Effect of pH, Temperature and Media on Acid and Alkaline Phosphatase Activity in "Clinical" and "Nonclinical" Isolates of *Bordetella bronchiseptica*

D.M. Mobley, M.M. Chengappa, W.L. Kadel and J.G. Stuart*

ABSTRACT

Twenty-two isolates of *Bordetella bronchiseptica* were studied to determine the effects of pH, incubation temperature and type of media on peak acid and alkaline phosphatase activity. The pH optimum for alkaline phosphatase activity was 9.0 for all of the isolates tested. The pH optimum for acid phosphatase activity was 5.8 for 67% of the isolates and 4.8 for 33%. All of the isolates showed peak phosphatase activity at 37°C. No preference was shown in 35% of the isolates between the types of media tested; however, 40% preferred tryptose broth, 20% preferred nutrient broth and 5% preferred brain-heart infusion broth. No relationship was shown between phosphatase activity and the mouse lethality of the isolates.

Key words: Acid phosphatase, alkaline phosphatase, *Bordetella bronchiseptica*.

RÉSUMÉ

Cette étude portait sur 22 souches de *Bordetella bronchiseptica* et elle visait à déterminer l'effet du pH, de la température d'incubation et du genre de milieu de culture, sur l'activité maximale de leurs phosphatases acide et alcaline. Le pH idéal pour l'activité de la phosphatase alcaline de toutes les souches expérimentales se situait à 9,0, tandis que, pour l'activité de leur phosphatase acide, il se situait à 5,8, pour 67% des souches précitées, et à 4,8, pour 33% d'entre elles. Toutes ces souches manifestèrent la plus grande activité de leurs phosphatases, à 37° C; 35% d'entre elles ne manifestèrent aucune préférence pour l'un ou l'autre des milieux de culture utilisés, tandis que 40% préférèrent le bouillon tryptosé, 20%, le bouillon nutritif, et 5%, le bouillon à l'extrait de cerveau et de coeur. On ne décela aucune relation entre l'activité des phosphatases des souches précitées et leur létalité pour les souris.

Mots clés: phosphatase acide, phosphatase alcaline, *Bordetella bronchiseptica*.

INTRODUCTION

Bordetella bronchiseptica is the only member of the genus *Bordetella* which is of significance in animal disease (1,2). It is commonly recovered during respiratory diseases from guinea pigs, rabbits, cats, dogs, horses and swine (2,3); however, it can often be isolated as part of the normal flora of the respiratory tract of these animals (4). *Bordetella bronchiseptica* is also considered to be a major contributor to atrophic rhinitis in swine (5). This disease is characterized by atrophy of the nasal turbinate bones and distortion of the nasal septum which may lead to shortening or twisting of the upper jaw (6). Recent investigation into changes in the acid and alkaline phosphatase activity of the nasal turbinates of young pigs inoculated with *B. bronchiseptica* has led to a possible correlation between enzymatic activity and morphological changes in the nasal turbinates (5).

Acid phosphatase production has been reported in *B. bronchiseptica* (4). The test for acid phosphatase production has proven to be of value with other organisms (7,8,9,10). It has been used to differentiate pathogenic from nonpathogenic staphylococci (10) and has also been used as a rapid means of identifying mycobacteria (9). This enzymatic activity has been shown to be produced by *Propionibacterium acnes, Escherichia coli, Streptococcus mutans, Serratia, Klebsiella, Proteus* and *Enterobacter* (7,8,10,11).

The purpose of the present investigation is to determine the effects of pH, incubation temperature and type of media on peak acid and alkaline phosphatase activity in *B. bronchiseptica* and to determine if there is a relationship between the phosphatase activity of the culture and its pathogenicity.

MATERIALS AND METHODS

BACTERIAL CULTURES

Twenty-two cultures of *Bordetella bronchiseptica* were examined. Thirteen were isolated from porcine nasals and nine from porcine lungs. Fourteen "clinical" isolates of *B. bronchiseptica* were recovered from animals submitted to the diagnostic laboratory showing signs of respiratory disease. Three "nonclinical" isolates were recovered from animals submitted to the diagnostic laboratory for reasons other than respiratory disease and the remaining five "nonclinical" isolates were recovered from nasal swabs of young pigs from three farms designated by a veterinarian as "Bordetella-free." The isolates were identified as *B.*

*Breathitt Veterinary Center, Murray State University, Hopkinsville, Kentucky 42240 (Mobley, Chengappa and Kadel) and Department of Biology, Murray State University, Murray, Kentucky 42071 (Stuart).

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bronchiseptica by the characteristics as described by Carter (1) and maintained by continual passage. The growth curve was plotted by the standard method, and the viable count was estimated by the spread plate technique.

DETERMINATION OF ENZYME ACTIVITY

The first assay procedure was performed according to the method of Bessey *et al* (12). Five-tenths of 1 mL of an 8 h tryptose broth culture (corresponding to late log phase) was added to 3.0 mL of a 0.1 M acetate buffer. The reaction was started by adding 0.5 mL of a 0.5% (W/V) solution of p-nitrophenyl phosphate (PNPP, Sigma). The solution was incubated for 12, 24 and 48 h and then centrifuged for 10 min at 3000 g. One mL of supernatant was added to 2.0 mL of 0.2 N NaOH to stop the reaction and develop the color. The p-nitrophenol released from the substrate was measured at 420 nm on a Coleman spectrophotometer (model 6/20), with reference to the standards. The standard curve was also prepared according to the method of Bessey *et al* (12).

The following media, buffer pH's, and incubation temperatures were used to determine the peak phosphatase activity using the substrate PNPP; media: tryptose, nutrient and brain-heart infusion broths (Difco); buffer pH's: 4.8, 5.2, 5.8, 7.0, 8.0, 9.0; temperatures: 25, 30, 37, 42, 50 and 55°C.

The second method used to determine phosphatase activity was that of Wolf *et al* (10) as described by Chengappa *et al* (4). Heavy inoculation of a 48 h sheep blood agar grown culture was added to 2.75 mL of 0.1 M sodium acetate buffer (pH 5.2 or 9.0), containing 2.0 mg of 5-bromo-4 chloro-3 indolyl phosphate substrate (Sigma). This substrate had previously been dissolved in 0.25 mL NN-dimethyl formamide (Sigma) and stored at 4°C until used. The tubes were incubated at 37°C, and phosphatase production was evidenced by a blue-green precipitate within 18 h.

PATHOGENICITY TEST

To compare the pathogenicity of the cultures, one month old Swiss Webster ICR albino mice of mixed sex were injected intraperitoneally with 0.25 mL of a 48 h sheep blood agar grown culture and culture dilutions (10^{-1}—10^{-9}). The culture was suspended in physiological saline to match the turbidity of McFarland tube #8. The mice were observed for seven days following injection of culture. Nine of the 22 isolates were used in the pathogenicity study, five "clinical" and four "nonclinical" isolates.

RESULTS

pH

The optimum pH for acid phosphatase activity was found to be 5.8 for 67% of the isolates. For the remaining 33%, the optimum pH was 4.8. The optimum pH for alkaline phosphatase activity was found to be 9.0 for all isolates tested.

TEMPERATURE

One hundred percent of the isolates showed peak activity at 37°C, for both acid and alkaline phosphatase.

MEDIA

Forty percent of the isolates showed higher phosphatase activity in tryptose broth, 20% in nutrient broth and 5% in brain-heart infusion broth, while the remaining 35% showed no preference for media.

PHOSPHATASE ACTIVITY

All isolates tested produced acid and alkaline phosphatase using PNPP as the substrate. The peak phosphatase activity was shown in 8 h broth cultures which corresponded with late log phase. The amount of p-nitrophenol (PNP) released during the acid reaction ranged from 0.22 μmol/0.5 mL to 10.5 μmol/0.5 mL (Table I). The average level of PNP produced by the "clinical" isolates during the acid reaction was 5.79 μmol/0.5 mL, while the average level produced by "nonclinical" isolates was 3.48 μmol/0.5 mL. In the alkaline phosphatase reaction, the amount of PNP released ranged from 0.19 μmol/0.5 mL to 0.42 μmol/0.5 mL (Table I). The average level of PNP produced by the "clinical" isolates during the alkaline reaction was 0.30 μmol/0.5 mL, while the average level produced by the "nonclinical" isolates was 0.31 μmol/

TABLE I. Acid and Alkaline Phosphatase Levels in "Clinical" and "Nonclinical" Isolates of *Bordetella bronchiseptica*

"Clinical" Isolates				"Nonclinical" Isolates			
Sample #	Origin	Acid Phosphatase μmol PNP[a]	Alkaline Phosphatase μmol PNP	Sample #	Origin	Acid Phosphatase μmol PNP	Alkaline Phosphatase μmol PNP
1	Nasal	10.4	0.40	1	Nasal	8.0	0.26
2	Nasal	10.2	0.31	2	Nasal	0.73	0.39
3	Lung	1.1	—[b]	3	Nasal	1.1	0.26
4	Lung	10.3	0.42	4	Nasal	4.4	0.27
5	Nasal	2.1	0.22	5	Nasal	1.4	0.33
6	Nasal	9.5	0.24	6	Lung	0.22	0.33
7	Lung	5.5	0.23	7	Nasal	10.5	—
8	Lung	1.2	0.33	8	Nasal	1.5	0.38
9	Lung	7.3	0.38				
10	Lung	8.0	0.37				
11	Nasal	—	—				
12	Lung	3.2	0.23				
13	Lung	—	—				
14	Nasal	0.75	0.19				

[a]μmoles PNP; μmoles p-nitrophenol released per 0.5 mL culture
[b]—; results not available

0.5 mL. Using 5-bromo-4 chloro-3 indolyl phosphate as substrate, 21 isolates were positive for acid phosphatase production and one isolate was negative. All isolates were positive for alkaline phosphatase production using this substrate.

PATHOGENICITY

Three of the five "clinical" isolates killed mice at the 10^{-1} dilution within 48 h, whereas the other two "clinical" isolates did not appear to be pathogenic, even in undiluted form. Three of the four "nonclinical" isolates were also pathogenic for mice at the 10^{-1} dilution within 48 h, while the fourth isolate was found to be nonpathogenic to mice either in diluted or undiluted form.

DISCUSSION

The optimum pH for acid phosphatase production was 5.8 for the majority of isolates (67%) and 4.8 for the remainder (33%). This generally agrees with the results of Luoma (13) using *Streptococcus mutans*, where the optimum activity was at pH 4.8, but the eluted factor showed optimum activity at pH 5.8. Similar variations in optimum pH for phosphatase activity in *S. mutans* was also noted by Greenman *et al* (7). The reasons for the variation in pH optima between isolates of *B. bronchiseptica* needs further investigation. The optimum pH for alkaline phosphatase activity was 9.0 which is in agreement with Greenman *et al* (7) for *S. mutans*. The optimum temperature was 37°C for both acid and alkaline phosphatase activity. This is probably due to the fact that 37°C provides suitable temperature conditions for *B. bronchiseptica*, allowing for production of maximum numbers of viable cells. In support of this, peak phosphatase activity corresponded to late log phase of growth, when the maximum numbers of viable bacterial cells were present. The majority of isolates preferred tryptose broth (40%) or showed no preference (35%) as to which broth was used. This suggests that the phosphatase production or activity in *B. bronchiseptica* may not be solely dependent on the type of growth media used; however, the authors feel that any media promoting the maximum level of growth of *B. bronchiseptica* would probably show maximum production or activity of the phosphatase enzyme.

Variations in the enzyme levels between different strains of *S. mutans* has been reported by Greenman *et al* (7). Similarly, the isolates of *B. bronchiseptica* used in this study showed a wide range of enzyme levels (Table I). Although there were apparent differences in the averages of enzyme levels between "clinical" (5.79 µmol/0.5 mL) and "nonclinical" (3.48 µmol/0.5 mL) isolates, it would be premature to draw any conclusions as there were insufficient numbers of isolates studied.

No correlation could be shown between the enzyme activity and mouse lethality. This, however, does not rule out the role of phosphatase in nasal turbinate atrophy. The authors' laboratory has presently undertaken a study to determine the significance of the phosphatase enzyme in the development of nasal turbinate atrophy, using a mouse model.

Bordetella pertussis has been shown to produce adenylate cyclase, a highly active enzyme which catalyzes the production of adenosine 3'-5' monophosphate (cyclic AMP) by phagocytic cells (14). Confer and Eaton (14) found a massive increase in intracellular cyclic AMP within neutrophils incubated with *B. pertussis* extracts, which caused disruption of normal cellular function. This finding led to the possible explanation of their previous observations of alveolar macrophage dysfunction in *B. bronchiseptica* infected rabbits. Cyclic AMP has been used as a substrate by alkaline phosphatase and was shown, at 10^{-3}M, to stimulate the activity of this enzyme by 25% (15). Recently, Silveira (5) demonstrated an increase in acid and alkaline phosphatase activity in turbinates of pigs with atrophic rhinitis, following experimental intranasal inoculation with *B. bronchiseptica*. The action of cyclic AMP on the phosphatase enzymes and a possible role of the enzymes in the development of atrophic rhinitis is worthy of further investigation.

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