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Is *Bordetella pertussis* clonal?

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Abstract

Objective—To establish whether *Bordetella pertussis* is essentially clonal.

Design—Analysis of restriction fragments of *Xba*I digests of DNA from clinical and control isolates of *B pertussis* by pulse field gel electrophoresis.

Materials—105 isolates of *B pertussis*: 67 clinical isolates from throughout the United Kingdom and 23 from Germany (collected during the previous 18 months); vaccine strains 2991 and 3700; and 13 control isolates from Manchester University's culture collection.

Main outcome measures—Frequency of DNA types according to country of origin and classical serotyping.

Results—17 DNA types were identified on the basis of the variation in 11 fragments, banding at 200-412 kilobases; 15 types were found in the clinical and control isolates from the United Kingdom and seven in those from Germany. There was no correlation with serotype. DNA type 1 was the commonest overall (22/105 strains, 22%), predominating in serotypes 1,2 and 1,2,3 and including the vaccine strains but not the isolates from Germany.

Conclusions—Current infections due to *B pertussis* are not caused by a clonal pathogen as multiple strains are circulating in a given population at one time. There is also considerable epidemiological variation in the pathogen population between countries. These findings may have implications for the design of acellular vaccines.

Introduction

Previous work based on examining isolates of *Bordetella pertussis* by analysing allelic variation in structural genes encoding 15 enzymes by electrophoresis concluded that this pathogen was clonal and that its genetic diversity was limited.¹ This is important in vaccine development as *B pertussis* is still a major pathogen, causing over 600 000 deaths annually, one every 52 seconds.^{2,3} Most deaths occur in unimmunised infants, and in the developing world only a third of children have been immunised. Control of the disease is mainly by immunisation, a process which is dependent on having a safe, effective vaccine. The original whole cell vaccine has been criticised for its associated persistent neurological effects,² and in the United Kingdom this has led to a fall in immunisation and the resurgence of epidemics in 1978-9 and in 1982.^{4,5}

These problems have been partially offset by the development of acellular vaccines, which have been introduced in Japan as both primary and booster doses.⁶ The incidence of adverse reactions was reduced, but estimates of the efficacy of these vaccines varied widely and were as low as 69%⁷ compared with that for the whole cell vaccine of 80-95%.⁸

The success of an acellular vaccine is linked with the idea that isolates are sufficiently similar that part of one isolate can produce adequate immunity against infection due to the clinically important strains of that species. If *B pertussis* is essentially clonal then this should be achievable.

This concept disagrees with the finding that *B pertussis* has three separate serotypes, designated 1,2; 1,2,3; and 1,3 according to the combination of agglutinogens demonstrated.⁹ Whole cell vaccines lacking agglutinin 3 did not protect against infection due to strains of serotype 1,3.¹⁰

We describe DNA fingerprinting of isolates of *B pertussis* by pulsed field gel electrophoresis, in which large DNA fragments are separated by alternatively switching the current between two sets of electrodes set at an obtuse angle.^{11,12} Simple electrophoresis of restriction fragments of whole DNA produced by the enzymes *Eco*RI, *Sma*I, *Nci*I, *Bam*HI, *Ava*I, or *Bgl*II had failed to discriminate between isolates (unpublished observations). The DNA was digested with *Xba*I as the genome of *B pertussis* has a G+C content of 67.7-68.0 mol%¹³ and the recognition sequence of this enzyme contains the rare tetranucleotide CTAG.¹⁴ Thus the enzyme was likely to produce DNA fragments of the appropriate size. The technique was applied to 105 isolates of *B pertussis* to determine whether the pathogen is clonal and whether variation in genotype correlates with variation in serotype. Isolates representing strains currently causing infection in the United Kingdom and Germany were examined and the results compared with 13 control isolates.

Materials and methods

Isolates—The following isolates were examined: 67 isolates from 14 public health laboratories and 16 district general hospitals throughout the United Kingdom, including four pairs of isolates from family clusters; vaccine strains 2991 and 3700; 13 isolates from this university's collection of bacteria (controls); and 23 isolates from Professor Enders, Stuttgart, Germany. The isolates were identified on the basis of

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colonial morphology on charcoal blood agar, Gram staining, and serotyping with monospecific agglutinating sera prepared at the Pertussis Reference Laboratory as previously described.⁹

Preparation of chromosomal DNA—The bacteria were grown for 48 hours at 37°C in 25 ml modified Stainer and Scholte broth with vigorous shaking. The cells were pelleted, washed twice with 10 ml 50 mM EDTA (pH 8.0) and the cell concentration adjusted at 600 nm to an optical density of 1.5, equivalent to 1.2×10^9 cells/ml. An equal volume of 2% low gelling temperature molten agarose was added and the mixture dispensed into a 10 plug mould (Bio-Rad). Plugs were solidified at 4°C for 10 minutes, and each plug was divided into four with a clean coverslip. The plugs were then incubated with shaking at 37°C in a sterile screw capped bottle containing EC lysis buffer (6 mM Tris-HCl pH 7.5, 1 M sodium chloride, 100 mM EDTA pH 8.0, 0.5% w/v Brij-58 (Sigma), 0.2% w/v sodium deoxycholate (BDH), 0.5% w/v N-lauryl sarcosine (Sigma), 1 g/l lysozyme (Sigma), and 20 g/l RNase (Sigma). They were further treated for 48 hours at 50°C in ESP solution (0.5 M EDTA pH 9.0, 1% w/v N-lauryl sarcosine, and proteinase K 1 g/l).¹⁵ Subsequently, the plugs were washed for at least 15 minutes three times in 3 ml 50 mM EDTA pH 8.0 and stored at 4°C until use.

Restriction digests of chromosomal DNA—The agarose plugs were washed twice with TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and three times in TE buffer without PMSF.¹⁴ Three plugs of each strain were collected in an Eppendorf tube and equilibrated in 300 µl of *Xba* I (Northumbria Biologicals) reaction buffer for 20 minutes on ice. Subsequently, 25 units of *Xba* I were

FIG 1—Pulsed field gel electrophoresis of DNA types 1-8, as described in table I; actual sizes of fragments given on left

FIG 2—Pulsed field gel electrophoresis of DNA types 9-16, as described in table I; actual sizes of fragments given on left

added and digestion carried out overnight at 37°C. The reaction was stopped by the addition of 50 mM EDTA. Other rare cutting restriction enzymes *Dra* I, *Ssp* I, and *Spe* I seemed to be worthwhile alternatives but were not examined extensively.

Pulsed field gel electrophoresis was performed with the CHEF-DR II System (Bio-Rad). Gels of 13.75×13 cm were made up of 1% agarose in TBE buffer (Tris-HCl 10.3 g, boric acid 5.5 g, EDTA 0.93 g/l). The plugs were loaded into separate wells; a bacteriophage DNA ladder (Promega) was included in each gel as molecular weight marker DNA. Electrophoresis was performed with a pulse time of 25 seconds for 40 hours and with a field strength of 4.5 V/cm.¹⁶ Gels were then stained with 0.5 mg/l ethidium bromide in distilled water for 20 minutes and photographed.

Results

All isolates were typable, and reproducibility as judged by visual comparison between gels run under the same conditions was excellent. Twelve *B pertussis* isolates were repeatedly subcultured on charcoal blood agar for 10 weeks and subsequently reanalysed. All subcultured isolates from the same origin produced identical DNA fingerprints, showing the stability of the DNA type on subculture.

Discrimination was much greater with DNA fingerprinting than serotyping, and 17 DNA types were identified; figures 1 and 2 show 16 of them. Cleavage of the *B pertussis* DNA with *Xba* I generated eight to 11 fragments per isolate in the size range 130-412 kilobases (kb). A cluster of smaller fragments in the 66-110 kb size range was also observed, but these separated suboptimally with the running conditions selected. The 17 different types were identified on the basis of the variation in 11 intense bands, ranging from 200 to 412 kb (table I). Bands at 130 and 155 kb were too conserved to be of value.

TABLE I—Characterisation of 17 DNA types of 105 isolates of *B pertussis*

DNA type	Size of DNA fragments (kb)										
	412	375	340	315	280	271	256	240	224	215	200
1	+	—	—	+	+	+	+	—	+	—	+
2	+	—	—	+	+	—	+	—	—	+	+
3	—	—	—	+	+	+	+	—	+	—	DB
4	—	+	—	+	+	+	—	+	+	—	DB
5	—	—	+	+	+	+	—	+	+	—	+
6	+	—	+	+	+	+	+	—	+	—	+
7	+	—	+	—*	+	—†	—	—	+	—	+
8	+	—	+	+	+	—	+	—	—	—	+
9	+	—	—	+	+	+	+	—	—	—	DB
10	+	—	+	+	+	+	+	+	+	—	+
11	—	+	—	+	+	—	+	+	+	—	+
12	+	—	—	+	—	+	+	—	—	—	DB
13	+	—	+	+	+	—	+	—	+	—	+
14	+	—	—	—‡	+	—	+	—	—	+	+
15	—	+	—	—	+	+	+	—	+	—	+
16	+	—	—	+	+	—	—	—	+	—	‡§
17	+	—	+	—‡	+	—	—	—	+	—	+

+ Band present.
— Band absent.

*Band runs at 297 kb.
†Band runs at 275 kb.

‡Extra band at 297 kb.
§Extra band at 178 kb.

DB=Double band.

TABLE II—Distribution of DNA types for each serotype of *B pertussis*

DNA types	Isolates from United Kingdom			Isolates from Germany			Isolates from Manchester (n=13)		
	1,2	1,3	1,2,3	1,2	1,3	1,2,3	1,2	1,3	1,2,3
1	10	2	0	0	0	0	2	2	7
2	8	1	1	0	0	1	0	0	0
3	8	5	2	0	4	1	0	0	0
4	2	2	0	0	0	0	0	0	0
5	4	1	0	3	1	0	0	0	0
6	0	1	0	0	0	0	1	1	0
7	1	0	0	0	0	0	0	0	0
8	1	0	0	3	0	0	0	0	0
9	1	1	0	0	4	2	0	0	0
10	6	0	0	2	1	0	0	0	0
11	1	2	0	0	0	0	0	0	0
12	0	4	0	0	0	0	0	0	0
13	0	1	0	0	0	0	0	0	1
14	1	0	0	0	0	0	0	0	0
15	0	0	1	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	1
17	0	0	0	0	0	1	0	0	0

DNA type 1 predominated in isolates of serotype 1,2 and serotype 1,2,3, accounting for 23 out of the 105 isolates (table II) and including the vaccine strains 2991 and 3700. DNA type 3 was commonest in serotype 1,3 and the second most common in serotypes 1,2 and 1,2,3. The other common DNA types were types 2, 5, and 10. DNA types 7, 14, and 17 displayed an extra band at 297 kb, which was absent from the rest of the DNA types, whereas types 3, 4, 9, and 12 showed a double band in the 200-207 kb range. The four pairs of isolates from the family clusters were identical in respect to both genotype and serotype (fig 3).

Isolates from the United Kingdom produced 15 separate DNA types whereas those from Germany produced seven. There was no obvious correlation with serotype with the possible exception that none of the isolates of serotype 1,2,3 were DNA type 1. This was probably a sampling error as seven of the control isolates of the serotype 1,2,3 from the university's culture collection were DNA type 1. Although DNA type 1 was the commonest type in both isolates from the United Kingdom and the controls, it was absent in the isolates from Germany.

Discussion

This paper reports the development of a genotype based typing system for *B pertussis*. The technique was highly reproducible and the results sufficiently stable for the technique to be applicable to large scale epidemiological investigations. Isolates from epidemiologically related cases were identical. The results show that *B pertussis* is highly genetically variable, proving that the idea of it being essentially clonal is too simple. Musser *et al* described only two closely related clones—one relatively specific to the United States and Mexico and the other specific to Japan.¹ Our study showed 15 separate types circulating in the United Kingdom and seven in Germany. It does, however, confirm that the predominant strain varies from country to country as the commonest DNA type (DNA type 1) was absent among the German isolates. Interestingly, the two vaccine strains (2991 and 3700)

were both DNA type 1 isolates, which may, in part, explain their efficacy as whole cell vaccines within the United Kingdom.

These findings are important when attempting to introduce a new vaccine. The genetic heterogeneity of the pathogen suggests that the response to the introduction of an acellular subunit vaccine derived from a single strain might be only a shift in the prevalent DNA type rather than the elimination of infection. It must be remembered, however, that the typing system reflects subtle changes in the DNA rather than immunologically significant changes in the proteins which are vaccine candidates. These are pertussis toxin, filamentous haemagglutinin, and the agglutinogens.¹⁷ Variation in these proteins was shown in this study to be independent of DNA type. An attempt to develop a typing system by immunoblot fingerprinting whole cell extracts against a rabbit hyperimmune antiserum raised against a strain of serotype 1,2,3 failed to distinguish between isolates of different DNA type (unpublished observations). There was no obvious correlation between phenotype determined by agglutination and immunoblotting and genotype determined by pulse field gel electrophoresis.

The primary role of the typing system will thus be to study the epidemiology of *B pertussis*. It could be used to trace the movement of clones within and between individuals and communities which may occur in response to the introduction of a new vaccine and to determine the clonal types associated with any immunisation failures and whether failure is due to pre-existing strains or the introduction of a new more virulent clone.

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FIG 3—Pulsed field gel electrophoresis of isolates from four pairs of siblings (lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8 respectively); actual sizes of fragments given on left

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