH 7.3, Oxoid) and anti-AC monoclonal McU, (1:4000 in PBS (Dulbecco A, pH 7.3, Oxoid); washed and finally probed with anti-rabbit immunoglobin serum conjugated to horseradish peroxidase (Scottish Antibody Production Unit, SAPU). Color development was performed with diaminobenzidine (DAB, Aldrich) substrate solution and results were recorded.

Intranasal passive protection test

B.pertussis 18-323, phase 1 (ATCC 9797) was grown on Bordet-Gengou (BG) agar (Difco) supplemented with 15% defibrinated sheep blood, at 37°C for 48 h. The growth was suspended in 1% Casamino acids (CAA) (Difco) in PBS (Dulbecco A, pH 7.3, Oxoid) and the cell suspension was made up to 10 opacity units corresponding to approx. 2 x 109 cfu/ml by comparison with 5th International reference of opacity standard developed by WHO.22 The suspension was diluted 1 in 5 and and 1 in 10 in PBS (Dulbecco A, pH 7.3) and designated as 'Dilution A' and 'Dilution B' corresponding to approximately 4 x 108 cfu/ml and 2x108 cfu/ml respectively. High challenge dose (HC) was prepared by mixing 0.5 ml of dilution A with 0.5 ml 1% CAA in PBS (Dulbecco A, pH 7.3) corresponding to approximately 2x108 cfu/ml. In the same way, low challenge dose was prepared by mixing 0.5 ml of dilution B with 0.5 ml 1% CAA in PBS (Dulbecco A, pH 7.3) corresponding to approximately 1x 108 cfu/ml.

Ms1 (0.5 ml) was heated at 56°C for 20 min to inactivate the complement activity, and 250 µl of this Ms1 was incubated at room temperature for 1 h with 250 µl of dilution A and designated as HC+Ms1. Similarly, LC+Ms1 was prepared by incubating at room temperature for 1 h a 250 µl of dilution B with the Ms1.

Groups of 10, 3-4 weeks old CD-1 strain mice for each dilution were anesthetized with ether and infected by intranasal administration of 25 µl of each preparation. First group of mice received 25 µl/mouse HC +Ms1 and its control group received 25 µl/mouse HC only. Second group of mice received 25 µl/mouse LC+Ms1 and its control group received 25 µl/mouse LC only. Third group of mice received 25 µl/mouse 1% CAA in PBS (pH 7.3). Fourth group of mice was left un-inoculated as control.

The mice were weighed every two days, and any fatalities noted over a period of 3 weeks.

Bacterial counts from the inoculum were performed by sampling 10-fold serial dilution on BG. Three weeks post infection; the mice were individually weighed and sacrificed. Leukocyte count (by coulter counter) and lung pathology were recorded. Lung pathology was determined by visual observation. The grading of consolidation as a score of the total lung area, was estimated. Lung score 4 represented edema of lungs /consolidation on both lungs (anterior posterior, top and bottom), 3 represented ¾ area of lungs consolidation, 2 represented 2/4 area of lung consolidation and 1 represented ¼ area of lung consolidation. Lungs were removed and weighed and the pieces were spread aseptically over BG plates, incubated at 37°C in a moist atmosphere. Bacterial counts were determined by 10-fold serial dilutions of homogenates of lungs on BG plates and counting colony-forming units after incubation at 37°C in a moist atmosphere.

Statistical analysis

Data are presented using the mean, SEM and SD of the values from 20 mice per group from two replicate experiments. The Student t-test test was used to assess statistical significance between groups of mice.

RESULTS

Immunoblot Study

The specificity of the antiserum raised against nitrocellulose-bound AC was tested by immunoblotting. AC from urea extracts of *B.pertussis* BP348 (pRMB1) was resolved by SDS and transferred to nitrocellulose membrane as described in the section of materials and methods. The AC band was excised, solubilized and used as immunogen in rabbits with Freund's incomplete adjuvant. Antiserum collected after two booster doses and characterized by immunolotting. Fig. 1 clearly demonstrates the formation of highly homologous antibodies against AC. To ensure that the antiserum recognize only the proteins of interest, an AC negative control *B.pertussis* BP348, a Tn5 insertion mutant for AC 17 was included in this study. The specificity was compared with anti-AC monoclonal antibody (McU), (Fig.1 panel D) which showed similar reaction profile. A cross reacting, 50KDa band was found in preimmune serum Fig.1 panel A and B), which was successfully adsorbed out by using the urea extracts of AC

negative strain BP348 (Fig.1 panel C). Urea extracts from recombinant *E.coli* H1469 (pRMB6); 16 harboring AC gene were also used to analyze the purity standard and specificity of the antiserum (Ms1) raised in this study.

FHA, a 220 KDa protein of *B.pertussis* resolves closely on gels with AC.17 In present study, FHA in AC preparations was not detected when probed with FHA monoclonal antibody (data not shown). Moreover, if FHA had been present with AC, antibodies should have been raised against it, which in turn could have recognized the FHA in urea extracts of BP348 (AC-). These finding suggest that highly specific, homologous antibodies against AC were raised by this novel method.

Passive-Protection Test

The protective activity of Ms1 was tested in mice against intranasal *B.pertussis* challenge. A sublethal dose was given to 3- week old mice with suitable controls and protective effect was monitored for 3- weeks. As is evident from Table 1, Ms1 partially protected the mice against infection.

Deaths occurred in mice treated with high challenge dose between 12th and 13th day but not in control group (p < 0.01). The weight gain in Ms1-treated mice was almost at control levels whereas that in challenge alone groups, weight was reduced (p>0.05). Similarly, there were clear differences in lung weight of challenge alone groups compared with Ms1-treated mice (p>0.01). Total leukocyte count did reveal differences although these were not very significant (Table 1). Viable counts of Ms1-treated and un-treated challenge were approximately equal (data not shown) confirming the viability of the challenge response showing that Ms1 itself was not bactericidal. Lung culture was positive in high challenge dose and low challenge dose + Ms1. Lung pathology determined as consolidation of lungs showed insignificant difference in high challenge dose and high challenge dose with Ms1, However, low challenge groups of mice that received Ms1 showed marginal significant difference (p=0.06), The data is summarized in Table 1 & Fig. 2.

Table 1. The Protective Effect of Anti-AC Monospecific Antibodies (Ms1) in Mouse Intranasal Challenge Test.

Challenge	Fatalities	Mouse, wt (g) Mean (SD; SEM)	Lungs as % body wt (g) Mean (SEM)	Score LP	TLC (log)	Lung-culture
Control (CAA)	0	22.5 (0.03,0.01)	0.74 (0.035, 0.01)	0	4.0	-
HC	2	17.4 (3.8,1.2)	3.11(1.8, 0.52)	3.3	4.5	+
HC+Ms1	0	22.0 (5.1,1.6)	2.18 (0.6,0.19)	3.2	4.3	+
LC	0	21.6 (3.7,1.8)	2.3 (0.5,0.17)	3.3	4.6	+
LC+Ms1	0	22.3 (4.1,1.3)	1.2 (0.9,0.26)	2.4	4.3	+

Mean and SEM values are from 20 mice of two separate experiments

CAA=Casamino acids (1% v/w in PBS)

HC=High challenge dose of *B.pertussis* 18-323 (5x106 cfu/mouse)

LC=Low challenge dose of *B.pertussis* 18-323 (2.5x106 cfu/mouse)

Ms1=Anti-AC monospecific antiserum raised in rabbits (see materials and methods)

LP=Lung pathology

TLC=Total Leukocyte count of WBCs /mm3 blood
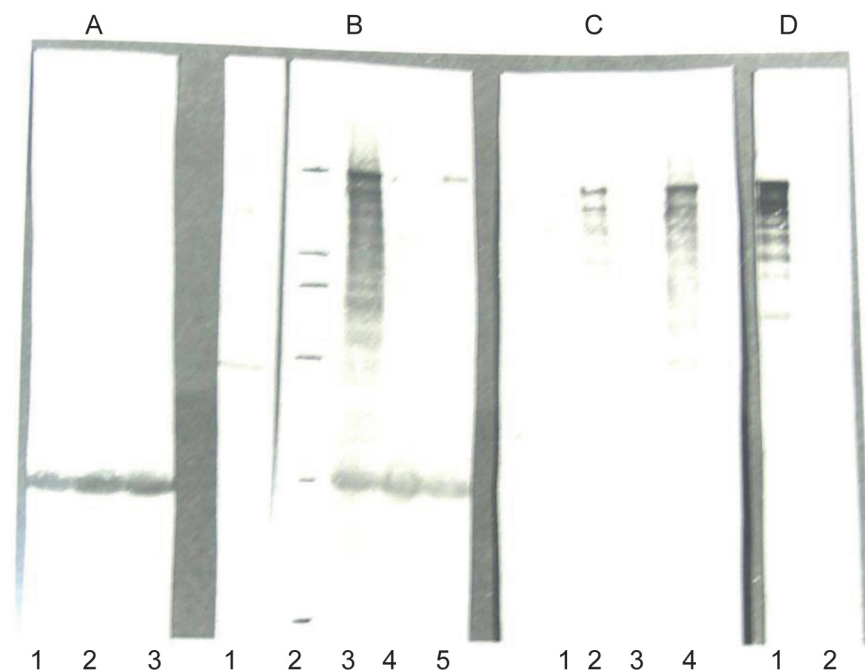
+ = growth (10/10), -= no growth

Figure 1. Immunoblot Assessment of Monospecific Antibodies Raised Against Nitrocellulose-bound AC from B. pertussis.

Urea extracts of native and recombinant AC from *B.pertussis* were separated by SDS-PAGE polyacrylamide (7.5%) resolving gels. Separated proteins were transferred to nitrocellulose and probed with pre-immune serum (Panel A), Ms1 (Panel B), adsorbed Ms1 (Panel C) and anti-AC monoclonal McU (Panel D).

Panel A, 1= *B.pertussis* BP348 (pRMB1); 2= *B.pertussis* BP348; 3= *B.pertussis* Tohama. Panel B, 1= *E.coli* H1469 (pRMB6) [34]; 2 =SDS-6H (MW standard; Sigma); 3= *B.pertussis* 348; 4= *B.pertussis* Tohama. Panel C, 1= *E.coli* H1469 (pRMB6); 2= *B.pertussis* Tohama; 3= *B.pertussis* BP348; 4= *B.pertussis* BP348 (pRMB1).Panel D, 1= *B.pertussis* BP348 (pRMB1); 2= *B.pertussis* BP348.
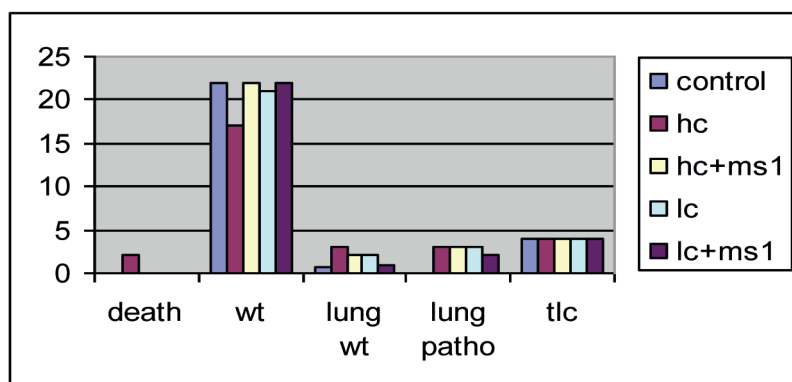

Figure 2. Passive-Protection against Patho- physiological Responses of Pertussis by MS1

The data is mean and SEM of two separate experiments.

Group of mice were challenged with wild-type *B.pertussis* 18-323 with Ms1-treated high challenge and low challenged doses as described in materials and methods. The legend describes: Control (Casamino acids 1% v/w in PBS)

hc= high challenge dose of *B.pertussis* 18-323 (5×10^6 cfu/mouse).

lc = low challenge dose of *B.pertussis* 18-323 (2.5×10^6 cfu/mouse)

hc + ms1= high challenge incubated with monospecific antiserum , lc + ms1= low challenge incubated with ms1.

LP= Lung pathology

TLC= Total Leukocyte count of WBCs /mm^3 blood

DISCUSSION

Immunoblot assessment of Ms1

Highly specific (monospecific) antibody against AC was prepared by using AC, transferred to nitrocellulose and used as an immunogen in rabbits.

Antibodies have become a useful reagent for the identification, localization and purification of biologically interesting molecules. However, their usefulness in providing reliable and credible information depends on their specificity. While monoclonal antibodies offer higher specificity, they sometimes can be difficult to produce because the procedure involved is expensive and time-consuming and requires well-equipped tissue culture facilities. Moreover, hybridoma cell lines are frequently unstable due to either chromosome loss or to tissue culture contamination. An alternate is the production of polyclonal antibodies from antigen separated by SDS-PAGE, still the separated proteins will contain both acrylamide and SDS, which may have detrimental consequences. However, transfer of SDS-PAGE separated AC to nitrocellulose in present study proved successful for raising monospecific antibodies against AC. This antibody (Ms1), although raised against denatured AC, was capable of recognizing epitopes in both native and denatured molecules. This method proved successful for antibody generation as a possible novel tool for AC detection or for AC neutralization for in vitro as well as in vivo studies. The multiband profile observed in the immunoblots was due to the breakdown product of AC possibly as a result of hydolytic action of proteases because the protease inhibitor was not added to the preparations to avoid any interference in the antibody probing. The detection of AC with Ms1 from wild type *B.pertussis*, recombinant AC expressed in *E.coli* and AC from recombinant strain of *B.pertussis* makes the antibody useful for detection of AC on immunoblotts and ELISA.

Passive-protection test

It is now well known that AC is one of the important virulence factors of *B.pertussis*. In a study, adenylate cyclase mutant BP348 was rapidly cleared from the lungs, with no viable bacteria suggesting adenylate cyclase a colonization factor required for the bacteria to initiate infection[22] therefore, neutralization of AC antigen at early stage of infection could help in protection mechanisms of a vaccine.

Guiso et al[24], Brezin et.al.[25] have reported an efficient protection of mice after active and passive immunization with monoclonal and polyclonal antibodies against AC. The results presented in this study elaborate the protective role of monospecific antibody against AC in passive protection test in murine model and supplement the previous findings.

Our study mainly focused on the effect of Ms1 on pathophysiological responses. Previous studies have reported the effect of polyclonal and monoclonal anti-AC antibodies on colonization and lung clearance. In this context, our study extends further the data on protection against various pathologies of *B.pertussis* infection.

Increased lung weight and oedema was reduced by the use of Ms1 but lung pathology was still present although it was not very prominent. The lung consolidation may have been due to other factors produced by *B.pertussis* such as LPS. The Leukocyte count did not show any difference. Preston 1 described this characterization of infection as a less reliable diagnostic feature since in many cases he found that true pertussis did not develop significant increase in circulating lymphocytes.

The possibility that Ms1 may have a bactericidal effect was clearly ruled out since the viable count performed before and after incubation with MS1 showed almost equal cfu/ml as those in Ms1 untreated controls. Furthermore, recovery of *B.pertussis* from the infected lungs proved that mice were infected with *B.pertussis*. These data indicate that AC could possibly act as one of the major virulence factors and protective antigens during infection with *B.pertussis* since its neutralization by Ms1 caused a reduction in the severity and rate of infection and the reason why a complete protection was not achieved may be due to the antibody (Ms1) used as that was raised against denatured AC. However, SDS-denatured AC has been shown to re-nature under hydrophobic condition.[26] The other possibility could be the titer of the antibody which may not have been high enough to neutralize the AC produced by *B.pertussis* 18-323 which has been reported to produce three times more AC and be more virulent than other wild-type *B.pertussis*15 and the data presented in this study support this view. In

the present work, this strain had 10-times more enzyme activity and 70% increased toxic activity than *B.pertussis* Tohama when determined by Hemolysis assay of sheep RBCs as described by Bellalou et al.[27] The partial protection also reflects the role of other virulence factors since, of course, the Ms1 neutralized the effects of AC, but *B.pertussis* has other factors involved in colonization of the host. However, in combination with other components of acellular vaccine, such as FHA, PT, PRN; AC may play a protective role against pertussis as suggested by previous studies.[16,25] The fact that AC represents one of the major virulence factors of *B.pertussis* was already shown by Goodwin and Weis,[23] when CyaA-deficient bacteria were unable to cause lethal infection and were cleared rapidly from the lungs in a mouse model. This strongly suggest that the effect in reduced pathology of the *B.pertussis* treated with Ms1 would most likely come from the neutralization of AC on the surface of *B.pertussis*28 thereby reducing the colonization of the lung in the first place.

Although there are some limitations in our study such as the data has been acquired from a smaller test population and the AC used in our study was crude and better response could have been achieved if purified AC would have been used. However, present study reports the neutalazation and protective effect of AC. No doubt, the current acellular pertussis vaccines prevent against full bloom disease, substantially exposed individuals become infected with *B.pertussis* and develop a milder form of the disease for which new vaccination strategies are under investigation [29] and here we suggest new formulation also. Significant human humoral antibody response to *B.pertussis* AC reported by Farfel et al.[15] and Arciniega et al.[30] and other published literature[16,23-25] supports our findings to include other antigens such as AC, in present formulation of DtaP because reports reveal that the efficacy of the various DTaP vaccines was not optimal in five trials and DTwP vaccines were more effective than DTaP vaccines.[31,32] Therefore, it is the high time to review the antibody responses of DTaP to other antigens including AC and ascertain their role for inclusion in DTaP along with PT, PRN and FHA. Moreover, the reports of AC of *B.pertussis* as an effective vaccine adjuvant in vivo, enhancing Ab responses to co- administered Ags[33,34] and; in-

duction of Th2 and regulatory T cells response[35] further qualifies AC as strong vaccine candidate.

In conclusion, significant protective response to *B.pertussis* AC has been observed after passive protection in animal model using monospecific anti-pertussis antibodies raised in present study. Our findings support the studies of Storsaeter[36] and Cherry[13] that DTaP vaccines should contain multiple antigens including AC.

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