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A reappraisal of serotype factors 4, 5 and 6 of *Bordetella pertussis*

BY N. W. PRESTON, NAREKUL SURAPATANA* AND E. J. CARTER
Department of Bacteriology and Virology, University Medical School, Manchester M13 9PT

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SUMMARY

Twelve strains of *Bordetella pertussis*, of various serotypes, were tested by agglutination with serial dilutions of six antisera which were thought by their donor to be monospecific for six distinct agglutinogens.

The first three sera were shown, by agglutination of the twelve donated strains and of standard pertussis serotypes, to be monospecific for the three well-recognized major agglutinogens (factor 1, factor 2, factor 3).

The 'factor 4' serum gave weak agglutination, but only of strains that possessed factor 2, though not all of such strains. This suggested that it may be a distinct minor component of factor 2.

The 'factor 5' serum was very weak, and gave agglutination only with a few of the factor 2 strains.

The titres of the 'factor 6' serum were very closely parallel to those of the factor 3 serum, and it may be inferred that these 'factors' probably constitute a single antigenic entity.

Seven of the twelve donated strains were shown to be mixtures of two or more serotypes, offering an explanation for discrepancies in serological tests involving the use of these strains on different occasions.

It seems likely that 'factors 5 and 6' do not actually exist; and, since 'factor 4' is only a minor component of factor 2, any protective role that it may have would probably be covered by the presence of factor 2 in vaccine.

INTRODUCTION

Numerous workers have recognized three major agglutinogens of *Bordetella pertussis*, and have designated serotypes of this species by the combinations of these factors that different strains possess: type 1,2,3; type 1,2; type 1,3; and the degraded type 1 organism that does not cause human infection. Preston (1979) has reviewed the vital role of the type-specific factors 2 and 3 in the natural human disease and in experimental infection of marmosets. More recently, their importance in experimental infection of rabbits also has been shown (Preston, Timewell & Carter, 1980). Their significance in the prevention of whooping-cough is recognized by the recommendation of the World Health Organization (1979) that vaccine should include all three agglutinogens.

* Present address: Bacteriology Section, Rajavithi Hospital, Phyathai, Bangkok 4, Thailand.

By contrast, few workers have claimed that more than three agglutinogens exist. Andersen (1953) mentioned factors 4 and 5; but she explained the subsequent failure of other workers to produce these sera, using her strains, as follows (Andersen, 1963): 'there is a great difference in the response of rabbits to factors 4 and 5 which means that it is rather difficult to get the specific sera sufficiently strong'. Eldering, Hornbeck & Baker (1957) added factor 6; but later Eldering, Eveland & Kendrick (1962) referred to factors 4 and 6 as 'minor antigens'. Dolby & Stephens (1973) examined 200 human sera for pertussis agglutinins: 'in none was agglutinin 4 found without 2, nor 6 without 3...Agglutinin 5 was found (with 2) in only one child. Since response to agglutinogens 2 and 3 is stronger than to 4, 5 and 6, the measurement and recording of agglutinins to agglutinogens 2 and 3 only are given'.

Nevertheless, the minor antigens (4, 5 and 6) were thought by Bronne-Shanbury, Miller & Standfast (1976) to be of possible value epidemiologically. However, their evidence is far from convincing (Preston, 1976*a*): in particular, there is no indication of the serological purity of their cultures; and Stanbridge & Preston (1974) had shown that mutation of pertussis serotypes was not uncommon, and that even fresh isolates of the organism may be mixtures of two or more serotypes. More recently, Toma, Lo & Magus (1978) abandoned further study of the 'minor factors 4, 5 and 6' after finding problems with cross-agglutination.

We realized the possibility that serological results may be influenced by the use of mixed cultures – either for injection into rabbits to produce sera, or for absorption of those sera, or as suspensions for testing the agglutinin-content of the sera. We felt it important to establish whether there may be more than three pertussis agglutinogens, with potential roles in immunity; and we therefore decided to study (*a*) the serological purity of some donated test-strains, and (*b*) the reproducibility of agglutination of these strains by six donated 'monospecific sera'.

MATERIALS AND METHODS

Strains of Bordetella pertussis

Standard strains. Three strains of known serotype (Preston, 1966) have been used in many previous studies: 360 E (type 1,2), H 36 (type 1,3), GL 353 (type 1).

Donated strains. Twelve strains were kindly supplied by Dr C. R. Manclark from the collection of the Bureau of Biologics (previously Division of Biologics Standards, National Institutes of Health), Bethesda, Md 20205, U.S.A. Their serotypes had been assigned by Dr Eldering, as follows: 105 (type 1,2,6), 112 and 125 (type 1,2,4), 114 and 129 (type 1,3,6), 122 (type 2,3), 131 and 160 (type 1,2,5,6), 133 (type 1,2,3,4), 140 and 163 (type 1,2,3,5,6), 155 (type 1,3).

Pertussis typing sera

Manchester sera. Three monospecific sera (factor 1, factor 2, factor 3) were prepared in this Department by the methods described previously (Preston, 1966). The factor 2 and factor 3 sera had titres of less than 20 against heterologous standard strains, and gave a negative reaction with those strains by slide-agglutination. All three of our typing sera were diluted to have a titre of 320 against the homologous standard strain, and, as reported previously (Preston, 1976*b*), they

gave the same typing results as the three Zakharova monospecific sera from the World Health Organization reference laboratory in Moscow.

Our three typing sera were used for assessing the serological purity of the twelve Eldering cultures.

Donated sera. Six sera, 'monospecific for factors 1–6', were kindly supplied from the Bureau of Biologics, having been 'prepared by Dr Eldering under contract'. They were titrated in the present study, in duplicate by each of two operators, against each of the twelve Eldering strains.

Serotyping of cultures by slide agglutination

Four drops of a single suspension of each culture were placed on a glass slide. To the first three was added a loopful of one of the three Manchester typing sera, whilst the fourth (control) drop detected any auto-agglutination. Degrees of agglutination were recorded as described in detail previously (Preston, 1966): +++, ++, +, −.

Typing of single-colony subcultures was performed as described by Stanbridge & Preston (1974).

Titration of sera

Two-fold dilutions of each serum, from 1 in 5, were made in the cups of a plastic haemagglutination tray. After addition of an equal volume of bacterial suspension to each cup, bringing the initial dilution of serum to 1 in 10, the tray was vibrated mechanically to mix the serum and bacteria, then incubated at 55 °C for 30 min, and examined under a plate-microscope after a further 15 min at room-temperature (see Preston, 1970, for full details).

RESULTS

Serological purity of twelve pertussis cultures

As shown in Table 1, all twelve strains agglutinated with our own factor 1 serum. The variation in strength of this reaction amongst the twelve strains caused no surprise, for type 1,2,3 and type 1,2 strains usually react strongly whereas type 1,3 strains react weakly. But weakness in the factor 2 or factor 3 reaction (for example, with strains 122 and 131) is usually an indication that only a few of the bacteria in the suspension possess the relevant antigen.

We therefore suspected that many of the initial cultures contained more than one serotype; and subsequent examination of individual colony subcultures showed that at least seven of the twelve cultures were mixtures (Table 1). For example, strain 122 was mainly type 1,3 but with some type 1,2,3 – explaining the weak factor 2 reaction noted above. And strain 131 was mainly type 1,2 but with some type 1,2,3 and some type 1 – explaining its weak factor 3 reaction.

Evaluation of six 'monospecific' sera

The identities of the sera for factors 1–3 were confirmed by agglutination of our standard strains: factor 1 agglutinated all three standard strains, factor 2 agglutinated only 360 E, and factor 3 agglutinated only H 36.

These three sera were then titrated against each of the twelve Eldering strains,

Table 1. *Agglutination reactions of twelve Eldering strains of* Bordetella pertussis *with three Manchester monospecific antisera*

Strain no.	Eldering serotyping of strain	Agglutination of strain* With Manchester monospecific serum 1	2	3	In saline control	Serotypes of 16–20 colonies of each Eldering strain tested with Manchester sera: type 1,2,3	1,2	1,3	1
105	1,2,6	+ + +	+ + +	+ +	–	17	3	0	0
112	1,2,4	+ + +	+ + +	–	–	0	17	0	3
114	1,3,6	+	–	+ + +	–	0	0	16	0
122	2,3	+	+	+ + +	–	2	0	18	0
125	1,2,4	+ +	+	tr	tr	0	16†	0	0
129	1,3,6	+	tr	+ + +	tr	0	0	16	0
131	1,2,5,6	+ +	+ + +	+	tr	2	13	0	1
133	1,2,3,4	+ +	+ + +	+ +	–	7	5	2	2
140	1,2,3,5,6	+ + +	+ + +	+ + +	–	20	0	0	0
155	1,3	+	tr	+ + +	tr	0	0	20	0
160	1,2,5,6	+ +	+ +	+	–	5	11	0	0
163	1,2,3,5,6	+ + +	+ + +	+ + +	—	16	0	0	0

* Agglutination of confluent growth of Eldering strain recovered from ampoule received. For degrees of agglutination, see Materials and Methods. 'tr' indicates a trace of agglutination, seen even in the control suspension (as evidence of auto-agglutinability).

† Of these 16 six were characteristic of type 1,2 (strong reactions with 1 and 2 sera); 10 of them were auto-agglutinable and had an unusually weak factor 2 reaction.

and the results of four tests performed by two operators showed remarkable consistency (Table 2). Occasional lack of reproducibility (more than two-fold deviation from the mean) is readily explicable in one of two ways: auto-agglutinability, with strain 129, causing difficulty in determination of the end-point; mixed serotypes also, with strain 131, so that the concentration of a particular antigen in one operator's suspension may be different from that in a culture grown on another occasion.

The three other Eldering sera, for 'factors 4–6', were titrated likewise and the results showed somewhat greater lack of reproducibility (Table 2). In addition to the points mentioned above, there was the problem that these sera gave only weak agglutination, so that there was greater difficulty in determination of the end-point and therefore greater likelihood of variation between the results recorded by two operators.

Nevertheless, broad general deductions can be made from the mean titres which are recorded in Table 3. For sera 1–3, there is a clear correlation between the titres of these sera and the antigenic composition of the twelve strains (often of mixed serotypes) against which they were titrated.

With 'factor 4' serum, a reaction was found only with strains for which factor 2 serum had a high titre, but not with all such strains. Although the factor 4 titres were relatively low, there does seem to be evidence that this is a distinct entity – a sub-unit of factor 2, found on some but not all strains that possess factor 2 as a major antigen.

With 'factor 5' serum, the reaction was always *very* weak, and again occurred

Table 2. *Agglutinin titres* of six Eldering sera against twelve Eldering strains of* B. pertussis

	Serum 1				Serum 2				Serum 3				Serum 4				Serum 5				Serum 6			
Strain	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
105	320	320	320	160	1280	1280	640	640	40	40	40	40	0	0	20	10	10	10	0	10	40	40	80	80
112	160	320	160	160	1280	1280	640	640	0	0	0	0	160	80	160	320	0	0	0	0	0	0	0	0
114	80	80	80	40	0	0	0	0	80	160	160	320	0	0	0	0	0	0	0	0	160	80	80	160
122	80	80	40	80	160	160	160	160	40	80	80	80	10	10	40	80	0	0	0	0	40	20	80	80
125	160	160	160	160	320	320	320	640	0	0	0	0	40	20	160	160	0	0	0	0	0	0	0	0
129	80	80	80	160	0	0	0	10	40	80	320	320	0	0	0	0	0	0	0	0	80	40	160	160
131	320	160	160	160	640	640	320	640	0	0	10	20	0	0	20	20	10	10	10	10	10	10	40	40
133	160	160	80	160	640	640	640	640	40	40	80	20	160	160	160	80	0	0	0	0	0	0	40	40
140	160	160	80	160	640	640	640	640	40	40	40	80	0	0	10	10	10	10	10	10	20	40	40	40
155	80	40	40	40	0	0	0	10	80	160	160	160	0	0	0	0	0	0	0	0	80	80	80	80
160	320	160	160	160	640	640	640	640	0	0	20	20	0	0	10	10	10	10	20	10	10	10	40	20
163	160	160	80	160	1280	1280	320	640	80	80	160	160	0	0	10	10	10	10	20	10	40	80	80	80

* Tests a and b were performed by one operator using one set of bacterial suspensions; tests c and d were performed by another operator with suspensions made from cultures grown on another occasion.

0 = < 10 (no visible agglutination at the initial serum dilution of 1 in 10).

Table 3. *Agglutinin titres of six Eldering sera with twelve Eldering strains of* B. pertussis

Strain no.	Eldering serotyping of strain	Serotypes detected with Manchester sera (see Table 1)	Titres of the six Eldering sera* (Geometric mean of four tests performed in Manchester – see Table 2)					
			1	2	3	4	5	6
105	1,2,6	1,2,3 (and 1,2)	270	910	40	0	0	57
112	1,2,4	1,2 (and 1)	190	910	0	160	0	0
114	1,3,6	1,3	67	0	160	0	0	110
122	2,3	1,3 (and 1,2,3)	67	160	67	24	0	48
125	1,2,4	1,2 and 1,[2]†	160	380	0	67	0	0
129	1,3,6	1,3	95	0	130	0	0	95
131	1,2,5,6	1,2 (and 1,2,3 and 1)	190	540	0	10	10	20
133	1,2,3,4	1,2,3; 1,2; 1,3 and 1	130	640	40	130	0	14
140	1,2,3,5,6	1,2,3	130	640	48	0	10	34
155	1,3	1,3	48	0	130	0	0	80
160	1,2,5,6	1,2 (and 1,2,3)	190	640	10	0	12	17
163	1,2,3,5,6	1,2,3	130	760	110	0	12	67

* 0 = < 10.

† 1,[2] indicates an unusually weak factor 2 reaction (see Table 1).

only with strains rich in factor 2. There is insufficient evidence that this is a distinct entity.

With 'factor 6' serum, the titres with the twelve strains were almost exactly parallel to those with factor 3, and this suggests that these two 'factors' are but one entity.

DISCUSSION

This study of twelve cultures of *B. pertussis*, and six Eldering 'monospecific' sera, gives ample evidence to support the validity of the existence of three major agglutinogens (factor 1, factor 2, factor 3). Indeed, with these three factors, there is surprisingly good agreement between our own typing results and those previously assigned to the cultures by Eldering; and this is so, in spite of our demonstration that many of the twelve cultures were mixtures of different serotypes.

With the minor factors, however, the discrepancies are more marked; and there seem to be several contributory causes. As we have noted, the reactions with these sera were weak and there was difficulty in specifying an end-point, so the reproducibility in the recorded results was poorer. These problems were only aggravated by the presence of mixed serotypes in the bacterial cultures, giving rise to the likelihood that the proportions of the type-specific antigens would vary in the suspensions prepared by different workers.

Our own results showed that 'factors 4 and 5' were detected only on strains possessing factor 2, of which 'factor 4' appears to be a minor component that is present only on some of the factor 2 strains. The weakness of the reactions with 'factor 5' serum leaves doubt as to whether such an entity actually exists; and it is noteworthy that Bronne-Shanbury *et al.* (1976) considered this an 'extremely

rare' component, for the existence of which they produced no convincing evidence. 'Factor 6' has been found by previous workers to be closely associated with factor 3; and our present detailed study has failed to differentiate this 'minor antigen' from factor 3.

Agglutinogens and immunity

In many countries, pertussis vaccine rich in factors 1 and 2, but lacking factor 3, has failed to protect children against infection with type 1,3 organisms, which have a predominance of factor 3 (Preston, 1976*b*). Conversely in Finland, where vaccine was made from a type 1,3 strain which had been thought to possess all three factors, the sera of vaccinated children contained antibody 3 but not 2, and the vast preponderance of strains from infected children became type 1,2 (Kuronen & Huovila, 1978).

With this and other evidence of the immunogenicity of these surface antigens, it is vital to know whether more than three important factors exist. As shown above, 'factors 5 and 6' can probably be discounted; and 'factor 4' is such a minor component of factor 2 that it is unlikely to act as a separate immunogen.

We may therefore rest content that there are only three important immunogens, and pertussis vaccine need contain only these three factors – together with adjuvant to ensure an adequate immune response (Preston, 1979).

Nevertheless, our present studies emphasise that old stock cultures, which have become auto-agglutinable, should not be used in standard serological tests for the presence of these three factors. However, growth on charcoal-blood-agar, rather than Bordet-Gengou medium, greatly reduces the tendency to auto-agglutinability (Preston, 1970).

Furthermore, cultures used for standard suspensions should be checked by single-colony subculture to ensure freedom from gross mutation of serotype.

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