MOLECULAR IDENTIFICATION AND TYPING

Restriction endonuclease analysis using *HhaI* and *HpaII* to discriminate among group B *Pasteurella multocida* associated with haemorrhagic septicaemia

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The purpose of this study was to improve and standardise restriction endonuclease analysis (REA) for discriminating isolates of serogroup B *Pasteurella multocida* associated with haemorrhagic septicaemia in wild and domestic animals and to create a reference database that can be used for epidemiological studies. Two techniques for extraction and isolation of chromosomal DNA were compared, a DNAzol[®] method and an enzymic lysis followed by a two-phase partition method. No differences were observed between DNA fingerprint profiles with either technique; however, the former technique was faster and easier to perform. *P. multocida* isolated from different animals in different countries representing serotypes B:2, B:3, B:3,4 and B:4 were subjected to REA with *HhaI* and *HpaII* endonucleases. Forty-eight fingerprint profiles were distinguished among 222 isolates when only *HhaI* was used. By combining the data from REA with *HhaI* and *HpaII* used separately, 88 different groups could be distinguished among the same isolates. Following digestion with *HhaI* and electrophoresis, the DNA of all serotype B:2 isolates produced fingerprint profiles characterised by two trailing bands at ~8.4–7.1 kb which have not been observed in any other serotypes of *P. multocida*. Passage of three serotype B:2 isolates on laboratory media or two serotype B:2 isolates through mice did not result in a change of DNA fingerprint profile detectable by REA. The findings with 59 isolates from Sri Lanka showed that REA was highly discriminative in determining the genetic diversity of serotype B:2 *P. multocida* in an area where haemorrhagic septicaemia is endemic.

Introduction

Haemorrhagic septicaemia (HS) is an acute and often fatal disease principally occurring in cattle and water buffalo, but occasionally other domesticated and wild mammals can be affected [1, 2]. The disease is particularly endemic in Asia and Africa, but outbreaks have occurred in Europe and North America. *Pasteurella multocida* serotypes B:2 and E:2 are the principal cause of HS. However, the E:2 serotype has been isolated only from animals in Africa. In recent years, serotypes B:3, B:4 and B:3,4 have been implicated as a cause of HS in cattle and wild ruminants such as deer, elk, and bison [3, 4, 5, 6, 7, R. B. Rimler unpublished

observations]. The clinical signs and lesions in ruminants such as cattle, bison and deer caused by serotypes B:3, B:4, and B:3,4 may appear similar, if not indistinguishable, to those seen in water buffalo and cattle caused by serotypes B:2 and E:2. Although most isolations of serogroup B *P. multocida* (other than serotype B:2) have been made in North America, isolations have been made occasionally from cattle in Australia [8] and fallow deer in Denmark [3] and the UK [5].

Environmental and other stresses that predispose domesticated and wild ruminants to outbreaks of HS are often similar. Anecdotal information suggests that selenium deficiency may have precipitated some outbreaks of HS caused by serotype B:3,4. *Pasteurella multocida* does not live freely in the environment, and there are no reports describing serogroup B or E *pasteurellas* being isolated from free-flying birds,

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rodents or man, suggesting that they are not intermediate hosts to the bacteria. Carrier animals are the reservoir for pasteurellas causing HS [1, 2], and the pharyngeal tonsils have been shown to be sites of carriage in healthy animals [1, 2]. In HS endemic areas, the number of carrier animals in buffalo and cattle herds may range from <1% to >40% [1]. Presumably, a similar situation also occurs in wild ruminant herds. It is not known whether diverse strains of the same serotype may be associated with an outbreak or occur in a single carrier animal. Also, it is largely unknown whether more than one strain of a particular HS serotype may occur within a specific endemic area.

Serogroups B and E of *P. multocida* are distinguished by indirect haemagglutination tests and by their inability to be affected by certain mucopolysaccharidases [9]. Somatic serotypes are distinguished by gel diffusion precipitin tests (GDPT) [10]. Of the serogroup B strains, only the B:2 serotype produces hyaluronidase and chondroitinase [11]. Although serological and conventional biochemical tests are still useful for disease diagnosis and designing vaccination programmes, they are limited in scope for epidemiological purposes, because they cannot differentiate isolates within a particular serotype.

Within the last decade, new and more powerful technologies have become available to discriminate among isolates of *P. multocida*. Among these technologies is restriction endonuclease analysis (REA), a technique capable of recognising differences in bacteria within the same serotype [12]. The objectives of this study were to improve and standardise REA for discriminating isolates of serogroup B *P. multocida* associated with HS in wild and domestic animals and to create a reference database that can be used for epidemiological studies.

Materials and methods

Bacteria

A total of 222 isolates of serogroup B *P. multocida* was used (Table 1). The isolates were from different mammalian species in different countries, except for one isolate from a chicken. The mammalian isolates originated from cases of HS or from carrier animals in areas where HS had occurred, except for one serotype B:3,4 strain isolated from an ox in Australia. Most isolates were submitted to the National Animal Disease Center for serotyping, and clinical case information (other than animal host of origin) often was not provided. The majority of the isolates were obtained from Sri Lanka and India, and most of those isolates were from cattle, water buffalo and swine. The capsule serogroup of an isolate was determined by indirect haemagglutination tests, and its somatic type was determined by gel diffusion precipitin tests [13]. The

ability of an isolate to produce hyaluronidase was tested as described previously [11].

REA

Bacteria were grown on blood agar medium base (without added blood; Difco) for 18 h at 37°C. Cells were suspended in either TE buffer (0.01 M Tris-0.001 M EDTA, pH 8.0) or saline and adjusted to 65%T at 600 nm with a 16 × 150-mm tube in a model 35 spectrophotometer (Perkin-Elmer). A 1.5-ml volume of the adjusted cell suspension was centrifuged to pellet the cells and chromosomal DNA was obtained from TE buffer-suspended bacteria by enzymic lysis followed by a two-phase partition method [12] or from saline-suspended bacteria with a DNAzol[®] procedure. For the DNAzol[®] procedure, after centrifugation of the saline-adjusted suspension the supernate was discarded and 1 ml of DNAzol[®] (Life Technologies, Inc., 9800 Medical Center Drive, Rockville, MD, USA) was added to the tube to resuspend and solubilise the pellet. The solution was allowed to stand for 15 min at room temperature before 500 µl of absolute ethanol were added. The contents of the tube were mixed by end-over-end rotation until a cloudy precipitate formed. The tube was centrifuged at 16 000 g for 2 min and the supernate was discarded. The pellet was washed twice by centrifugation with 800 µl of ethanol 95% and dried with a vacuum concentrator for 30 min.

The DNA extracted by two-phase partition or DNAzol[®] procedures was digested with HhaI or HpaII endonuclease (Life Technologies) as recommended by the manufacturer, and submarine electrophoresis in an agarose 0.7% gel was done as described previously [12]. A HindIII digest of λ phage was used as a standard at three positions on every gel (both sides and middle) so that gels could be normalised and comparisons of fingerprint profiles from different gels could be made with Gelcompar[®] (Applied Maths, Kortrijk, Belgium) computer software.

After electrophoresis, gels were stained with ethidium bromide and band patterns (fingerprint profiles) were viewed and photographed with ultraviolet light. Photographs were scanned with a Hewlett-Packard IICx flatbed scanner and Deskscan[®] software (Hewlett-Packard, Boise, ID, USA) to create a tagged image format file (tif file) for computer analysis. Images of fingerprint profiles were analysed and similarity between all possible pairs of fingerprint profiles within a serotype or between serotypes was calculated by the cluster analysis module of the Gelcompar[®] software using the Dice coefficient and a band position tolerance of 0.5%. Dendrograms were derived from a matrix of similarity values by the unweighted pair group method using arithmetic averages. Isolates that had >97.3% similarity based upon the data combined from analysis of fingerprint profiles produced by both HhaI and HpaII were considered identical.

Results

Serology and production of hyaluronidase

One hundred and ninety-two isolates were identified as serotype B:2 and 30 isolates were identified as serotype B:3, B:4, or B:3,4 (Table 1). None of the serotype B:3, B:4 or B:3,4 isolates produced hyaluronidase, whereas all the serotype B:2 isolates did.

Distinction between DNA fingerprint profiles and isolation of DNA by two-phase partition or DNAzol methods

Distinction between fingerprint profiles following electrophoresis of the endonuclease digests was determined by the number and position of bands in agarose gels. Although the same fingerprint profile of a particular isolate resulted from endonuclease digestion of DNA isolated by either the two-phase partition or the DNAzol method, the latter method was easier and more quickly performed. After adjustment of bacterial suspensions, 20 samples could be processed easily and made ready for electrophoresis within 5 h by the DNAzol method, in comparison with 7 h by the two-phase partition method.

Distinction among serotype B:2 isolates

For all 192 serotype B:2 isolates, distinction between fingerprint profiles produced by HhaI was determined with bands in the 8.4–3.35 kb range, and distinction between profiles produced by HpaII was determined with bands in the 28.3–5.25 kb range. Bands did not

occur above these ranges, and bands below these ranges were not well separated and defined.

Thirty-two different profiles were distinguished among the 192 serotype B:2 isolates after digestion with HhaI endonuclease. The DNA of five serotype B:2 *P. multocida* (isolated from buffalo, sheep and deer in India) could not be digested with HpaII endonuclease. Forty-two different DNA fingerprint profiles were distinguished among the remaining 187 isolates digested with HpaII. Fig. 1 shows nine of the most frequently observed DNA fingerprint profiles produced by HhaI and HpaII. Different isolates which had identical profiles with HhaI endonuclease did not necessarily have identical profiles with HpaII, and vice versa. Therefore, further discrimination among isolates could be made by combining the data from two separate endonuclease treatments.

Isolates with >97.3% similarity following both endonuclease treatments were considered identical and assigned the same descriptive identification epithet (DIE) code [12], i.e., B:2/HhaI 0018/HpaII 0008. Thus, excluding data from the five isolates whose DNA could not be digested with HpaII endonuclease (DIE code B:2/HhaI 0018/HpaII neg), the serotype B:2 isolates were separated into 70 different DIE coded groups. Percentage similarity among the different serotype B:2 isolates (as represented by distinct DIE codes) ranged from 67.1 SD 5.8% to 97.3%. There was very little evidence of clustering of isolates according to geographic origin. In fact, isolates with the same DIE code could originate from different continents. For example, B:2/HhaI 0018/HpaII 0020 represented isolates from Malaysia (one isolate), Egypt (one) and Zimbabwe (three) and B:2/HhaI 0020/HpaII 0025 represented isolates from Indonesia (two isolates), Malaysia (five) and Spain (six).

Table 1. Geographic origin and hosts of serotype B:2, B:3, B:3,4 and B:4 isolates of HS *P. multocida*

Serotype	Origin	Host	Number of isolates
B:2	Bangladesh	Cattle	1
	Burma	Buffalo	4
	China	Cattle, yak	3
	Egypt	Buffalo	1
	India	Buffalo, cattle, deer sheep, swine	63
	Indonesia	Buffalo, cattle	16
	Malaysia	Buffalo, chicken	6
	Philippines	Buffalo	5
	Romania	Buffalo, cattle	3
	Spain	Deer, sheep	7
	Sri Lanka	Buffalo, cattle, elephant, unknown	66
	Thailand	Buffalo	3
	USA	Bison, cattle	6
	Zimbabwe	Cattle	3
	Unknown	Unknown	5
B:3	USA	Bison, elk	4
B:3,4	Australia	Cattle	1
	Denmark	Deer	2
	Canada	Cattle	2
	UK	Deer	3
	USA	Bison, cattle, deer, elk	14
	Unknown	Unknown	2
B:4	Canada	Bison	1
	USA	Elk	1

The findings with 59 isolates from animals in Sri Lanka (kindly provided with information on province of origin by Dr M. De Alwis) produced the best example of the discriminative power of REA and the genetic diversity of serotype B:2 in an area where HS is endemic. Buffalo and cattle were the main sources of these isolates, and isolations were made over a period of c. 12 years. With combinations of serology and REA with the two separate endonucleases, the 59 isolates could be assigned to 34 DIE codes. Similarity between non-identical isolates ranged from 83.6 SD 3% to 97.3%. Fig. 2 shows a map of Sri Lanka (66 000 km²) and the number of different DIE codes that could be found among isolates from each of its eight provinces. As an example of serotype B:2 diversity within a specific geographic region, Fig. 3 shows percentage similarity of DIE coded *P. multocida* in the North-western province isolated over a 12-year period; similarity ranged from 87.9 SD 2.6% to 97.3%. Strains with the same DIE code could originate from different animal species within Sri Lanka and occur in more

Fig. 1. DNA fingerprint profiles most frequently observed among *P. multocida* serotypes B:2 and B:3,4 produced by (a) *Hha*I and (b) *Hpa*II endonucleases. Each lane shows the representative fingerprint profile for a number of isolates as follows: lane 1a, 3 isolates; 2a, 76; 3a, 3; 4a, 19; 5a, 14; 6a, 7; 7a, 6; 8a, 4; 9a, 6; 10a, 5; 11a, 5; 12a, 2; 13a, 3; 1b, 26; 2b, 44; 3b, 8; 4b, 20; 5b, 6; 6b, 3; 7b, 13; 8b, 14; 9b, 3; 10b, 3; 11b, 2; 12b, 3; 13b, 2. Arrow shows two trailing bands in the 8.4–7.1-kb range which are characteristic of all serotype B:2 isolates. *Fingerprint profile of serotype B:2 type strain M-1404.

Fig. 2. Map showing number of DIE codes (numerator)* per number of isolates (denominator) originating from cases of HS in eight administrative provinces of Sri Lanka: North and East (1), North Central (2), North-western (3), Central (4), Western (5), Sabaragamuwa (6), Uva (7) and Southern (8).

than one province; e.g., B:2/HhaI 0018/HpaII 0007 was isolated from elephant (one isolate) and buffalo or cattle (six) and was associated with five of eight provinces. Interestingly, B:2/HhaI 0018/HpaII 0007 was the DIE code for 21 serotype B:2 *P. multocida* isolated from a different species in the neighbouring country of India (19 isolates from swine and two from unknown hosts).

Distinction among serotype B:3, B:4 and B:3,4 isolates

Twenty-four isolates were identified as serotype B:3,4, four as serotype B:3 and two as serotype B:4. For all of these serogroup B strains, distinction between fingerprint profiles with HhaI was determined with bands in the 9.78–2.72-kb range, and distinction between fingerprint profiles with HpaII was determined with bands in the 32.73–4.5-kb range. Bands did not occur

Fig. 3. Dendrogram showing percentage similarity between HS *P. multocida* isolated in the Northwestern province of Sri Lanka.

above these ranges, and bands were not well separated and defined below these ranges. Fig. 1 shows four of the most frequently observed DNA fingerprint profiles of serotype B:3,4 obtained with HhaI and HpaII.

Digestion with HhaI endonuclease resulted in 12 different DNA fingerprint profiles among the serotype B:3,4, two among the B:3 and two among the serotype B:4 isolates. The same DNA fingerprint profile did not occur between isolates of a different serotype. With HpaII endonuclease, 13 different profiles were found among the serotype B:3,4 isolates, two among the B:3 and two among the serotype B:4 isolates. Again, the same profile did not occur between isolates of a different serotype.

Except for serotype B:3,4 isolated from fallow deer in Denmark and the UK, isolates that had identical profiles with HhaI endonuclease also had identical profiles with HpaII. By serology and REA with the two separate endonucleases, 17 DIE code groups were recognised among the 30 isolates that comprised serotypes B:3, B:4 and B:3,4 (Fig. 4). The similarity between strains with different DIE codes ranged from 57.4 SD 5.9% to 96.3%. There was no evidence of clustering based upon geographic origin or host species.

Comparisons between DNA fingerprint profiles representing classical HS *P. multocida* and non-classical HS *P. multocida*

The DNA of all serotype B:2 isolates (classical HS *P. multocida*) produced fingerprint profiles characterised by two distinct bands at the top of the gel (~ 8.4–7.1 kb) after digestion with HhaI endonuclease

Fig. 4. Dendrogram showing percentage similarity between serotypes B:2, B:3, B:3,4 and B:4 *P. multocida* associated with HS.

and electrophoresis (Fig. 1). Fingerprint profiles with two top bands at this position were not found with HhaI endonuclease for any of the other group B serotypes (B:3, B:3,4 and B:4; non-classical HS *P. multocida*), and they have not been found with serotypes within groups A, D, E and F of *P. multocida* (unpublished observations). Except for those isolates whose DNA could not be digested with HpaII, two clusters were seen when all fingerprint profiles of the serogroup B *P. multocida* were compared (Fig. 4). One cluster consisted of the serotype B:2 isolates; the other cluster consisted of serotypes B:3, B:3,4 and B:4. Similarity between the two clusters was 45.1 SD 6.5%.

Stability of DNA fingerprint profiles

Because of the large number of DIE groupings that could be made within the serotype B:2 *P. multocida*, particularly in countries where HS is endemic (i.e., India and Sri Lanka), it was deemed necessary to determine whether REA fingerprint profiles were relatively stable following laboratory passage of the bacteria. Strain M-1404, the type strain for serotype B:2, has been maintained by the US Department of Agriculture as a lyophilised stock culture since 1948. Subcultures have been passaged intermittently by single colony picks on various media for >50 years, totalling at least 100 in-vitro passages. When profiles of a single passage of this strain from a stock culture lyophilised in 1948 were compared with the same strain passaged 100 times, there were no differences in fingerprint profiles. Similarly, no differences were seen in fingerprint profiles between one and 30 in-vitro

passages of another well-known serotype B:2 isolate, the Insein strain [14].

No influence on DNA fingerprint profiles of *P. multocida* passaged through experimental infection of animals was observed for two serotype B:2 strains. Strain M-1404 re-isolated from the livers of experimentally infected mice did not produce different profiles from the multi-passaged laboratory culture above. Similarly, a Sri Lankan strain (R-39) did not produce different profiles after 10 serial mouse passages.

Discussion

A previous study showed that REA with HhaI endonuclease could discriminate among isolates of the serogroup B *P. multocida* that cause HS [12]. The current study, with a larger number of isolates from more varied sources, showed that REA with two endonucleases (HhaI and HpaII) could result in better discrimination among these bacteria. Whereas 48 fingerprint profiles were distinguished among 222 isolates with HhaI, 88 different groups (including the isolates that were not digested by HpaII) were recognised among the same isolates when the findings with both HhaI and HpaII were combined. As determined by percentage similarity of the fingerprint profiles, the B:2 serotype constituted a more homogeneous cluster than that seen with the other HS serotypes tested (B:3, B:4 and B:3,4). Particularly interesting was the fact that all serotype B:2 fingerprint

profiles were characterised by two trailing bands at ~8.4–7.1 kb after digestion with HhaI endonuclease and electrophoresis. A profile characterised by these particular bands has not been seen in any other *P. multocida* serotypes treated with the same endonuclease and thus, like the ability to produce hyaluronidase, may constitute another property that can be used as a diagnostic test to recognise members of the B:2 serotype.

Documentation on the host species and geographic origin of the Sri Lankan isolates provided an opportunity to determine the diversity among serotype B:2 that may occur within particular areas of a country where HS is endemic. Although the data show that a large number of genetically different isolates could occur within a specific province (i.e., 14 DIE codes in the Northwestern province), the importance of this finding to the epidemiology of serotype B:2 and HS in Sri Lanka is not immediately apparent. However, as a result of this finding, questions arose as to whether serotype B:2 is subject to fingerprint profile change due to either animal or laboratory passage, and whether some strains may be more stable than others. In contrast to the findings reported by Townsend et al. [15], where field alternation gel electrophoresis (FAGE) detected genetic alterations in subcultures of a North American serotype B:2 bison strain (Buffalo B), the findings reported here indicate that repeated subculture of strain M-1404 (a derivative of Buffalo B also used in that study) did not result in genetic alterations detectable by REA. Furthermore, laboratory passage of the Insein strain and mouse passage of strains M-1404 and R-39 did not result in new and different fingerprint profiles with REA. Laboratory and mouse passages obviously do not represent all the environmental pressures which may initiate genetic changes. Whether transmission of HS *P. multocida* among the same and diverse natural hosts or immunological pressure from a carrier or vaccinated animal can result in a genetic alteration recognisable by REA remains unknown.

Other molecular methods, including ribotyping and FAGE, have been used to discriminate among *P. multocida*. Comparison between FAGE and ribotyping showed that the former method had greater discriminating power than ribotyping for HS *P. multocida* [15]. Studies on other *P. multocida* serotypes where both ribotyping and REA methods were included resulted in better discrimination among strains by REA [16, 17].

REA is a sensitive and relatively uncomplicated method of DNA fingerprinting that directly detects differences between strains of *P. multocida* which appear similar by traditional serotyping methods. Detection of differences with a small number of strains can be accomplished without much difficulty by visual comparison of profiles within a gel. However, as in this study with a large number of strains, computer methods

are necessary to simplify gel-to-gel comparisons and storage of reference fingerprint profile images. Images that represent the distinctive HhaI and HpaII fingerprints analysed in this study have been archived, forming a reference database that can be used for comparison with images of fingerprints of unknown HS strains. Thus, following methods outlined in this study, it is possible for fingerprint images of HS *P. multocida* created in another laboratory to be sent electronically (in a tif format) for comparisons.

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