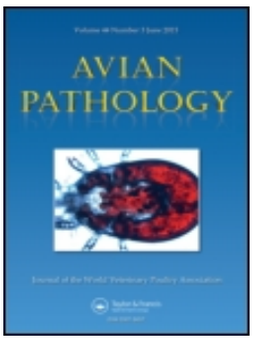

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TECHNICAL REVIEW

Identification and typing of *Pasteurella multocida*: a review

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Pasteurella multocida is an important pathogen of many avian species. This review critically examines recent developments in new-generation tests for the identification and typing of this bacterium. Two polymerase chain reaction (PCR) tests have been reported for *P. multocida*. Both tests show promise as diagnostic tests that could be considered for routine use. However, there have not yet been effective evaluation studies that examine the ability of these new tests to distinguish between *P. multocida*, both typical and atypical isolates, and the range of other *P. multocida*-like organisms found in avian species. One PCR, reported by Townsend *et al*. (*Journal of Clinical Microbiology, 36*, 1096–1100), has been the more fully evaluated and is the better choice, at this stage, for laboratories considering the use of PCR technology for detection of *P. multocida*. An important point is that the PCR tests have been validated by use on pure cultures or enrichment broths – not on direct examination of body tissues. To date, there have been five different technologies used to type avian *P. multocida*: restriction endonuclease analysis (REA), ribotyping, pulsed field gel electrophoresis, repetitive extragenic palindromic-PCR (REP-PCR) and multi-locus enzyme electrophoresis (MLEE). The methodology underlying these techniques is briefly explained and the performance of these techniques with regards to the typing of avian *P. multocida* is critically examined. For smaller laboratories that are investigating outbreaks of fowl cholera, it would appear that REA and REP-PCR are the typing methods of choice. For central reference laboratories that are considering studies of large collections of isolates, MLEE supported by two of the other methods would appear the most suitable techniques.

Introduction

Pasteurella multocida is a well-recognized pathogen of a range of avian species. In chickens and turkeys, *P. multocida* is the causative agent of fowl cholera (Rimler & Glisson, 1997). In this review, new techniques for the identification and differentiation of avian *P. multocida* will be covered. As there are excellent reviews/overviews on traditional culture methods and such well-established typing methods as serotyping (Rhoades & Rimler, 1989; Rimler, 1992; Christensen & Bisgaard, 1997; Rimler & Glisson, 1997; Rimler *et al.*, 1998), the current review will not attempt a coverage of these traditional methods. This review will concentrate on detection and typing of avian *P. multocida* – a detailed review of the general molecular biology of *P. multocida* has been recently published (Hunt *et al.*, 2000). The intention of this review is to critically examine the effectiveness of the new-generation identification and typing systems. The final section of the review is a section on recommendations of what typing methods are best suited for particular applications.

Background

Fowl cholera is an important disease of both domesticated and wild birds (Rimler & Glisson, 1997). As noted by Rimler & Glisson (1997), the disease is often classified into two forms, acute and chronic, despite the fact that field outbreaks often present clinical signs and lesions that are intermediate between these two forms. In the classic acute form, death may be the only indication of disease noted (Rimler & Glisson, 1997). Astute observation of birds in the hours before death can reveal signs such as fever, anorexia, ruffled feathers, mouth discharge, diarrhoea and increased respiratory rate. In the chronic form, signs are typically limited to localized infections – swelling

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Table 1. *The species and un-named taxa of the genus* Pasteurella *that have been isolated from birds*

Species/taxon	*Reference*
P. anatis	Mutters *et al.* (1985a)
P. avium	Mutters *et al.* (1985b)
P. gallinarum	Hall *et al.* (1955)
P. haemolytica-like (*Actinobacillus salpingitidis*)	Bisgaard (1982)
P. langaa	Mutters *et al.* (1985a)
P. multocida subsp. *gallicida*	Mutters *et al.* (1985a)
P. multocida subsp. *multocida*	Mutters *et al.* (1985a)
P. multocida subsp. *septica*	Mutters *et al.* (1985a)
P. volantium	Mutters *et al.* (1985b)
Pasteurella spp. A	Mutters *et al.* (1985b)
Bisgaard Taxon 2	Bisgaard (1982)
Bisgaard Taxon 3	Bisgaard (1982)
Bisgaard Taxon 14	Bisgaard & Mutters (1986)
Bisgaard Taxon 22 (*P. pneumotropica*-like)	Bisgaard (1982)
Bisgaard Taxon 26	Piechulla *et al.* (1985)
Bisgaard Taxon 32	Bisgaard & Mutters (1986)

of sinuses, wattles and leg and/or wing joints (Rimler & Glisson, 1997).

Fowl cholera is widely accepted as being of major economic importance wherever intensive poultry production occurs, e.g. in the US (Rimler *et al.*, 1998). However, the disease also has a significant impact in less intensive systems, e.g. fowl cholera is among the main diseases seen in African village chickens (Guèye, 1999). Furthermore, fowl cholera is a significant cause of mortality in waterfowl, e.g. causing an estimated 5.3% of all non-hunting mortalities in North American waterfowl (Stout & Cornwell, 1976).

The primary method of control of fowl cholera should be good sanitary practice and effective biosecurity (Rimler & Glisson, 1997). In areas where endemic fowl cholera is a problem, vaccines are used. Both inactivated vaccines, typically based on the prevalent serovars in the target poultry population, and live attenuated vaccines are available (Rimler & Glisson, 1997). The accepted belief is that the inactivated vaccines protect only against the serovars present in the vaccine, while the live vaccines give broad cross-serovar protection (Rimler & Glisson, 1997).

P. multocida is named in honour of Louis Pasteur who, in classic experiments in the early 1880s, attenuated the agent and thus produced the first deliberately developed vaccine (Rimler & Glisson, 1997). While a range of different names were used, the organism we now know as *P. multocida* remained the sole recognized species within the genus *Pasteurella* until the 1930s (Mutters *et al.*, 1985a). From the 1930s onwards, a range of new avian *Pasteurella* species as well as some un-named taxa have been described. Table 1 shows the formally recognized species as well as the various un-named taxa that have been recorded as being present in birds.

Hence, the identification of a *Pasteurella*-like isolate from an avian host is a challenging task – the complexity of which is not often appreciated by those who think of the genus *Pasteurella* as consisting of two to three species.

DNA-Based Identification Methods

Given the complexity associated with conventional identification methods already described, DNA-based identification methods, if fully validated, clearly are an attractive option. To date, two polymerase chain reaction (PCR) tests that claim species specificity have been described (Kasten *et al.*, 1997; Townsend *et al.*, 1998).

PCR technology, which has been whimsically described as having a "moonlight conception in the mountains of northern California in 1983" (Podozorski & Persing, 1995), has revolutionized almost all aspects of biology. The basic steps in PCR have been well described in a vast array of texts. The technique uses two primers, short stretches of DNA, each of which is specific for one end of the target DNA sequence. This target sequence has been exposed by heating the double-stranded DNA to the point where the strands separate, typically around 95°C ("denaturation"). The temperature is then lowered, to around 52 to 55°C, allowing DNA–DNA binding to occur. The complementary nature of DNA binding (guanine with cytosine, and adenine with thymine) means the two primers bind to each end of the target ("annealing"). The temperature is then raised again, often to around 72°C, and a heat-resistant DNA polymerase then starts filling in the bases to convert the stretch of single-stranded DNA ahead of the primer back to double-stranded DNA. After one cycle, there are now two copies of the sequence of interest. The cycle is then repeated – denaturation, annealing and extension – giving four copies of the sequence of interest. Each subsequent cycle gives another doubling of the target sequence – a 20-cycle run in theory being sufficient to generate 1 million copies of the target sequence.

The PCR described by Kasten *et al.* (1997) is based on the outer membrane protein that is known as the P6-like (PSL) protein (Kasten *et al.*, 1995). This PSL protein is known to share significant similarities with the P6 protein of *Haemophilus influenzae* (Kasten *et al.*, 1995). The PSL *P. multocida* PCR has been shown to detect each reference strain of the 16 Heddleston somatic serovars, one field isolate of *P. multocida* and one live vaccine strain (Kasten *et al.*, 1997). The PSL PCR has not been checked for specificity with any of the other avian *Pasteurella* other than the type strain of *P. avium*, which gave a negative reaction

(Kasten *et al.*, 1997). Due to the high homology between the PSL and P6 proteins, the PSL *P. multocida* PCR also gives a positive reaction with *H. influenzae* (Kasten *et al.*, 1997). As *H. influenzae* has not been found in avian hosts, this false-positive reaction is not a practical concern with avian specimens.

The PSL *P. multocida* PCR, as reported by Kasten *et al.* (1997), used PCR followed by hydridization involving detection of a positive by chemiluminescence. This is a complicated and time-consuming process that is unlikely to be attractive to routine diagnostic laboratories. Using the full assay (PCR and hybridization), the detection limit of the assay is 24 femtograms of purified *P. multocida* DNA (Kasten *et al.*, 1997). Kasten *et al.* (1997) compared the use of mouse inoculation and the full PSL *P. multocida* PCR. For both techniques, the samples examined were turkey oropharyngeal swabs that had been incubated overnight in brain heart infusion broth. Formal statistical analysis of the results of using the two methods on six outbreak flocks (total of 178 birds examined) showed poor agreement between the methods, with the mouse method yielding 23 positives and the PCR method 11 positives. Kasten *et al.* (1997) found that only the use of both mouse inoculation and the PSL PCR ensured that all six infected flocks were detected.

The PCR for *P. multocida* described by Townsend *et al.* (1998) is based on a DNA sequence obtained by a fortuitous finding. The DNA sequence was encountered in work directed at looking for differences between haemorrhagic septicaemia and non-haemorrhagic septicaemia isolates of *P. multocida*. Using one of the three clones obtained during this work, the KMT1 clone, Townsend *et al.* (1998) were able to develop a PCR. This PCR, termed the KMT PCR for the purpose of this review, has been shown to give a positive signal with 13 field isolates of *P. multocida* (representing capsule types A, B, D and F) and the type strains for the three *P. multocida* subspecies (Townsend *et al.*, 1998). Of the avian *Pasteurella* known to occur in birds, the KMT PCR has only been tested with *P. anatis* and *P. langaa*, with the type strain of both species giving a negative reaction. The KMT PCR has been tested with the type strains of *P. canis* biovar 1, *P. dagmatis, P. stomatis* and *Pasteurella* sp. B, and has given negative reactions with all four strains (Townsend *et al.*, 1998). The KMT PCR has been shown to give a positive reaction with the reference strain for *P. canis* biovar 2 (Townsend *et al.*, 1998).

In a recent study, the KMT PCR has been reported to have a high sensitivity, with the assay being able to detect as few as 100 cells of *P. multocida* in seeded, autoclaved chicken intestinal contents (Lee *et al.*, 2000). However, this detection was not achieved by direct detection. Rather, the 100 cells of *P. multocida* were mixed in the autoclaved colon contents and then incubated in brain heart infusion broth overnight, and the PCR then used on the subsequent growth (Lee *et al.*, 2000). The KMT PCR has also been applied to the detection of *P. multocida* in the tissues of chickens artificially infected with *P. multocida* (Lee *et al.*, 2000). The KMT PCR was not used directly on the tissues (crop, various intestinal sites, liver and blood), but was used after the samples were enriched overnight in either brain heart infusion broth or cooked meat medium (Lee *et al.*, 2000). Of the 162 tissues examined by both the KMT PCR and selective culture, 132 tissues were negative in both assays, 24 tissues were positive in both assays, five tissues were positive in only the KMT PCR and one tissue was positive by selective culture only (Lee *et al.*, 2000). These results suggest that the KMT PCR when used on enrichment broths is as good as selective culture for the detection of *P. multocida* in infected chickens.

There are a number of areas where there is important information lacking on the performance characteristics of the PSL and KMT PCR tests for *P. multocida*. Neither test has been extensively evaluated with a collection of diverse field isolates of avian *P. multocida*. Neither PCR has been checked for specificity with representatives, ideally both type strains and field isolates, of all the known avian *Pasteurella* species and un-named *Pasteurella*-like taxa. The KMT PCR is known to give a false-positive reaction with the type strain of *P. canis* biovar 2 – whether field isolates would uniformly give the same reaction is not known. The PSL PCR has not been evaluated with *P. canis* biovar 2. As *P. canis* biovar 2 was once known as Bisgaard Taxon 13, it is important that the other sub-species that was once also in Taxon 13, *P. avium* biovar 2 (Mutters *et al.*, 1989), be tested in both the PSL and KMT PCR tests.

The known "false-positive" reaction of the KMT PCR with *P. canis* biovar 2, and the possibility/likelihood of a false positive with *P. avium* biovar 2, should not be regarded as major drawbacks for the use of this test in avian bacteriology. To date, there have been no reports of avian isolates of *P. canis* biovar 2. Similarly, there have been no formal reports of avian isolates of *P. avium* biovar 2. However, it is worth noting that isolates identified as nicotinamide adenine dinucleotide (NAD)-independent *P. avium*, and which therefore meet the definition of *P. avium* biovar 2, have been reported in South African poultry (Bragg *et al.*, 1997). It would be beneficial to have these South African NAD-independent isolates of *P. avium* examined in the KMT and the PSL PCR tests.

Overall, despite the lack of extensive evaluation, the KMT and PSL PCR tests do offer potential advantages to diagnostic laboratories. The range of avian *Pasteurella* spp. means that many diagnostic laboratories are either not capable or not willing to routinely use the extended battery of phenotypic

tests necessary to achieve a confident identification of an avian *Pasteurella* isolate. As PCR technology becomes widespread in veterinary diagnostic laboratories, it will be more feasible to perform a PCR test than maintain the media and other reagents associated with extended conventional identification. At this stage, the KMT PCR is the more characterized of the two available PCR tests for *P. multocida*, and thus is the test that should be considered ahead of the PSL PCR when a laboratory is considering adopting PCR technology for *P. multocida*.

An important consideration is that there has been little published evaluation on the applicability of the *P. multocida* PCR tests for use directly on tissues such as lung or liver. While some PCR tests for avian bacteria have been validated as direct detection methods, e.g. the *Haemophilus paragallinarum* PCR (Chen *et al.*, 1998), this is not the case for the *P. multocida* PCR tests. The tests have, in general, been examined with pure cultures or with enrichment broths. Hence, the major application of these PCR tests is, at this stage, as a replacement for conventional identification methods and not as a total replacement for conventional detection methods.

Typing – Some Background Comments

Within the context of this review, typing is based on the theory that isolates of *P. multocida* which have a common source will share properties that allow these related isolates to be differentiated from other non-related *P. multocida* isolates. A clear implication underlying this hypothesis is that the species *P. multocida* consists of genetically divergent lineages. There is considerable evidence that *P. multocida* is indeed genetically diverse; for example, on the basis of DNA:DNA hybridization and phenotypic properties, three subspecies, *gallicida, multocida* and *septica*, have been recognized (Mutters *et al.*, 1985a), NAD-dependent isolates of *P. multocida* have been recognized (Mutters *et al.*, 1985a) and 16 different biochemical types, termed biovars, have been recognized within the species (Fegan *et al.*, 1995; Blackall *et al.*, 1997). Hence, there is potential for typing systems to achieve the goal of both grouping related isolates and separating unrelated isolates.

It is important to understand that in evaluating typing methods, there are no "gold standards". Hence, the usual laboratory test based criteria of "sensitivity" (a measure of the number of true positives detected) and "specificity" (a measure of the number of false positives detected) cannot be used. A more relevant method of evaluating typing systems has been suggested by Arbeit (1995), who has proposed the criteria of typeability, reproducibility, discriminatory power and ease of use. Typeability means the ability of a typing system to produce a result with all isolates examined. Systems that have good typeability do not yield negative or uninterpretable results that result in non-typeable isolates. Reproducibility is the capacity of a technique to give the same result when repeatedly used on the same isolate. Discriminatory power is, in many ways, a measure of the specificity and sensitivity of typing systems. A typing system with high discriminatory power will always allocate unrelated isolates to unique types. The use of a numerical index of discrimination, termed Simpson's Index of Diversity, has been suggested as a means of comparing typing methods (Hunter & Gaston, 1988). An effective typing system should have an index of greater than 0.9 (Hunter & Gaston, 1988). Ease of use is an important consideration, particularly for laboratories that are not central reference laboratories.

A review of selected newer typing systems for *P. multocida* is presented in the following sections. The techniques are reviewed in terms of the criteria suggested by Arbeit (1995). The realities of limited resources and funding for veterinary research mean some of the published typing methods have been proposed or applied without a full evaluation of their effectiveness. While no published study has used Simpson's Index of Diversity to assess the effectiveness of a typing system, the Index has been retrospectively applied where the original publication contains the required details and where at least 50 isolates have been examined.

Restriction Endonuclease Analysis

Restriction endonuclease analysis (REA) uses restriction endonuclease enzymes that are a part of the normal bacterial cell "house maintenance" system. The enzymes are capable of cutting double-stranded DNA whenever the specific recognition sequence of the enzyme occurs. As an example, the enzyme *Hpa*II will cut DNA every time the following sequence of bases occurs in the DNA strand: cytosine–cytosine–guanine–guanine (C↓CGG), with the cut occurring between the two cytosine bases. This means that if, for example, the DNA contains only one *Hpa*II site, then two fragments will be produced when the DNA is digested with this enzyme. The reality is that the size of the typical bacterial chromosome is such that an enzyme such as *Hpa*II can produce hundreds of fragments. By subjecting the resultant fragments to electrophoresis in a gel (typically agarose), it is possible to sort the fragments on the basis of size – the smaller the fragment, the further it can migrate in the gel. The gel is stained and the DNA fragments visualized, typically under UV light. A typical gel showing avian *P. multocida* isolates that have been digested with the enzyme *Hpa*II is shown in Figure 1.

Several factors influence the complexity of an REA pattern. *Hpa*II has a recognition sequence that consists of only guanine and cytosine. Hence, bacterial DNA that has a relatively low overall

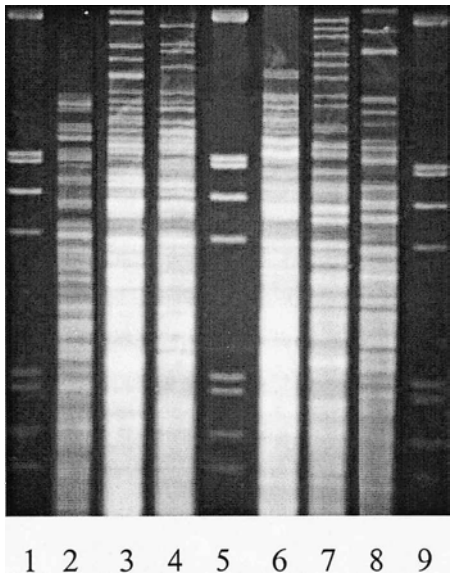

Figure 1. *Example REA patterns, generated using the enzyme* HpaII *of* P. multocida *isolates. Lanes 1, 5 and 9, Molecular weight marker; lane 2, REA pattern A; lane 3, REA pattern B; lane 4, REA pattern C; lane 6, REA pattern D; lane 7, REA pattern E; lane 8, REA pattern F.*

percentage of guanine and cytosine, such as *P. multocida*, is cut less frequently, and therefore yields fewer and larger fragments than if an enzyme containing only adenine and thymine in the recognition sequence were used. Restriction enzymes also vary in the number of bases in the recognition sequence – some being four-base cutters, some six-base cutters and some eight-base cutters. *Hpa*II is a four-base cutter as it has four bases in the recognition sequence (C↓CGG). As a general rule, a four-base cutter will cut DNA more frequently, and hence generate more and smaller fragments, than an eight-base cutter.

Typically, REA of avian *P. multocida* has been performed using enzymes that are either six- or four-base cutters. A list of the enzymes that have been used to date is shown in Table 2. As REA can be applied to all *P. multocida* isolates, the technique has good typeability, although some exceptions are noted in the relevant areas in the following.

REA using the enzyme *Bgl*II has only been reported in one study (Kim & Nagaraja, 1990). This lack of detailed study means that it is difficult to assess the reproducibility and discriminatory power of *Bgl*II-based REA. An important finding is that *Bgl*II-based REA does result in the CU and M9 live vaccine strains showing different patterns (Kim & Nagaraja, 1990). However, *Bgl*II-based REA as used by Kim and Nagaraja (1990) used polyacrylamide gel electrophoresis – a more complex form of electrophoresis than the traditional agarose method. Hence, this form of REA has not been widely used.

In a similar manner, only limited studies have been reported on the use of REA of avian *P. multocida* using the enzymes *Eco*RI, *Pst*I, the combined use of *Sma*I/*Sal*I (Snipes *et al.*, 1989) and *Xho*I (Christiansen *et al.*, 1992a), meaning that any formal evaluation of these enzymes is not possible.

REA using the enzyme *Sma*I has been reported in several studies on fowl cholera outbreaks in California turkeys (Snipes *et al.*, 1990; Christiansen *et al.*, 1992a,b). Although not formally studied, REA by *Sma*I appears to have good reproducibility as all three studies (Snipes *et al.*, 1990; Christiansen *et al.*, 1992a,b) have reported that the live *P. multo-*

Table 2. *Restriction endonuclease enzymes that have been used for REA of avian* P. multocida

Enzyme	Recognition sequence	Reference
*Bgl*II	A↓GATCT	Kim & Nagaraja (1990)
*Eco*RI	G↓AATTC	Snipes *et al.* (1989)
*Hha*I	GCG↓C	Wilson *et al.* (1993), Wilson *et al.* (1995a,b), Christensen *et al.* (1998)
*Hpa*II	C↓CGG	Wilson *et al.* (1993), Diallo *et al.* (1995), Blackall *et al.* (1995), Wilson *et al.* (1995a,b), Christensen *et al.* (1998)
*Pst*I	CTGCA↓G	Snipes *et al.* (1989)
*Sma*I	CCC↓GGG	Snipes *et al.* (1990), Christiansen *et al.* (1992a), Christiansen *et al.* (1992b)
*Sma*I/*Sal*I	CCC↓GGG/G↓TCGAC	Snipes *et al.* (1989)
*Xho*I	C↓TCGAG	Christiansen *et al.* (1992a,b)

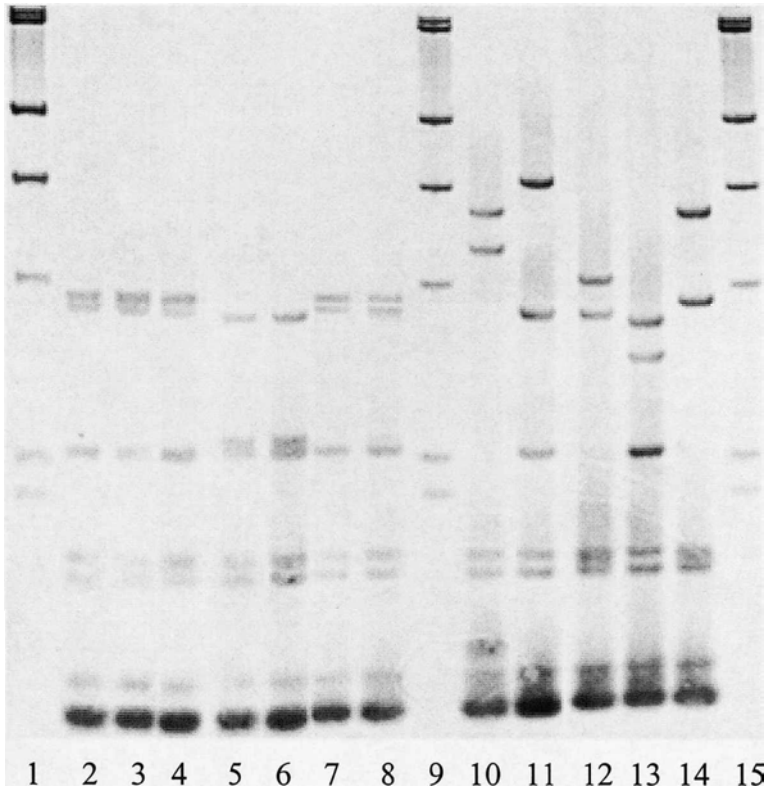

Figure 2. *Example ribotype patterns of* P. multocida *isolates. Ribotyping was performed using the enzyme* HpaII *and a PCR-generated probe that contained the* rrs *gene of* P. multocida. *Lanes 1, 9 and 15, Molecular weight marker; lane 2, ribotype pattern A; lane 3, ribotype pattern A; lane 4, ribotype pattern A; lane 5, ribotype pattern B; lane 6, ribotype pattern B; lane 7, ribotype pattern C; lane 8, ribotype pattern C; lane 10, ribotype pattern D; lane 11, ribotype pattern E; lane 12, ribotype pattern F; lane 13, ribotype pattern G; lane 14, ribotype pattern H.*

cida vaccine strain M9 gave the same REA pattern – termed pattern 5. There are some strains of *P. multocida* that cannot be typed using *Sma*I as some isolates are not cut by this enzyme (Christiansen *et al.*, 1992a). There has been no formal estimation of the discriminatory power of REA using *Sma*I. However, Christiansen *et al.* (1992a) note that the relatively low number of bands which form the basis of *Sma*I REA patterns mean that the technique has a relatively poor discriminatory power. Indeed, these authors suggest that a second REA should be performed, using *Xho*I, to attempt to subdivide SmaI patterns (Christiansen *et al.*, 1992a). *Sma*I-Based REA typing cannot separate the CU and M9 live vaccine strains of *P. multocida*. *Sma*I-based REA patterns are not as easy to read as those generated by enzymes such as *Hpa*II and *Hha*I. This is due to the fact that the bands used to differentiate isolates by *Sma*I REA typing are typically in the 5 to 2 kilobase (kb) range, with the top part of the gel usually consisting of a smear of larger-sized fragments.

REA using *Hha*I has been used in a series of studies on avian *P. multocida* (Wilson *et al.*, 1993, 1995a,b). The 16 Heddleston serovar reference strains have been shown to give unique *Hha*I profiles (Wilson *et al*., 1992). The reproducibility of REA using *Hha*I appears high as all seven strains of the live attenuated *P. multocida* CU vaccine strain, collected from four different vaccine companies, gave the same profile (Wilson *et al.*, 1993). When *Hha*I typing was used on 63 avian *P. multocida* isolates, a total of 28 different profiles were recognized (Wilson *et al.*, 1993). If this data is analyzed using Simpson's Index of Diversity (Hunter & Gaston, 1988), the result is an index of 0.865, somewhat lower than the minimum recommended index of greater than 0.9 (Hunter & Gaston, 1988). REA typing by *Hha*I, supported by follow-up typing with *Hpa*II to further subdivide the *Hha*I REA types, has been suggested as an alternative tool to serotyping (Wilson *et al.*, 1993). However, even the combination of *Hha*I and *Hpa*II cannot separate the CU and M9 vaccine strains from each other. The banding patterns generated by *Hha*I are easy to read, with Wilson *et al.* (1992) claiming the patterns for this enzyme, as well as those generated using *Hpa*II, are easier to read than those generated by a battery of other restriction enzymes.

REA using the enzyme *Hpa*II appears to generate a finer subdivision than that achieved with the use of *Hha*I. As an example, 314 *P. multocida* isolates from wild birds that had a single *Hha*I pattern were allocated to three different *Hpa*II profile groups (Wilson *et al.*, 1995a). Similarly, 19 raptor *P. multocida* isolates with a single *Hha*I pattern were allocated to four different *Hpa*II profiles (Wilson *et al.*, 1995b). In Denmark, Christensen *et al.* (1998)

found that 30 avian *P. multocida* isolates, obtained from both wild and domestic birds, were allocated to 15 profile types by *Hha*I and 17 profile types by *Hpa*II.

Overall, *Hha*I and *Hpa*II appear to be the most suitable enzymes for use in REA of *P. multocida*. While there have been no formal estimations of the discriminatory power of typing by either enzyme, the literature clearly indicates that *Hpa*II gives a finer typing than *Hha*I. While the suggestion of a combined *Hha*I/*Hpa*II REA typing has been made, it would appear that laboratories facing increasing cost pressures would be best served by performing REA with *Hpa*II. As the differentiation of the M9 and CU vaccine strains is not a major issue, M9 being a slow-growing mutant of CU (Snipes *et al.*, 1990), the inability of *Hpa*II-based REA to separate these two strains is not a major drawback. There is a need for good electrophoresis conditions and careful examination of REA patterns to obtain fine and reproducible typing when using *Hpa*II (Christensen *et al.*, 1998).

Ribotyping

Ribotyping is a typing technique based on REA. However, rather than looking directly at all of the DNA fragments generated in a typical REA gel, only selected DNA fragments are visualized. This is achieved by completing the gel electrophoresis of a typical REA analysis and then transferring the DNA fragments out of the gel onto the surface of a membrane, typically nylon in nature. This membrane is then exposed to a nucleic acid probe that binds only to those DNA fragments which contain segments homologous to the probe. Hence, most of the DNA fragments present in an REA gel are not detected in this type of analysis. Ribotyping is named because the probe used for visualization is based on either ribosomal RNA (rRNA) itself or the genes that code for rRNA. As shown in Figure 2, ribotyping results in a much simpler banding pattern than that generated by REA.

There are several important variables within ribotyping – the main ones being the restriction enzyme and the probe. The same comments that have been made for the restriction enzyme used in REA (see earlier) apply for ribotyping.

To gain an appreciation of the issues that surround the choice of a ribotyping probe, it is necessary to have some understanding of rRNA, whose key role is protein synthesis, and the genes that code for rRNA. There are three forms of rRNA – in increasing size, they are 5S rRNA, 16S rRNA and 23S rRNA. The genes that code for the forms of rRNA are termed *rrf, rrs* and *rrl*, coding for 5S, 16S and 23S rRNA, respectively. These genes are organized in a grouping termed an operon and are organized in the sequence *rrs–rrl–rrf* in *P. multocida* (Hunt *et al.*, 1998). There are five copies of this operon (Hunt *et al.*, 1998).

Across the broad sweep of Gram-negative bacteria, the following types of ribotyping probes have been used – a mixture of the 16 and 23S rRNA of *Escherichia coli* (Grimont & Grimont, 1986), a cloned copy of the *rrs* and *rrl* genes of *E. coli* (Altwegg & Mayer, 1989), PCR amplified copies of parts or all of the *rrs* gene (Stanley *et al.*, 1993), and either a single oligonucleotide probe or a mix of oligonucleotide probes that recognize regions within the *rrs* and *rrl* genes (Göbel *et al.*, 1987; Jannes *et al.*, 1993).

Regnault *et al.* (1997) have clearly shown that this use of diverse probes can make the comparison of ribotyping results difficult as different probes can yield different results. In particular, Regnault *et al.* (1997) suggested that the use of cloned or PCR-generated probes or some oligonucleotide probes can result in ribotyping patterns that are only a subset of those which could have been obtained with the use of a 16S+23S rRNA probe. In an elegant study, Regnault *et al.* (1997) showed that a mix of five oligonucleotide probes (two that target the two ends of the *rrs* gene, two that target the two ends of the *rrl* gene, and one that targets the middle of the *rrl* gene) gives ribotype patterns which are equivalent to the use of 16S+23S rRNA. As these probes, termed OligoMix5, can be easily ordered from a number of commercial sources and can be chemically labelled, the use of this mixture is both safe and easy. While the use of OligoMix5 with *P. multocida* (avian or otherwise) has not yet been evaluated, it would appear that this is an approach which is worth pursuing.

The various enzyme and probe combinations that have been used in ribotyping avian isolates of *P. multocida* are shown in Table 3, which highlights the difficulties in comparing and contrasting the results of ribotyping methods as applied to avian *P. multocida*. While the most commonly used probe has been the 16S+23S rRNA from *E. coli*, two other probes, one a cloned product and the other a PCR amplified product, have been used. Indeed, the enzyme *Hpa*II has been used in combination with all three different probes.

All studies have indicated that the typeability of avian *P. multocida* by ribotyping is high as no strains that cannot be ribotyped have been reported.

Ribotyping performed using *Eco*RI or *Pst*I digestion and a 16S+23S rRNA probe has been shown to generate the same degree of discrimination among field isolates as REA performed using the enzymes *Sma*I (Snipes *et al.*, 1990) or a combined *Sma*I/*Sal*I digest (Snipes *et al.*, 1989). There appears to have been no formal estimation of reproducibility, although Snipes *et al.* (1990) imply that isolates of the live attenuated M9 vaccine strain always give the same ribotype pattern. This method has always been used with radioactive labelling techniques (Snipes *et al.*, 1989, 1990; Carpenter *et al.*, 1991; Morishita *et al.*, 1996a,b), making it a method that

Table 3. *Restriction endonuclease enzymes and probes that have been used in ribotyping avian* P. multocida

Enzyme (recognition sequence)	Probe	Reference
*Eco*RI (G↓AATTC)	16S+23S rRNA (*E. coli*)	Snipes *et al.* (1989), Snipes *et al.* (1990), Carpenter *et al.* (1991), Morishita *et al.* (1996a,b)
*Hha*I (GCG↓C)	16S+23S rRNA (*E. coli*)	Christensen *et al.* (1998)
*Hin*DIII (A↓AGCTT)	16S+23S rRNA (*E. coli*)	Petersen *et al.* (1998), Christensen *et al.* (1998), Morishita *et al.* (1996a,b)
*Hpa*II (C↓CGG)	16S+23S rRNA (*E. coli*)	Petersen *et al.* (1998), Christensen *et al.* (1998)
*Hpa*II (C↓CGG)	PCR amplified 16S gene (*P. multocida*)	Blackall *et al.* (1998)
*Hpa*II (C↓CGG)	cloned 16S gene (*H. paragallinarum*)	Blackall *et al.* (1995)
*Pst*I (CTGCA↓G)	16S+23S rRNA (*E. coli*)	Snipes *et al.* (1989), Snipes *et al.* (1990), Carpenter *et al.* (1991)

would not be easy to implement for many laboratories. However, there is no need for radioactive labelling to be used, and the method could certainly be adapted to chemical labelling techniques, such as the use of digoxigenin, that are more suited to the majority of veterinary laboratories.

In studies on small numbers of *P. multocida* isolated from raptors and psittacines (five isolates in both cases), ribotyping performed by either *Hin*DIII digestion or *Eco*RI digestion, in combination with a 16S+23S rRNA probe, have been shown to achieve the same typing results (Morishita *et al.*, 1996a,b). In a study of a larger number of isolates, 15 in total, of maltose-positive *P. multocida*, most of which were avian in origin, the use of *Hin*DIII digestion gave 10 ribotype patterns, while the use of *Hpa*II digestion gave 11 ribotype patterns (Petersen *et al.*, 1998).

Ribotyping using *Hpa*II digestion has been the most extensively studied method for ribotyping avian *P. multocida*. A total of 110 field isolates and 19 reference strains were examined by this method using a PCR amplified copy of the *rrs* gene from *H. paragallinarum* (Blackall *et al.*, 1998). A retrospective evaluation of this study (Blackall *et al.*, 1998) indicates that *Hpa*II-based ribotyping has a good discriminatory power with a diversity index of 0.899, very near to the recommended minimum of greater than 0.9 (Hunter & Gaston, 1988). As the probe used in this study (Blackall *et al.*, 1998) was a PCR amplified copy representing only the *rrs* gene, it is possible that the use of a 16S+23S rRNA probe or the Oliogmix5 already mentioned would have resulted in a greater discriminatory power. When *Hpa*II digestion was used with a 16S+23S rRNA probe, all 31 isolates from two outbreaks in wild birds gave the same ribotype pattern, while 30 other isolates from backyard poultry gave 12 patterns, one of which was the same as the wild bird isolate pattern (Christensen *et al.*, 1998). While not a formal estimation of discriminatory power, these Danish results suggest that this technique has good typing capacity (Christensen *et al.*, 1998). This Danish study also found that REA using *Hpa*II, which gave 17 patterns among the 30 backyard poultry isolates, gave a higher level of sub-typing than given by ribotyping with *Hpa*II, which found only 12 patterns among the same 30 isolates (Christensen *et al.*, 1998). This contrasts with an Australian study that found, among 22 isolates from seven turkey farms, that REA and ribotyping, both performed with *Hpa*II digestion, gave the same allocation of isolates to the same pattern groups – a total of seven patterns (Blackall *et al.*, 1995). It is probable that the greater number of locations (20 different backyards) and host species (five) in the Danish study (Christensen *et al.*, 1998) make the Danish results more representative than the Australian study, which was based on only seven farms and one host species (Blackall *et al.*, 1995). There has been no formal evaluation of the reproducibility of *Hpa*II-based ribotyping. All studies on this technique have used a chemical labelling technique, meaning that the technique is suited for non-specialist laboratories.

Ribotyping using the enzyme *Hha*I and a 16S+23S rRNA probe has only been used in a single study to date (Christensen *et al.*, 1998); hence, formal estimations of discriminatory power and reproducibility are not available. It is notable that in this single study, *Hha*I-based ribotyping gave fewer patterns among the 30 isolates examined (10 patterns) than *Hpa*II-based ribotyping, which gave 12 patterns (Christensen *et al.*, 1998).

Overall, the enzyme of choice for ribotyping studies of avian *P. multocida* appears to be *Hpa*II. The most suitable probe used to date has been 16S+23S rRNA from *E. coli*. However, it would appear that the OligoMix5 probe approach of Regnault *et al.* (1997) would be an easier alternative which would give equally effective results. As a technique, ribotyping has been shown to give a good level of discrimination. In the few direct comparisons of REA and ribotyping performed to date, the overall evidence suggests that ribotyping will give fewer patterns than REA. Certainly, ribotyping requires additional technical steps, and an additional level of equipment and expertise associated with activities such as probe labelling, the transfer of the DNA to a membrane and the subsequent probing. While not yet applied to *P. multocida*, there is potential for a computerized approach that allows the creation of a database and then a comparison of field isolates with this database, and thus possible identification of a particular ribotype. This type of approach has been recently demonstrated with *E. coli* (Machado *et al.*, 1998).

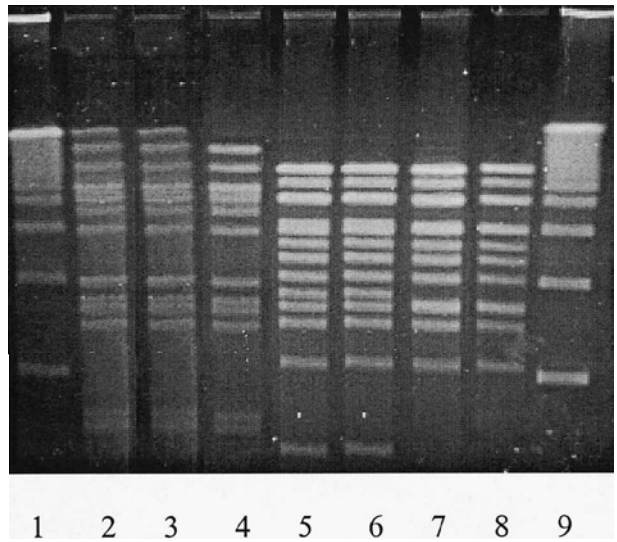

Figure 3. *Example PFGE patterns generated among porcine* P. multocida *isolates using the enzyme* ApaI. *Lanes 1 and 9, Molecular weight marker; lane 2, PFGE pattern A; lane 3, PFGE pattern A; lane 4, PFGE pattern A1 (differs in only one band from pattern A); lane 5, PFGE pattern B; lane 6, PFGE pattern B; lane 7, PFGE pattern B1 (only two band differences from pattern B); lane 8, PFGE pattern B1 (only two band differences from pattern B).*

Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is essentially a specialized form of REA. In PFGE, the restriction endonuclease enzyme used is best described as a "rare cutter". As a consequence, the resultant digest contains few fragments, typically five to 20, with most of these fragments being in the size range of 10 to 800 kb, much larger than the fragments present in typical REA. As the fragments are, relatively speaking, few in number, it is important that the DNA used for PFGE is high-quality material that has not already been fragmented by random shearing forces. Hence, DNA for PFGE is typically prepared by embedding intact bacterial cells in agarose plugs. The subsequent steps of lysis of the bacterial cells, removal of contaminant proteins and restriction digestion are all performed *in situ*. Conventional electrophoresis, which uses a constant electric current, cannot resolve the large fragments generated by "rare cutting" restriction enzymes. However, a modified electrophoresis technique in which the electric field across the gel is changed periodically ("pulsed") can effectively separate, on size basis, the large fragments typical of PFGE analysis. An example of PFGE of porcine isolates of *P. multocida* is shown in Figure 3.

While widely regarded in clinical microbiology as the typing method coming closest to being the most suitable (Tenover *et al.*, 1995), PFGE has only been used, to date, on avian *P. multocida* in a single study (Gunawardana *et al.*, 2000). The enzyme used in this study was *Apa*I. While there was no examination of reproducibility, the general literature on PFGE indicates that PFGE patterns are highly reproducible (Arbeit, 1995). The study of Gunawardana *et al.* (2000) examined 73 Australian avian isolates and 22 Vietnamese avian isolates. The 73 Australian isolates were allocated to 21 PFGE pattern types (Gunawardana *et al.*, 2000). A retrospective calculation establishes a diversity index of 0.899, close to the score of greater than 0.9 needed for an effective typing scheme (Hunter & Gaston, 1988). The results for the 22 Vietnamese isolates have not been included in this retrospective evaluation as there is evidence that many of these isolates are clonal in nature with limited diversity, meaning that including them in calculating the diversity index would bias the calculation. There are two limitations to PFGE-based typing from the view of ease of use. First, the most commonly used DNA extraction methods require periods of 2 to 4 days to complete (Maslow *et al.*, 1993), although a rapid 1-day method has been described for *E. coli* (Gautom, 1997). The second limitation is that PFGE requires specialized and expensive electrophoresis equipment which is generally not available in routine veterinary diagnostic laboratories.

PCR-Based Typing Methods

A range of PCR-based typing techniques has been developed in recent years for a number of both human and veterinary pathogens. One of the most widely used has been repetitive extragenic palindromic (REP)-PCR. REP elements are 33- to 40 base pair repeats that are present as 500 to 1000 copies accounting for up to 1% of the genome (Stern *et al.*, 1984), and are present in a wide range

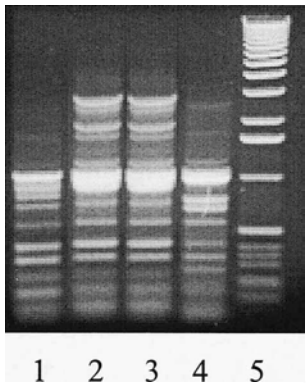

Figure 4. *Example ERIC-PCR patterns generated among avian* P. multocida *isolates. Lane 1, ERIC-PCR pattern 1; lane 2, ERIC-PCR pattern 2; lane 3, ERIC-PCR pattern 2; lane 3, ERIC-PCR pattern 2; lane 4, ERIC-PCR pattern 3; lane 5, molecular weight marker.*

of enteric bacteria (Olive & Bean, 1999). In REP-PCR, amplified products result whenever two correctly orientated primers are close enough for the PCR amplification process to occur efficiently. As the REP elements are distributed widely across the genome, the resultant products are not a single size – unlike a species-diagnostic PCR, where a single band is typically generated. In REP-PCR, a multiplicity of bands can be produced, depending on the distribution/location of the REP elements across the genome. The palindromic nature of the REP elements means that it is possible to perform REP-PCR with a single primer or, more commonly, with two primers. Figure 4 shows the results of a PCR-based fingerprinting of *P. multocida*. The technique used in Figure 4 is not REP-PCR, but a related technique termed ERIC-PCR (Versalovic *et al.*, 1991). ERIC-PCR differs from REP-PCR only in terms of the primers used; those used in ERIC-PCR target enterobacterial repetitive insertion consensus (ERIC) sequences (Versalovic *et al.*, 1991). There has been no reported use of ERIC-PCR on avian *P. multocida*.

To date, REP-PCR has only been used on avian *P. multocida* in a single study, the same study that evaluated PFGE (Gunawardana *et al.*, 2000). While there was no examination of reproducibility in this study, it has been claimed by other workers that REP-PCR has excellent reproducibility (Arbeit, 1995). The study of Gunawardana *et al.* (2000) examined 44 Australian avian isolates and eight Vietnamese avian isolates, with the 44 Australian isolates being allocated to 21 REP-PCR pattern types. A retrospective calculation of discriminatory power, using only the Australian isolates, results in a diversity index of 0.95, above the minimum score of greater than 0.9 needed for an effective typing scheme (Hunter & Gaston, 1988). It is possible that the relatively low number of isolates examined (44) may have resulted in an inflated discriminatory power. In the same study, a further 29 Australian isolates were available but not examined by REP-PCR (Gunawardana *et al.*, 2000). As many of these isolates had been allocated to common PFGE patterns, it is likely that REP-PCR would have done the same, i.e. allocated these additional isolates to existing REP-PCR patterns. Hence, the most likely situation is that the discriminatory power of REP-PCR is similar to that of PFGE and would have a diversity index of around 0.9. In the study of Gunawardana *et al.* (2000), REP-PCR consistently found indistinguishable isolates that showed minor differences by PFGE. PCR-based typing methods offer a considerable practical advantage to all of the DNA-based typing methods already described. PCR-based tests do not necessarily need high-quality DNA preparations (a must for REA, ribotyping and PFGE). Hence, REP-PCR can be performed directly on colonies without any need for extensive DNA extraction, as carried out by Gunawardana *et al.* (2000). This direct use of colonies also means that REP-PCR has a time advantage over the other DNA-based typing methods – the results can be available within 1 day. There is a need for a thermal cycler to perform the PCR reaction; however, this type of equipment is becoming widely available in many diagnostic laboratories.

Multi-Locus Enzyme Electrophoresis

Multi-locus enzyme electrophoresis (MLEE) differs from all the other typing methods covered in this review in that this method is not a genotypic method. MLEE is, in fact, a phenotypic method that examines variation in the electrophoretic mobility of water-soluble enzymes. Full technical details of MLEE are available from a methodological review by Selander *et al.* (1986).

The enzymes examined in MLEE are typically housekeeping or cell-maintenance enzymes. All isolates should contain the enzyme, as the diversity detected by MLEE is not the presence or absence of the enzyme. In fact, the diversity is the variation in the amino acid sequence that makes up the enzyme. There is considerable potential for variation in the amino acid sequence of any particular enzyme. As the electrophoretic mobility of an enzyme is determined by the amino acid sequence, changes in this sequence will result in variation in the mobility of the enzyme. The electrophoretic mobility of the various enzymes is established by horizontal gel electrophoresis. In general, MLEE is performed using simple starch gels. After electrophoresis, the gels are sliced horizontally, creating a number of thin gels that are duplicates of each other. Each slice is then specifically stained for one enzyme – meaning that a single original gel can be used for a number of different enzymes – depending on the number of slices. This staining results in a coloured spot of enzyme activity. The various mobility

Table 4. *Electrophoretic types, as detected by MLEE, of six avian* P. multocida *isolates*[a]

Isolate (ET)	EST	MPI	APK	ADK	PGM	G6PD	6GPD	PGI	NP	GDH	GP	HEX	MDH	ME	GOT	LT	FUM	CAT
PM27 (A19)	6[b]	9	6	6	3	2	4	3	3	2	3	3	2	2	2	2	2	2
PM63 (A1)	9	6	4	4	3	2	4	3	3	2	3	3	2	2	2	2	2	2
PM68 (A1)	9	6	4	4	3	2	4	3	3	2	3	3	2	2	2	2	2	2
PM79 (A39)	11	7	3	3	3	2	4	5	2	2	3	3	2	2	1	2	2	2
PM133 (A35)	6	6	4	4	4	2	4	4	3	2	3	3	2	2	2	1	2	2
PM138 (A52)	5	5	3	3	3	1	4	2	3	2	3	3	1	1	2	2	2	2

[a]Data from Blackall *et al.* (1998). EST, Esterase; MPI, mannose-6-phosphate isomerase; APK, arginine phosphokinase; ADK, adenylate kinase; PGM, phosphoglucomutase; G6PD, glucose-6-phosphate dehydrogenase; 6GPD, 6-phosphogluconate dehyrogenase; PGI, phosphoglucose isomerase; NP, nucleoside phosphorylase; GDH, NADP-dependent glutamate dehydrogenase; GP, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase; HEX, hexokinase; MDH, malate dehyrogenase; ME, malic enzyme; GOT, glutamate-oxaloacetate transaminase; LT, L-leucyl-L-tyrosine; FUM, fumarase; CAT, catalase.

[b]The values indicate different mobility variants of each enzyme.

variants of each enzyme, often called electromorphs, are numbered, with the lowest number, 1, being allocated to the form that moves furthest in the gel. Examples of the electrophoretic types of six avian *P. multocida* isolates, extracted from the data of Blackall *et al.* (1998), are shown in Table 4. Isolates with the same electromorphs across all examined enzymes are allocated to the same electrophoretic type (ET). In the examples shown in Table 4, isolates PM63 and PM68 show the same electromorphs across all 18 enzymes, and hence form one ET (ET A1) while the other four isolates form unique ETs. Using some standard statistical packages (see the methodological review of Selander *et al.* (1986)), it is possible to express the extent of similarity between two strains as the genetic distance between the pairs. A matrix of genetic distance for a collection of isolates can then be used to construct a phenogram that relates genetic distances between pairs of isolates to illustrate the relationships between clusters of isolates (Selander *et al.*, 1986). A published phenogram that shows the genetic distance amongst 71 ETs of Australian avian *P. multocida* and 15 ETs formed by the 16 Heddleston serovar reference strains and the type strains for the three subspecies of *P. multocida* is presented in Figure 5 (Blackall *et al.*, 1998).

Like the molecular typing methods already reviewed, MLEE has the capacity of being applied to all isolates of a species. MLEE analysis of 81 Australian avian isolates of *P. multocida* resulted in the recognition of 56 ETs (Blackall *et al.*, 1998). A retrospective analysis of this data yields a diversity index of 0.97, well above the minimum required level of greater than 0.9 (Hunter & Gaston, 1988). There has been no formal assessment of the reproducibility of MLEE as a typing tool for avian *P. multocida*. The finding that all 12 strains of an outbreak previously shown to be identical by two genotyping methods (REA and ribotyping) were also allocated to the same ET indicates that MLEE appears to have good reproducibility (Blackall *et al.*, 1999). MLEE is not a technique that would suit routine laboratories. While the equipment used in MLEE is quite basic, an electrophoresis power supply being the main equipment, the use of starch gels and extensive enzyme staining techniques are not common features of many veterinary laboratories. Hence, MLEE studies are generally performed in centralized, well-equipped laboratories.

Emerging Typing Technologies

While not yet reported to have been used on avian *P. multocida*, there are a number of emerging typing technologies that are being applied to a range of clinical and veterinary bacterial pathogens. Of these emerging technologies, those that appear to have the most potential are amplified fragment length polymorphism (AFLP) (Savelkoul *et al.*, 1999) and multi-locus sequence typing (MLST) (Spratt, 1999).

AFLP is a technique combining REA and selective PCR that amplifies some of the fragments generated during the REA stage (Savelkoul *et al.*, 1999). AFLP is regarded as having high reproducibility and good discriminatory power, and can be applied to all organisms (Savelkoul *et al.*, 1999). AFLP is most frequently performed using fluorescent labelling techniques and automated DNA sequencing equipment (Savelkoul *et al.*, 1999). This use of sequencing equipment means that, at this stage, AFLP is best suited for central reference laboratories and not routine veterinary diagnostic laboratories.

MLST is a technology that takes the phenotypic method of MLEE to the level of a genotypic method (Spratt, 1999). Rather than indirectly detecting amino acid variation by electrophoretic mobility variations, as occurs with MLEE, MLST uses DNA

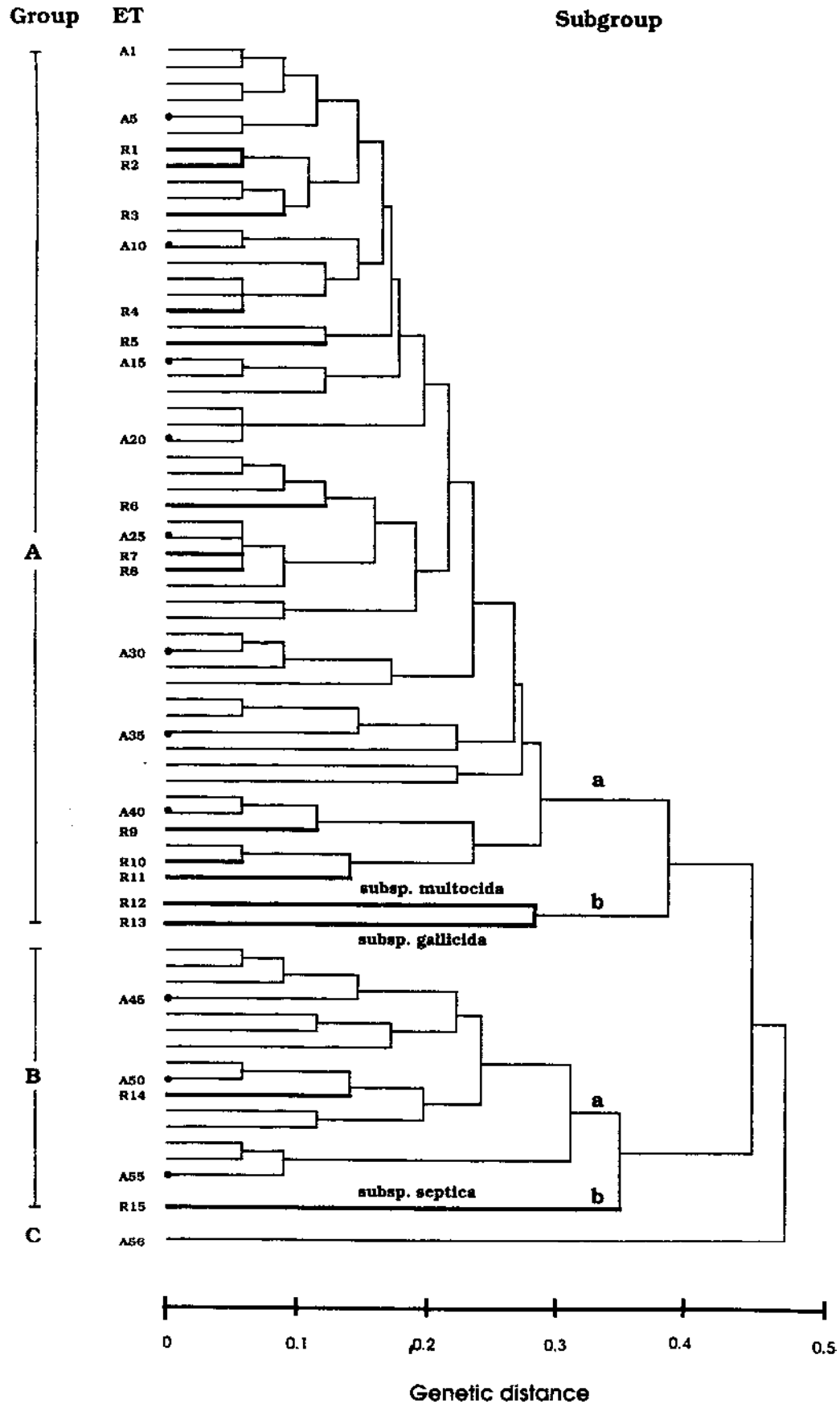

Figure 5. *Phenogram of genetic distance among 71 ETs formed by poultry isolates of* P. multocida. *ETs formed by Australian field isolates are labelled A and marked by thin bars. ETs formed by reference strains are labelled R and marked by thick bars. Figure reproduced, with permission, from the journal* Microbiology.

sequencing of the gene loci to directly detect the genetic variation that results in amino acid sequence variation in the enzymes (Spratt, 1999). The method is regarded as highly reproducible, has high discriminatory power and can be applied to all isolates of any species for which there is sufficient knowledge of relevant gene loci to allow DNA sequencing (Spratt, 1999). A major advantage of MLST is that data is highly portable. Indeed, it is possible to compare typing results from laboratory to laboratory and use a centralized Web-based databank (Enright & Spratt, 1998).

Interpretation of Typing Results

A major problem that confronts all typing methods is the interpretation of when patterns, whether from REA or ribotyping and so on, are different. This is an important issue – is a single band difference sufficient to allocate isolates to different REA patterns or should single band differences in REA be regarded as subtypes of the one pattern? Should a universal rule, e.g. a four band or greater difference means a different pattern, be applied to REA patterns generated by such different enzymes as *Sma*I and *Hpa*II, or such different techniques as ribotyping and REP-PCR as well as PFGE?

As most typing studies are performed to help clarify the epidemiology of disease outbreaks, there is great value in adopting the suggestion of Arbeit (1995) and Tenover *et al.* (1995) to link the concepts of epidemiological relatedness and genetic relatedness. Epidemiologically related isolates are isolates that, on the basis of field information such as time of isolation, location of isolation, disease history and so on, are most likely to be derived from a common source (Tenover *et al.*, 1995). Genetically related isolates are isolates that are either indistinguishable or show only minor differences by a variety of typing methods, and hence can be presumed to have a common parent (Tenover *et al.*, 1995). On this basis, for PFGE typing, Tenover *et al.* (1995) suggested that isolates indistinguishable by PFGE are part of the outbreak. Isolates that have one genetic difference (which converts to a two to three band difference in PFGE patterns) are close genetic relatives and are probably part of the outbreak. Isolates that have two genetic differences (which results in a four to six band difference in PFGE patterns) are possibly genetic relatives and possibly are part of the outbreak. Isolates that have three or more genetic differences are genetically distinct and are not part of the outbreak (Tenover *et al.*, 1995). As PFGE and REA are essentially different versions of the same technology, the PFGE-linked rules of Tenover *et al.* (1995) can also be applied to REA.

There is, at present, no universally agreed or applied guidelines for interpreting REP-PCR and ribotype patterns. Use of the available epidemiological information to guide the interpretation of REP-PCR and ribotyping patterns is probably the best approach that can be recommended at this stage.

It is possible to use numerical and graphical methods (similarity matrix, Dice coefficients and phenograms) to interpret typing results. To meet the underlying assumptions of these analytical techniques, it is necessary that the data being used represent multiple distinct characteristics that vary independently (Arbeit, 1995). Of the typing techniques reviewed, only MLEE- and MLST-generated data undoubtedly meet these criteria. There is considerable debate on whether data from REA, PFGE and ribotyping analysis can be used in this manner (Arbeit, 1995). For avian *P. multocida*, it has been shown that this form of numerical and graphical analysis of ribotyping data does yield results that are broadly similar to the same analysis performed on MLEE data (Blackall *et al.*, 1998).

A Direct Comparison of Typing Methods

All five of the typing methods reviewed in this review, REA, ribotyping, PFGE, REP-PCR and MLEE, have been used on the same set of 22 avian *P. multocida* obtained from outbreaks of fowl cholera on seven Australian turkey farms. As the investigation of the relationships or otherwise among outbreaks of fowl cholera is a major reason for typing *P. multocida*, this multiple application of typing methods to a single set of *P. multocida* isolates with a detailed field history is a good opportunity to directly compare typing methods in a relevant setting. The REA and ribotyping work, along with the original field epidemiological investigation, were reported by Blackall *et al.* (1995). The retrospective use of PFGE and REP-PCR was reported by Gunawardana *et al.* (2000), and the retrospective use of MLEE was reported by Blackall *et al.* (1999). Table 5 shows the direct comparison of all five typing techniques. For Outbreaks II, VI, VII and VIII, all five typing methods agree that the outbreaks are distinct and not connected. All five typing methods agree that the two outbreaks seen on Farm 1 are different. Four of the five methods, REA, ribotyping, PFGE and REP-PCR, indicate that Outbreaks I and V are connected. PFGE and REP-PCR typing found subtle differences between these outbreaks – but not enough to allocate the isolates to different pattern types. In contrast, MLEE found the two outbreaks to be caused by distinctly different strains. As shown in Figure 5, the two ETs involved in Outbreaks I and V, ET A53 and A34, are separated by a genetic distance of 0.45. Four of the five typing methods, REA, ribotyping, REP-PCR and MLEE, agree that Outbreaks III and IV are distinct and unconnected. However, PFGE typing suggested that the strains involved in these two outbreaks had identical types and thus were directly connected to a common strain.

Table 5. *Comparison of REA, ribotyping, PFGE, REP-PCR and MLEE typing of 22 avian isolates of* P. multocida

Farm[a]	Outbreak[a]	Isolates[a]	REA Type[a]	Ribotype[a]	PFGE Type[b]	REP-PCR Type[b]	MLEE Type[c]
1	I	PM137	I	i	19ii	20ii	A53
	II	PM1	VI	vi	4	4	A47
		PM12–14	VI	vi	4	4	A47
2	III	PM2–10	VII	vii	20	21	A38
		PM11	VII	vii	20ii	21ii	A38
		PM15, 16	VII	vii	20	ND	A38
3	IV	PM132	II	ii	20	22	A35
4	V	PM133	I	i	19i	20i	A34
5	VI	PM134	III	iii	18	19	A32
6	VII	PM135	IV	iv	17	17	A43
7	VIII	PM136	V	v	16	16	A30

[a] As defined by Blackall *et al.* (1995).
[b] As defined by Gunawardana *et al.* (2000).
[c] As defined by Blackall *et al.* (1999).

Overall, there was strong agreement among all five typing methods. The lack of total agreement for all methods across all outbreaks indicates that epidemiological studies are best performed using at least two different typing methods.

Which Typing Method?

There is no simple or single answer to the question of which typing method should be used for studies on avian *P. multocida*. The factors to be considered include the technical capacity of the various typing methods (typeability, discriminatory power, reproducibility and ease of use) as reviewed in this article. Also, the reason for the typing study is an important factor. A comparison of a relatively small number of isolates and outbreaks in a routine diagnostic laboratory may result in the selection of different typing methods compared with a study of a large, disparate culture collection being performed in a central reference laboratory. The final set of factors to be considered is the level of expertise and equipment available within a given laboratory.

For a relatively simple disease investigation situation, the two techniques that appear most suitable are REA and REP-PCR. These methods require minimal equipment and allow direct visual comparison of typing patterns. For many bacteria, PFGE is recommended above REA. However, for *P. multocida*, REA analysis using *Hpa*II or *Hha*I generates such crisp and clear typing patterns that there is no need to resort to PFGE and the high cost of PFGE electrophoresis equipment. The recommended methods, REA and REP-PCR, use totally different technologies and thus maximize the likelihood of detecting the maximum possible diversity present in the isolates being examined.

For a complex study looking at large numbers of isolates and requiring data analysis, the method of choice is MLEE or, more likely, MLST in the near future. MLEE/MLST should be supported by at least two of the other techniques – REA, PFGE, ribotyping or REP-PCR. For a well-equipped laboratory, ribotyping, performed with *Hpa*II and the OligoMix5 probe (or 16+23S rRNA), has the advantage of generating patterns that are easily digitized and analyzed. There is a need for caution in the interpretation of such data analysis as already noted.

Conclusions

There are considerable potential advantages for those laboratories that are considering the adoption of the newer methods for identifying and differentiating avian *P. multocida*. However, these new-generation tests are not without limitations and constraints. The PCR tests require further rigorous evaluation to conclusively demonstrate their suitability for use. In particular, the use of PCR directly on clinical material remains a significant challenge. A strong message that should emerge on the adoption of the newer molecular typing techniques is that these tests cannot be used in isolation. The tests are best used in parallel with other conventional technologies, and at least two distinctly different methods should be used. The power of these tests is still very much improved by using these typing techniques as part of an integrated laboratory and field investigation. The importance of this integration between field and laboratory remains as great as ever.

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RÉSUMÉ

Identification et typage de *Pasteurella multocida*

Pasteurella multocida est un agent pathogène important pour de nombreuses espèces aviaires. Cette synthèse examine de façon critique les développements récents des tests de nouvelle génération pour l'identification et le typage de cette bactérie. Deux tests d'amplification en chaîne par polymérase ont été décrits pour *P. multocida*. Les deux tests semblent prometteurs et pourraient bien être utilisés comme tests de routine. Cependant, il n'y a pas encore eu d'études d'évaluation pour savoir si ces nouveaux tests permettraient la distinction entre les isolats typiques et atypiques de *P. multocida*, et entre tous les autres organismes *P. multocida*-like, trouvés chez les espèces aviaires. Le test PCR décrit par Townsend et al. (*J. Clin. Microbiol*. 36 : 1096–1100), a été davantage évalué et serait, à ce niveau, à recommander aux laboratoires désirant utiliser la PCR pour la détection de *P. multocida*. Un point important est que les tests PCR ont été validés en utilisant des cultures pures ou des bouillons d'enrichissement – et non directement à partir de tissus d'animaux. Jusqu'à présent il y a 5 techniques différentes permettant de typer les *P. multocida* aviaires – restriction enzymatique (REA) – ribotypage – électrophorèse en champ pulsé (PFGE) – PCR – Palindromique extragénique (REP-PCR) et électrophorèse enzymatique en points multiples (MLEE). La méthodologie de ces différentes techniques est brièvement expliquée et la performance de ces techniques vis-à-vis du typage des *P. multocida* aviaires est examinée de façon critique. Pour de petits laboratoires qui étudient les cas de choléra aviaire, il semblerait que la REA et le REP-PCR soient des méthodes de choix pour le typage. Pour les laboratoires de référence qui étudient des collections importantes d'isolats le MLEE en plus de deux des autres méthodes apparaîtraient être les techniques les plus appropriées.

ZUSAMMENFASSUNG

Identifizierung und Typisierung von *Pasteurella multocida*

Pasteurella multocida ist ein wichtiger Krankheitserreger vieler Vogelarten. Die vorliegende Übersicht befasst sich mit einer kri-

tischen Untersuchung der jüngsten Entwicklungen bei Tests der neuen Generation zur Identifizierung und Typisierung dieses Bakteriums. Zwei Polymerase-Kettenreaktion (PCR)-Tests sind für *P. multocida* beschrieben worden. Beide Tests berechtigen zu den besten Hoffnungen als diagnostische Tests, die für den routinemäßigen Einsatz in Betracht gezogen werden könnten. Es gibt allerdings noch keine effektiven Bewertungsstudien, die die Eignung dieser neuen Tests für die Differenzierung zwischen typischen und atypischen *P. multocida*-Isolaten und anderen, in Vogelarten nachgewiesenen *P. multocida*-ähnlichen Mikroorganismen geprüft haben. Eine PCR, und zwar die von Townsend *et al.* (*J. Clin. Microbiol.* 36:1096–1100) beschriebene, wurde gründlicher geprüft und ist zum gegenwärtigen Zeitpunkt für Laboratorien, die den Gebrauch der PCR zum Nachweis von *P. multocida* in Erwägung ziehen, die bessere Wahl. Ein wesentlicher Gesichtspunkt ist, dass die Eignung der PCR-Tests mit Reinkulturen oder Anreicherungsnährböden beurteilt worden ist und nicht durch ihre Benutzung zur unmittelbaren Untersuchung von Körpergeweben. Bis heute gibt es fünf verschiedene Technologien, die für die Typisierung von *P. multocida* des Geflügels verwendet werden: Restriktionsendonuklease-Analyse (REA), Ribotypisierung, Pulsfeld-Gelelektrophorese (PFGE), Repetitive Extragene Palindrom-PCR (REP-PCR) und Multilocus-Enzymelektrophorese (MLEE). Die diesen Techniken zugrunde liegende Methodologie wird kurz erklärt, und die Leistungsfähigkeit dieser Techniken im Hinblick auf die Typisierung von *P. multocida* des Geflügels wird kritisch geprüft. Für kleinere Laboratorien, die Geflügelcholera-Ausbrüche untersuchen, sind offenbar REA und REP-PCR die Typisierungsmethoden der Wahl. Für zentrale Referenzlaboratorien, die an Untersuchungen von großen Ansammlungen von Isolaten denken, scheint die MLEE, unterstützt durch zwei der anderen Methoden, die geeignetste Technik zu sein.

RESUMEN

Identificación y tipificación de *Pasteurella multocida*

Pasteurella multocida es un patógeno importante de muchas especies de aves. Esta revisión examina de forma crítica el desarrollo reciente de nuevas generaciones de técnicas de identificación y tipificación de esta bacteria. Se han descrito dos técnicas de reacción en cadena de la polimerasa (PCR) para detectar *Pasteurella multocida*. Ambas técnicas son prometedoras como técnicas diagnósticas y podrían ser consideradas de uso rutinario, pero áun no se han llevado a cabo estudios de evaluación de la capacidad de estas nuevas técnicas para distinguir entre cepas típicas y atípicas de P. multocida, y otros microorganismos *Pasteurella*-like aislados de diferentes especies aviares. Una de las técnicas de PCR, publicada por Townsend *et al.* (*J.Clin.Microbiol.* 36:1096–1100), es la que se ha evaluada de forma más completa y es, por lo tanto, la técnica de elección, actualmente, para los laboratorios que estén considerando el uso de la técnica de PCR para la detección de *P.multocida*. Un dato importante es que las técnicas de PCR han sido validadas para su uso en cultivos puros o caldos de enriquecimento-no para un examen directo de tejidos. Hasta el momento, se han utilizado cinco técnicas diferentes para tipificar *P.multocida* de origen aviar-análisis de restricción con endonucleasas (REA), ribotipificado, electroforesis de campo pulsado (PFGE), PCR de palindrómicos repetitivos extragénicos (REP-PCR) y electroforesis con enzimas multi-locus (MLEE). La metodología utilizada en estas técnicas se explica de forma breve y la realización de las técnicas con respecto a la tipificación de *P.multocida* de origen aviar se examina de forma crítica. Para laboratorios pequeños que investigan epidemias de cólera aviar, los métodos de elección serían la técnica de REA y la técnica de REP-PCR. Para laboratorios centrales de referencia que estan dispuestos a realizar estudios de grandes colecciones de cepas, la técnica de MLEE apoyada por dos de los métodos restantes sería el protocolo diagnóstico más apropiado.