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Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*

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Abstract

*Pasteurella multocida* is responsible for major animal diseases of economic significance in both developed and developing countries whereas human infections related to this bacterium are infrequent. Significantly, development of a carrier status or latent infections plays a critical role in the epidemiology of these diseases. Aiming at increased knowledge of these infections, we examine potential diagnostic and selected typing systems for investigating diseases caused by *P. multocida*. Detection of *P. multocida* from clinical specimen by; i) isolation and identification, ii) polymerase chain reaction (PCR), iii) specific hybridisation probes, iv) serological tests and v) other alternative methods is critically evaluated. These detection systems provide a wide spectrum of options for rapid diagnosis and for detecting and understanding of latent infections in herd/flock health control programmes, though PCR methods for detecting *P. multocida* in clinical specimen appear increasingly preferred. For establishing the clonality of outbreak strains, we select to discuss macromolecular profiling, serotyping, biotyping, restriction enzyme analysis, ribotyping and multiplex PCR typing. Although *P. multocida* infections can be rapidly diagnosed with molecular and serological tests, isolation and accurate species identification are central to epidemiological tracing of outbreak strains. Our review brings together comprehensive and essential information that may be adapted for confirming diagnosis and determining the molecular epidemiology of diseases associated with *P. multocida*.

Keywords: *Pasteurella multocida*, diagnostic tests, PCR, ELISA, typing.
1. Introduction

The genus Pasteurella has recently been outlined (Mutters et al., 2005; Anon, 2007) and the taxonomical position of P. multocida has been defined (Mutters et al., 2005, Christensen and Bisgaard, 2003; Christensen et al., 2005). P. multocida is the type species of the genus including the three subspecies; P. multocida subsp. multocida, P. multocida subsp. gallicida and P. multocida subsp. septica. In addition to P. multocida, Pasteurella sensu stricto also includes the species; P. canis, P. stomatis, P. dagmatis and the unnamed taxon Pasteurella species B and two new species-like taxa related to P. multocida (Christensen et al., 2005).

The pathogenic potential of P. multocida in vertebrate animals was recognized over a century ago and infections are broadly termed pasteurelloses. P. multocida infects a wide range of animal hosts causing specific infections that manifest differently. Indeed, P. multocida has a broad host range, but this peculiar property is poorly understood. Potential virulence factors of P. multocida have recently been reviewed (Hunt et al., 2000; Christensen and Bisgaard, 2000; 2003, Harper et al., 2006) but no host-specific factors have been identified as yet.

The major diseases of economic significance include porcine progressive atrophic rhinitis (PAR; de Jong 1999), haemorrhagic septicaemia (HS) of cattle and water buffaloes (De Alwis, 1992), fowl cholera of poultry (Christensen and Bisgaard, 2000) and snuffles in rabbits (DeLong and Manning, 1994). These infections can vary from slow or latent infections observed with PAR to rapidly developing fatal septicaemias seen with fowl cholera and HS. Additionally, P. multocida also plays a significant role in increasing the severity of primary lung lesions in pigs (Pijoan, 1999) and ruminants (Frank, 1989) caused by other pathogens, though little is known
of the pathogenesis of these infections. Like any other diseases, clinical signs may be suggestive of the aetiology, but obtaining a definitive diagnosis provides essential guidance for effective treatment and for instituting successful control measures. In this respect, diagnostic tests play a pivotal role in confirming clinical cases and in detecting healthy carriers or reservoirs of infection(s). Healthy carriers or latent infections are common to all *P. multocida* infections and play a significant role in the epidemiology of these infections.

PAR, a severe disease of pigs characterized by stunted development and turbinate atrophy is caused by toxinogenic strains of *P. multocida* (de Jong, 1999). Piglets often acquire the infection from their carrier dams. Hence identification of carrier sows in breeding herds by bacteriological procedures and other specific diagnostic tests like PCR and a commercial ELISA kit (DAKO) with subsequent removal of them play key roles in establishing infection-free pig herds (cited by De Jong, 1999).

HS is a significant form of septicaemic pasteurellosis affecting predominantly cattle and water buffaloes (Carter and De Alwis, 1989). In many South East Asian countries, this disease is endemic and sporadic outbreaks are often witnessed following introduction of index cases, which are often healthy carriers within the same herd (De Alwis, 1992). Due to the short incubation period and the fact that symptoms may assume a peracute nature, treatment is often of limited value (Carter and De Alwis, 1989; De Alwis, 1992). Detection with subsequent removal of carriers significantly contributes to the control of HS (De Alwis, 1992).

Fowl cholera, is another significant septicaemic and worldwide disease of severe economic importance (Christensen & Bisgaard, 2000). Both healthy carrier birds within a flock and infected wild birds can act as sources of infection (Glisson et
al., 2003). In peracute or acute cases, chemotherapy may provide limited success, hence rapid detection and subsequent elimination of reservoir birds interrupts the transmission cycle (Glisson et al., 2003).

Snuffles, a highly contagious pasteurellosis of rabbits primarily affects the upper respiratory tract with potential fatal consequences (DeLong and Manning, 1994). Rabbits often get colonized with *P. multocida* for long durations without clinical signs, and the prevalence of this organism in clinically healthy animals has been estimated to range from 20 to 90% depending on the detection method employed (cited by Sanchez et al., 2004). Infection is often acquired from a carrier dam, and the disease develops when the animals are subjected to some form of stress like transportation. Profound losses in both commercial and research breeders are often inevitable as vaccination (cited by Ruble et al., 1999) and antimicrobial therapy have been found to be largely ineffective (Gaertner, 1991; Mahler et al., 1995). Hence the detection of *P. multocida* in clinically healthy rabbit colonies is important for the control of this disease (Ward, 1973).

Human infections are, in most cases, of animal origin and most often related to bites or scratches by carnivores, though other types of infections have also been reported (Hubbert and Rosen, 1970; Frederiksen, 1993; Liu *et al*., 2003; Christensen *et al*., 2005, Polzhofer *et al*. 2004).

Clearly, diseases caused by *P. multocida* impose a huge economic burden on the livestock industry. This has led to intensive research efforts to understand mechanisms by which this organism invades and causes disease(s). Parallel to this, the desire for rapid diagnostic tests to either complement or substitute traditional methods rose to unprecedented levels. Consequently, a vast amount of literature now exists on diagnostic tests and epidemiology of *P. multocida* and the pathogenetic mechanisms of
this organism are slowly being elucidated. Without doubt, very little has been published on the epidemiology and significance of infections caused by *P. multocida* in developing countries. This review aims at generating preconditions for increased knowledge on these infections in developing as well as developed countries by examining potential diagnostic and typing options that may be adapted for their investigations.

2. Isolation of *P. multocida* from clinical specimen

Though *P. multocida* can grow on basic laboratory media like nutrient agar, blood and chocolate agar are preferentially favourable. In our hands, 5% or 10% bovine or ovine blood agar has consistently yielded reasonable success in isolating *Pasteurella* species (Muhairwa et al., 2000; 2001a; Dziva et al., 2000; 2001). An unquestionable advantage of laboratory isolation is that strains can be collected and archived for further characterisation, confirmation and also for epidemiological studies. Besides, these can also act as vaccine seed strains for the control of respective infections as is the case with autogenous vaccines or bacterins for HS (cited by Verma and Jaiswal, 1998). The source or tissue to be sampled for isolation of *P. multocida* depends on the specific disease. Generally, swabs from the naso-pharynx or tonsillar tissue are most appropriate specimen for isolating *P. multocida* associated with carriage or upper respiratory infections (Lariviere et al., 1993; DeLong and Manning, 1994; de Jong, 1999; Jamaludin et al., 2005). For septicaemic conditions like HS and fowl cholera, heart blood or visceral organs of newly dead animals readily yield pure cultures of *P. multocida* (De Alwis, 1992; Christensen and Bisgaard, 2000; Glisson et al., 2003).
However, if fresh samples from septicaemic diseases cannot be obtained as is often the case in rural areas of developing countries, bone marrow and/or brain may be appropriate for inoculation of blood agar.

Primary isolation of *P. multocida* from clinical specimen may be complicated by overgrowth of other host microflora. Selective culture media generally remove this obstacle and numerous of these have been developed for *P. multocida*. A modified Knight’s medium was described to be the best method for isolating *P. multocida* from nasal cavities of piglets (Lariviere et al., 1993), but there is no evidence of its wider application. Double selective treatment (*Pasteurella multocida* selective enrichment broth; PMSB and selective agar; PMSA) was claimed to be proficient in isolating *P. multocida*, but this tended to reduce the isolation rate of *P. multocida* from both pure and contaminated samples (Moore et al., 1994). Though this selective enrichment procedure provided successful isolation of *P. multocida* from deliberately-infected pond water, testing of PMSA as primary isolation medium for *P. multocida* from suspect avian cholera cases produced a lower detection rate than standard blood agar (Moore et al., 1994). Antimicrobials such as clindamycin, gentamycin, neomycin, amikacin, vancomycin and kanamycin, singly or in combination have been added to agar-based media for isolating *Pasteurella* (Morris, 1958; Smith and Baskerville, 1983; Avril et al., 1990) and yielded inconsistent results. Our own experience with selective media revealed variable success in isolating *P. multocida* ssp. *multocida* from ducks, suggesting that host microflora might influence the selectivity of the media (Muhairwa et al., 2000; 2001a). Comparison of different isolation media by Baldrias et al. (1988) remarkably revealed that conventional sheep blood agar, was by far, the most efficient choice for isolating various species of *Pasteurella* than selective media and mouse inoculation. In addition to blood agar, dextrose starch agar or
trypticase soy agar have recently been recommended for primary isolation
(Christensen and Bisgaard, 2000).

Mouse inoculation selectively enriches *P. multocida* (Lariviere et al., 1993; Muhairwa et al., 2001a) but is not strain specific. Samples from infected animals are inoculated into *Pasteurella*-free mice intraperitoneally, subcutaneously or even intramuscularly. Most strains of *P. multocida* will kill mice within 24-48 hours and pure cultures can be obtained from spleen, liver and heart blood (Chandrasekaran and Yeap, 1982; Muhairwa et al., 2001a). Ability to kill mice may depend on the virulence status of the *P. multocida* strain (Rutter, 1983; Lariviere et al., 1993). Mouse inoculation is most sensitive for surveillance and detection of carrier animals (Christensen and Bisgaard, 2000), but should be disfavored on animal welfare grounds and only used when other methods are not available.

3. Phenotypic identification of *P. multocida*

3.1. Colony and biochemical characteristics

Though the detection of *P. multocida* in clinical specimen can be achieved by rapid alternative tests like PCR, standard phenotypic identification techniques have remained trusted in providing a definitive diagnosis. Following isolation, a presumptive identification of *P. multocida* is often made from growth characteristics on blood agar plates, where pure colonies are round, gray in colour, nonhaemolytic, mucoid or non-mucoid with a typical sweetish smell of indole. However, major variations in colony morphology have been observed for *P. multocida*, some of which are host-related. Mucoid colonies are often obtained from pneumonic lesions in cattle, pigs and rabbits while non-mucoid colonies most often are recovered from poultry. It should be remembered that even V-factor dependent isolates have been reported.
(Krause et al., 1987). A characteristic bipolar staining feature, frequently observed in Gram-stained smears of fresh isolates, is often abolished following serial laboratory subculture. This staining feature is not fully understood, though we can speculate it to be linked to expression of capsule material. It is well-established that serial subculture often results in reduced capsular material (Heddleston et al., 1964) and previously this has been the basis for generating non-capsulated mutants of *P. multocida* (Tsuji and Matsumoto, 1989). By this way, the importance of encapsulation in the virulence of *P. multocida* was earlier established. A presumptive diagnosis of *P. multocida* from cases of fowl cholera has been suggested based on observing colonies on dextrose starch agar using a stereomicroscope with an oblique source of light (Heddleston et al., 1964; Bond et al., 1970). Highly encapsulated colonies, often from clinical specimen, assume an iridescent phenotype whereas those from a serial laboratory passage appear blue or take an intermediate range.

A wide range of biochemical tests are available for a definitive identification of *P. multocida* (see for example Christensen and Bisgaard, 2003), but these are rarely done in most laboratories except in those engaged in an extended phenotypic typing scheme or national culture collection (Christensen et al., 2007). A presumptive diagnosis of *P. multocida* is often made following associating disease syndrome and host and minimal laboratory findings that include growth characteristics, colonial morphology, odour, bipolar staining, positive catalase and oxidase reactions and failure to grow on MacConkey agar. However, this compromise imposes a huge risk of mis-identification. The easiest solution toward a safer identification is to combine initial phenotypic testing with a genotypic test. And to aid in the definitive identification, it is imperative to include reference strains of *P. multocida* and those with a public access are given in Table 1.
3.2. Semi-automated identification systems

Semi-automated identification systems including the analytical profile index (API) system were developed in the 1980s (Collins et al., 1981; Collins and Swanson, 1981; Oberhofer, 1981; Groom et al., 1986), but there appears to be no evidence of their routine use for the identification of *P. multocida*. Though rapid and easy to use, the associated high costs may be prohibiting routine use in most ordinary diagnostic laboratories. Besides, mis-identification of strains appears to be of major concern. Certain biotypes of *Haemophilus influenzae* and *H. parainfluenzae* were identified by the API system as *Pasteurella* species (Hamilton-Miller, 1993). Recently, identification of 40 *Pasteurellaceae* strains to the species level using the API 20NE system was found to be unreliable (Boot et al., 2004). Despite this, there is some evidence on confirmation of *P. multocida* strains by the API 20NE alongside standard sugar fermentation methods (Samuel et al., 2003a).

4. Genotype-based detection and identification of *P. multocida*

4.1. Species-specific PCR

PCR-based methods employ specific primers targeting a conserved gene within the genome. Development of a species-specific PCR was indeed a significant step in the diagnosis of some *P. multocida* infections. Rapid confirmation of suspected cases of pasteurellosis was facilitated. A recently developed 5′ Taq nuclease assay (Corney et al., 2007) promises to be far superior in detecting *P. multocida* in field samples than culture-based methods. Though initially described for detecting *P. multocida* from cases of fowl cholera, the potential for a wider application in diagnosing pasteurellosis in other host species has
been reported (Corney et al., 2007). In addition to type and reference strains of *P. multocida*, isolates from bovine, porcine and avian sources were all detected in the assay. Specificity was confirmed by negative results obtained with 27 other taxa within the *Pasteurellaceae* family and some selected bacterial species and viruses.

Although the newest PCR test (Corney et al., 2007) promises to be the required solution to rapid diagnosis of *P. multocida* infections, earlier PCR tests may also be considered. Information gleaned from genomic subtraction studies enabled identification of a unique chromosomal region, which upon subsequent amplification produced a 460 bp product from all *P. multocida* strains (PM-PCR) tested (Townsend et al., 1998). This test has successfully been used to detect *P. multocida* from tonsils of slaughtered pigs (Townsend et al., 2000). From 36 tonsil swab samples, 16 gave a positive PCR test, whilst 17 strains were eventually isolated. However, *P. multocida* could not be isolated following mouse inoculation of 5 samples that were PCR positive (Townsend et al., 2000) reflecting a higher sensitivity of the PCR test. A modified PM-PCR assay allowed detection of *P. multocida* in intestinal contents of orally infected chickens (Lee et al., 2000), further confirming the potential for this PCR test to substitute culture-based detection methods.

The PCR of Miflin and Blackall (2001) amplified a product of 1,432 bp, which was present in avian and porcine strains of *P. multocida*. The authors recommended the test to accurately diagnose fowl cholera and porcine pasteurellosis, but there appears to be no evidence of its wider application in clinical diagnosis. However, this PCR test gave a positive result with biovar 2 variants of *P. canis* and *P. avium*, which together with results from DNA-DNA hybridizations provided the basis of including these variants under *P. multocida* (Christensen et al., 2004).
A PCR test based on two putative transcriptional regulators (Pm0762 and Pm1231) has recently been described for the detection of \textit{P. multocida} (Liu et al., 2004). These genes appeared to be unique to \textit{P. multocida} and PCR based on these gave products of 567 bp and 601 bp, respectively (Liu et al., 2004). Confirmation of PCR amplicons was achieved by DNA-DNA hybridization or alternatively by PCR-ELISA (Sanchez et al., 2004). These findings suggest that this could be a species-specific detection test for \textit{P. multocida}, but there is no evidence for further applicability.

A PCR assay based on the \textit{pls} (P6-like) gene (PCR-H) was earlier developed (Kasten et al., 1997), but not widely used. The \textit{pls} gene encodes for a protein that is unique to \textit{P. multocida} and \textit{Haemophilus influenzae} and a positive sample gives an amplicon of 453 bp in size. The test can be undertaken with mixed cultures or clinical specimen like pharyngeal swabs from infected birds, thus offering a distinct advantage. However, in a subsequent test, whilst mouse inoculation detected 5 out of six infected flocks, the PCR-H assay only detected 4 of the same six infected flocks (Kasten et al., 1997), probably due to the presence of inhibitors of PCR in the clinical specimen. Though this PCR-H assay seems to be easily adaptable for the confirmation of pasteurellosis, evidence is lacking on its wider application in detecting naturally infected birds.

tRNA-intergenic spacer PCR has been shown to discriminate members of the \textit{Pasteurella sensu stricto} (Catry et al., 2004) by producing specific patterns for each species, but there is lack of evidence of its practical application.

\subsection*{4.2. Disease specific PCRs}

\subsubsection*{4.2.1. PCR methods for PAR}
The *P. multocida* toxin (PMT) is the single most important virulence factor responsible for atrophic rhinitis in pigs. Development of several PCR assays for the detection of toxinogenic *P. multocida* followed identification, successful cloning and sequencing of the entire *toxA* gene (Petersen and Foged, 1989; Lax et al., 1990; Buys et al., 1990). An early PCR assay targeting the *HindIII-HindIII* 1.5 kb region of the *toxA* gene was shown to be highly specific (Nagai et al., 1994), but this appeared not to have been widely used. An alternative PCR assay amplifying a 846 bp fragment of the *toxA* gene was shown to give equally sensitive and specific differentiation of toxinogenic from nontoxinogenic *P. multocida* strains (Lichtensteiger et al., 1996). We and others elsewhere have confirmed the reproducibility of this PCR assay (Amigot et al., 1998; Townsend et al., 2000; Dziva et al., 2004). A nested PCR protocol based on this PCR was reported to offer even a more sensitive and reproducible alternative to the conventional test (Choi and Chae, 2001).

To cater for large-scale screening, a PCR test adaptable to a microtitre plate format was developed (Kamp et al., 1996). The test employed 2 primer sets derived from the *toxA* gene sequence and was validated to be suitable for large scale screening of nasal and tonsillar swabs from clinically affected animals. The advantage of using 2 primers sets was not very obvious. However, it has been suggested that 2 primer sets avoid cross reactions with genes encoding for cytotoxic necrotizing factors which may be found in *E. coli* (cited by Kamp et al., 1996). Consistent with an earlier notion of synergistic contribution of *Bordetella bronchiseptica*, toxinogenic and nontoxinogenic *P. multocida* to PAR in pigs, a multiplex PCR that simultaneously identifies these pathogens has recently been reported (Register and DeJong, 2006). These PCR tests have brought an added advantage for the detection of *P. multocida* in PAR-infected swine herds. A major advantage of these procedures lies in direct
detection of the toxA gene in nasal swabs, thus shortening the diagnostic process. Today, some control and surveillance programmes rely on these successful, rapid, easy and cheap diagnostic PCR assays (cited by de Jong, 1999).

4.2.2. HS PCR

Insights from subtractive hybridization studies provided the basis of a serotype B-specific PCR (Townsend et al., 1998). Primers based on a clone designated KMT1 generated an amplicon of 590 bp from only type B strains irrespective of the somatic antigen type, indicating the specificity of the assay. Recently, another PCR based on information gathered from amplicon patterns generated by 16S-23S rDNA universal primers has been described (Brickell et al., 2002). This region was found to be unique to pathogenic type B:2 strains of P. multocida, thus potentially becoming a diagnostic marker for HS-causative agents in Asia. To the authors’ knowledge type E strains have not yet been associated with HS in Asia. However, it should be notified that capsular type B strains have infrequently been reported from poultry (Rhoades & Rimler, 1987; Jonas et al., 2001).

4.2.3. Fowl cholera PCR

Taking advantage of that hyaluronic acid is the predominant component of the capsule material in capsular type A P. multocida strains, a PCR assay based on a section of the hyaluronic acid encoding region (hyaC-hyaD) has been reported (Townsend et al., 2001) and this gives a 1044 bp DNA product. Recently, a PCR test targeting a shorter region of the same locus gives a 564 bp amplicon (Gautam et al., 2004). Validation of the specificity and sensitivity of this assay was provided by a successful nested PCR designed alongside this initial PCR (Gautam et al., 2004). This
test has been extended to confirm suspected fowl cholera cases in chickens using morbid tissues (Shivachandra et al., 2004). By virtue of targeting a universal component of capsule, these serotype-specific PCR assays are expected to detect all serotype A strains irrespective of the disease condition or host species. However, associations between serotype, diseases and hosts should not be strict since serotype A strains can also cause other diseases in other animals for example snuffles in rabbits and besides, fowl cholera can be caused by capsular types D and F strains that lack this gene.

Serotype 1 is one of the more frequent serotypes associated with fowl cholera in both wild and domesticated birds (Botzler, 1991; Gunawardana et al., 2000). A PCR assay based on a unique 490 bp arbitrarily amplified fragment detected Heddleston serotypes 1 and 14 reference strains (Rocket et al., 2002). Although the assay inevitably gave amplicons in both serotypes 1 and 14, the authors (Rocke et al., 2002) did not consider this to be a drawback since serotype 14 strains are hardly encountered in birds (Botzler, 1991).

4.3. DNA-DNA hybridisation

The first application of this technique for the diagnosis of PAR followed the identification of the toxA gene (Petersen and Foged, 1989; Kamps et al., 1990). Several hybridization probes were evaluated by colony hybridization, but only two (an EcoR1-Xba1 fragment of 2000 bp; a HindIII fragment of 1500bp) of the five probes were considered to be of diagnostic value (Kamps et al., 1990). There is lack of evidence in relation to successful field application of these probes. A fluorescein- or biotin-labelled probe based on the amplified region of toxA (Nagai et al, 1994) was
reported to offer higher sensitivity and specificity (Register et al., 1998). Based on an earlier notion of dual causation of PAR, a two-colour hybridization assay for simultaneous detection of *B. bronchiseptica* and toxinogenic *P. multocida* was developed and subsequently evaluated with 84 primary isolation plates generated from clinical cases of PAR (Register et al., 1998). Direct detection of *P. multocida* on mixed cultures removes the need for purification of colonies which may be time consuming.

A 1,200 bp *Hpa*I fragment from the coding region of adenylate cyclase was shown to specifically hybridise with only *P. multocida* among other *Pasteurella* species, *Actinobacillus ureae* and group EF-4 bacteria (*Neisseria* spp.) (Escande and Crasnier, 1993). Recently, *in situ* hybridization with fluorescent-labelled rRNA has been described and evaluated using tissues from chickens with fowl cholera and deliberately-infected pig lung tissues (Mbuthia et al., 2001). Following sequence comparison of the 16S rRNA, a region that separated *P. multocida* from other members of the *Pasteurellaceae* was identified and labelled with Cy3 or fluorescein. The authors recommended this test to be a supplementary tool for the diagnosis of *P. multocida*.

In essence, amplified products of PCR can easily be converted into probes to allow hybridization studies once conjugated to specific fluorescein dyes or isotopic compounds. With the public availability of the whole genome sequence of *P. multocida* (May et al., 2001), development of new probes and specific PCRs for rapid diagnosis of pasteurellosis is bound to be made easier and faster.

### 4.4. DNA-sequence comparison
The potential of using gene sequence comparison in the identification of *P. multocida* has increased with decreased costs of nucleotide sequencing. DNA sequencing mostly has focused on conserved genes aiming for characterization at species and subspecies levels (Kuhnert *et al.*, 2000; Petersen *et al.*, 2001; Davies *et al.* 2004; Gautier *et al.*, 2005; Kuhnert and Korczak, 2006). Targeting a higher resolution, MLST methods have been developed based on partial sequencing of seven genes of *P. multocida* (*adk, aroA, deoD, gdhA, g6pd, mdh* and *pgi*) (Davies *et al.* 2004). It remains to be shown if sequence types identified correlate with virulence, type of lesions or host association.

5. Antibody-based detection and identification of *P. multocida*

Immunological assays are rarely undertaken for routine diagnosis of infections caused by *P. multocida*. Evidently serological tests are practically valueless in diagnosing rapidly fatal septicaemic forms of pasteurellosis (HS and fowl cholera) and mucosal infections (PAR and snuffles) may be characterized by low level systemic immunity in early stages of infection. Moreover, a positive antibody test should not be interpreted as presence of active infection. Consequently, the majority of serological tests for *P. multocida* can be regarded as research support tools though some have found their way into herd/flock health screening and monitoring schemes.

5.1. Disease specific ELISAs

5.1.1. PAR

As mentioned earlier (4.2.1), differentiation of toxinogenic from nontoxinogenic strains became crucial following identification of *P. multocida* toxin
(PMT) to be a significant mediator of PAR. Monoclonal antibodies against purified PMT allowed development of a sandwich ELISA (Foged et al., 1988) for confirming diagnosis and herd health screening (Foged et al., 1990). Field-based serological surveys identified infected pigs that were also confirmed by culture of nasal swabs and nasal secretions (Foged et al., 1990; Bowersock et al., 1992). Today, a commercially available P. multocida toxin ELISA kit (DAKO, Glostrup, Denmark) is widely used for diagnosis and surveillance of PAR including creation of PAR-free sow herds (cited by de Jong, 1999). A distinct advantage of this test is that the culture does not necessarily have to be purified. However, most laboratories often combine ELISA with toxA PCR (section 4.2.1) thus confirming expression of the gene. This test has also been adapted for the detection of anti-PMT antibodies in sow colostrum in a PAR control program of breeding pigs (Levonen et al., 1996).

Monoclonal antibodies directed at specific outer membrane proteins of porcine P. multocida, designated H and W, have been evaluated for specific identification of type D strains (Marandi and Mittal, 1995). Though capable of specific detection of type D strains by dot-ELISA (Vasfi Marandi et al., 1997), the test failed to distinguish toxin-producing strains which are central to the development of PAR. But peroxidase-labeled monoclonal antibodies against the PMT specifically detected toxigenic P. multocida in primary cultures from experimentally infected gnotobiotic pigs by colony-blotting (Magyar and Rimler, 1991), suggesting a potential use of this assay under field conditions.

5.1.2. HS

To identify HS-causing organisms, an ELISA test using a live or formalin-inactivated suspension of P. multocida was developed (Dawkins et al., 1990).
Regardless of the capsular serogroup associated with HS, the assay was reported to have a specificity of 99% and a sensitivity of at least 86%. The authors asserted that this serodiagnostic tool enables assessment of the impact of HS in endemic countries. A similar technique confirmed the passive transfer of antibodies from HS-vaccinated dams to calves (el-Eragi et al., 2001) and antibody responses in buffaloes (Chandrasekaeran et al., 1994) following vaccination against HS. Recently, an outer membrane protein-based ELISA enabled estimation of the levels of maternally and naturally acquired *P. multocida* antibodies in beef calves (Prado et al., 2006).

5.1.3. Fowl cholera

The need to accurately monitor antibody responses to *P. multocida*-derived vaccines in turkeys gave rise to the development of an ELISA test for fowl cholera (Marshall et al., 1981). Using sonicated whole cells of *P. multocida* as the antigen source, ELISA was shown to be far more superior to previously described agglutination tests (Marshall et al., 1981). Recently, an ELISA using purified bacterial cellular constituents prepared by a cell disrupter (French press), was found to be accurate in estimating the prevalence of *P. multocida* in wild birds (Samuel et al., 1999), though subsequent tests showed little association between ELISA-determined antibody levels and carrier status in waterfowls (Samuel et al., 2003a). Today, a commercial ELISA kit (IDEXX FlockChek, Westbrook, Maine) for the detection of *P. multocida* antibodies is available for large-scale screening of poultry sera. The FlockChek *P. multocida* Antibody Test Kits have been employed in serosurvey studies for pathogens of ostriches (Cadman et al., 1994) and backyard chicken flocks (Kelly et al., 1994). In both instances, the authors identified *P. multocida* to be a prevalent pathogen for the respective birds. The availability of this commercial test is
a huge asset allowing rapid and accurate detection of fowl-cholera-infected flocks. However, when compared with dot immunobinding assay (DIA) in an unrelated study, the ELISA protocol was found to be less specific (Choi et al., 1990). The authors concluded that DIA offered several distinct advantages over ELISA that included more uniform binding of coating antigen, but these findings have not yet been commercially exploited.

5.1.4. Snuffles

Previous work (Marshall et al., 1981) provided the basis for the development of an ELISA test for the detection of *P. multocida* antibodies from infected but clinically healthy rabbits (Klaasen et al., 1985). Despite differences in the nature of the antigen, ELISA-based detection of *P. multocida* in apparently healthy but consistently nasal culture-negative rabbits was confirmed by several independent workers (Holmes et al., 1986; Hwang et al., 1986; Lukas et al., 1987; Zaoutis et al., 1991; Kawamoto et al., 1994). Intriguingly, the majority of these ELISA tests detected antibodies to cross-reacting antigens, thus lacking specificity. Furthermore, some of the assays tended to be serotype-specific, thereby limiting their applicability under field conditions; snuffles is also caused by either serotype A or D (DeLong and Manning, 1994) and potentially serotype F strains (Jaglic et al., 2007). To address these limitations, more sensitive and specific ELISA tests targeting conserved factors among strains associated with snuffles in rabbits have been reported. A 37kDa protein-based capture ELISA (Peterson et al., 1997) and a NanH sialidase-based ELISA (Sanchez et al., 2004) have been commended to be sensitive and specific in detecting infected rabbits, though field applicability remains to be ascertained.
Collectively, these findings suggest that an ELISA-based technique could be an extremely valuable serological tool for the diagnosis of pasteurellosis.

5.2. In situ detection using antibodies

Consistent with the finding that HS ‘carrier animals’ harbour \textit{P. multocida} in their tonsils (De Alwis, 1992), immunoperoxidase and peroxidase anti-peroxidase (PAP) techniques successfully revealed this organism in the organs of naturally and experimentally challenged buffaloes (Horadagoda et al., 1990; 1998). And using an immunohistochemical technique, \textit{P. multocida}-specific staining was demonstrated in the kidneys of pigs that manifested with dermatitis and nephropathy syndrome (Thomson et al., 2001). In a separate study, a modified immunoperoxidase assay was used to diagnose rabbit pasteurellosis (Takashima et al., 2001). Importantly, \textit{in situ} detection is feasible in disease conditions that result in localization of the bacteria, its products or immune complexes. It has been reported that antibody-coated staphylococci could detect soluble group antigen in the plasma and liver extracts of mice experimentally infected with HS strains of \textit{P. multocida}, and that the two serotypes (B and E) could also be differentiated by the same coagglutination test (Rimler, 1978).

5.3. Other antibody detection tests

Potential serological techniques that have not widely been used for diagnosis but typing and epidemiological studies include the; haemagglutination assay (Carter, 1955), indirect haemagglutination (Sawada et al., 1982), agar gel diffusion precipitation (Hedleston \textit{et al.}, 1972), mouse protection assay (Carter, 1964) and
counterimmunoelectrophoresis (Carter and Chengappa, 1981; Chengappa et al., 1986). Employing passive immunization of mice and by indirect haemagglutination and agglutination test (IHAT), naturally acquired antibodies against *P. multocida* types B and E were successfully detected in the sera of calves (Sawada et al., 1985) suggesting a potential use of this test in diagnosis. However, the reliability of this test could be highly questionable. A 23.5% correlation with positive nasal culture results was obtained with IHAT in naturally infected rabbits and no antibodies were demonstrable in experimentally infected rabbits (Kawamoto et al., 1994). It is most probable that the sensitivity of the serological test is dependent on the *P. multocida* antigen used. IHAT employs a crude capsular extract that is coated on fixed sheep red blood cells and may therefore not detect OMP or LPS antibodies which will be detectable when whole cells are used as an antigen source in tests like ELISA.

Counter-immunoelectrophoresis (CIE) appears to be useful as a confirmatory test. It has been successfully applied for the identification of types B, D and E strains of *P. multocida* (Carter and Chengappa, 1981; Chengappa et al., 1986). And when used in conjunction with IHA test, a 100% correlation was observed, presumably due to the nature of the antigen, i.e. crude capsular extract. However, the potential use of this test in routine detection of *P. multocida* appears highly unlikely.

A dot-immunobinding assay using LPS as antigen has also been reported to be efficient in detecting *P. multocida* infection in laboratory rabbits (Manning et al., 1987), but there is lack of its proper assessment.

5.4. Challenges to serological diagnosis

Although serology may be a valuable diagnostic alternative, potential complications arise when the epidemiology of the disease changes. Indeed, in recent
years a decrease in the incidence of type E strains of *P. multocida* in southern African countries has been noted (Lane et al., 1987; Voigts et al., 1997; Dziva et al., 2000). To our knowledge, serotype E has never been reported outside Africa and there is lack of evidence that this serogroup still poses a disease threat in domestic animals. This decline in the incidence of serogroup E has been gathered from few reports linking HS with serotype B in regions previously known to harbour serotype E strains. Surprisingly, this apparent change in the prevalent serotype has attracted very little attention. And perhaps the authors can be given some freedom of postulating some probable reasons for this scenario. Firstly, it is known that many African cattle breeds are of Asian origin brought over years of historical trade between the two continents. It is likely that these cattle brought along capsular serotype B carried in their tonsils, which is now encountered in most recent HS outbreaks in Africa. Improved changes in husbandry systems could have promoted reversion to a gene arrangement that encode for type B capsular antigen, thus leading to the disappearance or low incidence of type E strains. Strictly, no major differences exist between these two HS causing serotypes apart from: i) the capsular antigen (Carter, 1955, 1961), ii) that serotype B strains produce hyaluronidase (Carter and Chengappa, 1991), and iii) the electrophoretic position of one major outer membrane protein (Johnson et al., 1991).

Intriguingly, a parallel change in prevalent serotypes has also been reported in *Avibacterium [Haemophilus] paragallinarum* strains in South Africa (Bragg et al., 1996).

The other hurdle seems to be poor elicitation of systemic immune responses particularly by infections occurring at mucosal surfaces. One such example already discussed is PAR, where it takes up to 3 months for serum detectable levels of antibodies to develop following infection (Levonen et al., 1996). Although sow
colostrum provides a concentrated source of antibody, PAR is predominantly a
disease of growing piglets that are direct targets for diagnosis in cases of outbreaks
rather than sows. However, sow colostrum becomes necessary when establishing
PAR-free sow herds. Following intranasal instillation of purified PMT, van Diemen et
al. (1994) were able to demonstrate significant differences, but weak humoral
responses in piglets, further indicating that serological diagnosis might be
inappropriate for this disease.

6. Alternative detection techniques

Observations that mice and guinea-pigs were extremely susceptible to some
serotypes of \textit{P. multocida} provided the first animal models for pasteurellosis (de Jong
et al., 1980). Intraperitoneal injection of mice with suspected clinical specimen is
often used as a purification procedure for isolating \textit{P. multocida} and for confirmation
of the virulence status of some strains. It has been reported that toxinogenic strains of
\textit{P. multocida} are often lethal for BALB/c mice whilst non-toxinogenic strains may
cause mild disease (Rutter, 1983). Furthermore, toxinogenic strains of \textit{P. multocida}
cause skin necrosis when injected intradermally into guinea-pigs (de Jong et al.,
1980). Use of live animals has huge implications on animal welfare hence the
development of cell-based assays for the detection of toxinogenic \textit{P. multocida}.

Various cell lines have been shown to produce results comparable to data generated
by other alternative tests; mouse inoculation, guinea pig skin test, ELISA and \textit{toxA}
PCR (Rutter and Luther, 1984; Pennings and Storm, 1984; Amigot et al., 1998). The
agar overlay method was reported to shorten the identification of toxinogenic strains
by 48 hours (Chanter et al., 1986). Radiographic examination of the snout, rhinoscopy
and computerized tomography have been described for clinical diagnosis of PAR (cited by de Jong, 1999), but applicability on a wider scale has been hampered by technical difficulties.

Certain serotypes of *P. multocida* exhibit distinctive features which have been exploited for their rapid identification. Serotype B:2 strains from cases of HS produce hyaluronidase that depolymerizes hyaluronic acid found in encapsulated streptococci or type A *P. multocida* (Carter and Chengappa, 1991). Similarly, Type A strains can easily be identified by cross-streaking with a hyaluronidase-producing *Staphylococcus aureus* (Carter and Rundell, 1975). By an unknown mechanism, serotype D strains typically produce a coarse flocculation when acriflavine dye is added to a broth culture (Carter and Subronto, 1973). Additionally, a presumptive identification of non-HS serotypes of *P. multocida* can be obtained by enzymatic digestion with mucopolysaccharidases (Rimler, 1994).

7. Typing methods for *P. multocida*

7.1. Biotyping and macromolecular profiling

Strains from different disease conditions or hosts are often indistinguishable by simple biochemical tests. Biotyping seems to be of little value in epidemiological investigations but remains one of the trusted traditional methods of identifying bacterial species following primary isolation. For *P. multocida*, an extended phenotyping scheme (Bisgaard et al., 1991; Muhairwa et al., 2001a) offers a comprehensive classification technique for this pathogen. In laboratories where resources are limited, five key differentiation sugars described by Biberstein et al. (1991) often yield information that is essential for grouping *P. multocida* into biotypes or subspecies. We have employed these and obtained reasonable success in
differentiating isolates from clinical cases (Muhairwa et al., 2001a; Dziva et al., 2001; 2004). However, the limiting factor in this scheme is the frequent encounter of unassigned biotypes as previously reported by Fegan et al. (1995). Although biotyping remains one of the key phenotypic typing schemes of *P. multocida*, variation in the utilization of sugars often confounds clear strain differentiation.

Outer membrane protein (OMP) profiling offers a relatively quick alternative way to establish relationships between strains. In *P. multocida*, the electrophoretic mobility of 2 outer membrane proteins, designated H and W, provided the basis of typing strains from atrophic rhinitis cases (Lugtenberg et al., 1984). A close association between an OMP profile and pathogenicity as evidenced by the guinea-pig skin test was established (Lugtenberg et al., 1984). Based on the electrophoretic migration of protein H (OmpH), different OMP patterns were demonstrated among capsular serotype strains from various hosts and geographical origins (Vasfi Marandi et al., 1997). OmpH and a heat-modifiable outer membrane protein of *P. multocida* (OmpA) provided another OMP typing scheme. Based on the electrophoretic separation of these 2 major outer membrane proteins and other minor ones, Davies et al. (2003) demonstrated up to 19 OMP types among avian strains of *P. multocida*. However, the variable molecular mass exhibited by OmpA when solubilised at different temperatures (Marandi and Mittal, 1996) suggests that this could not be a consistent typing technique despite a strong correlation between certain capsular types and specific OMP-types (Davies et al., 2003). High resolution OmpA and OmpH profiling of bovine isolates from England and Wales recently revealed no correlation with disease-status and geographic origin (Davies et al., 2004). But electrophoretic protein profiles had previously correlated well with capsular serotype and country of origin (Johnson et al., 1991) suggesting that OMP profiling could still provide a non-
serological technique for identifying HS strains of *P. multocida*. Furthermore, minor variations between field and vaccine strains were reported following OMP typing of B:2 isolates (Tomer et al., 2002). Although classifying strains on the basis of electrophoretic mobility of proteins provides a simple typing alternative, the possibility of unrelated proteins migrating at the same rate should be considered as a potential risk.

Interestingly, lipopolysaccharide (LPS) profiling was reported to correlate well with OMP profiles (Lugtenberg et al., 1984), but a subsequent study revealed different profiles for the 16 Heddeston somatic serotypes (Rimler, 1990).

### 7.2. Serological typing

Over a century, researchers have relied primarily on serological typing to classify and/or identify bacterial strains. The first serological classification of *P. multocida* was based on agglutination and adsorption tests (Cornelius, cited by Rosenbusch and Merchant, 1939) and Khalifa was the first to correlate serological results with the fermentation of some sugars; xylose, arabinose and mannitol (cited by Rosenbusch and Merchant, 1939). Since then, several schemes have been developed for serological and epidemiological studies of *P. multocida* and among them are; specific agglutination, passive haemagglutination, passive protection of mice and agar gel diffusion precipitin tests (reviewed by Rimler & Rhoades, 1987). The typing scheme developed by Carter (1955; 1961) facilitated the first recognized grouping of *P. multocida* into capsular serotypes (A, B, D and E). Decades later, an improved indirect haemagglutination (IHA) assay that employed glutaraldehyde-fixed sheep red cells was developed (Sawada et al., 1982) and identified a fifth capsular serotype F.
In the early days, a trend associating a particular capsular serotype with a specific disease in a distinct host and even geographical location was widely accepted. Typically, serotype B strains were reported to cause HS only in cattle and water buffaloes and restricted to the Asian continent whilst serotype E strains caused HS in Africa (Carter, 1961; Carter and De Alwis, 1989). And despite isolated incidences of acute septicaemic pasteurellosis in pigs due to capsular type B, in India (Murty and Kaushik, 1965) and in Australia (Cameroon et al., 1996), serotype B strains had remained associated with HS in cattle and buffaloes mostly in Asia. However, it is becoming increasingly unsafe to use these associations. Most HS outbreaks in North, Central and Southern Africa are associated with serotype B (Shigidi and Mustafa, 1979; Lane et al., 1991; Martrenchar & Njanpop, 1994; Voigts et al., 1997), previous enclaves for serotype E strains (Carter, 1961; De Alwis, 1992). To further complicate the initial generalizations, capsular serotypes B and D have now been recovered from poultry disease conditions in addition to the usual serotypes A and F (Rhoades and Rimler, 1987; Davies et al., 2003). And in recent years, serotype E strains are hardly isolated from any animal species around the world (Dziva et al., 2000; Ewers et al., 2006). The epidemiology of *P. multocida* is probably changing and in this regard serotyping is not always a good predictor of host-disease relationships.

IHA assay enjoyed immense popularity worldwide and became the gold standard for capsular typing (Rhoades and Rimler, 1987; Rimler and Rhoades, 1987) until the advent of PCR-based typing technique (Townsend et al., 2001). Although capsular distribution in various animals could be investigated, some problems existed in the readily dissociation of the isolates that rendered them untypable. Using a tube agglutination test (Namioka and Murata, 1961a) first demonstrated that a single capsular type could have two somatic antigens and that untypable dissociation
variants still possessed the same somatic antigen as the parent strain. The somatic antigen typing scheme of Namioka and Murata (1961b) recognized only 6 groups, which appeared to offer very restricted differentiation. Further differentiation of somatic serotypes of *P. multocida* is possible with an agar gel diffusion precipitin test (Heddleston et al., 1972) and identifies 16 somatic antigens (designated 1 to 16). To date, no molecular typing technique has substituted this somatic typing scheme. Due to the laborious nature of the test, researchers more than often rely on capsular typing, which tends to correlate well with some infections.

7.3. Genotyping methods

Aiming at tracing outbreak strains or simply sorting of isolates, nucleic acid-based methods are now the cornerstone of typing bacteria. Molecular typing is generally accomplished using; i) restriction enzyme digestion with or without subsequent hybridization with a standard probe (7.3.1) or ii) PCR-based methods (7.3.2), iii) sequencing of multiple loci of predominantly house-keeping genes. The choice of a typing tool depends on available resources. In view of detailed reviews of typing methods for *P. multocida* given elsewhere (Blackall and Miflin, 2000; Christensen and Bisgaard, 2003), we will only give an overview of a selected few including those we have recently employed in our studies.

7.3.1. Restriction enzyme digestion with or without subsequent hybridization with a standard probe

Restriction endonuclease analysis (REA) is based on specific cleavage of DNA by a restriction enzyme (often derived from different bacterial species) providing a basis for typing. Restriction enzyme digestion of chromosomal DNA produces fragments of different sizes which upon electrophoresis generate a specific
pattern that can be visually inspected or computed for analysis. The power of REA was shown when porcine strains *P. multocida* belonging to the same somatic and capsular serotypes were differentiated (Harel et al., 1990), and likewise strains causing atrophic rhinitis in pigs (Gardner et al., 1994). The discriminatory power of REA is dependent upon the restriction enzyme used. In addition to pulse-field gel electrophoresis (PFGE; Boerlin et al., 2000; Liu et al., 2003; Pedersen et al., 2003), REA typing has been one of the most frequently used methods for epidemiological investigations of pasteurellosis (Olson and Wilson, 2001; Samuel et al., 2003b; Weiser et al., 2003; Pedersen et al., 2003).

Ribotyping involves an initial digestion of genomic DNA with a restriction enzyme, followed by transfer onto a nitrocellulose membrane that is reacted with a 16S or 23S rRNA-based probe. Specific patterns generated are compared among the strains under study for similarity and these are dependent on the restriction enzyme applied. Significantly, information on the genetic diversity and population structure within *P. multocida* has been unraveled using this typing tool (Blackall et al., 1998). We have employed this method in characterizing *P. multocida* strains from Zimbabwe (Dziva et al., 2004), Tanzania (Muhairwa et al., 2001a; 2001b) and Denmark (Petersen et al., 1998). However, when compared to RAPD (see below), we observed that ribotyping offered a limited discrimination capability among *P. multocida* strains from cases of atrophic rhinitis. Typically, strains from Zimbabwe clustered with a reference toxinogenic strain from Denmark. The same strains were shown to be distinct when analyzed by RAPD. Indeed, it has been reported that ribotyping findings should be validated by RAPD in epidemiological surveys of *Pasteurella* from animals (Chaslus-Dancla et al., 1996), hence it should continue to provide a complimentary
service to other genotyping methods. The enzymes \textit{HpaII} and \textit{HhaI} have been found most suitable for \textit{P. multocida} (Christensen and Bisgaard, 2000; Rimler, 2000).

7.3.2. \textit{Multiplex PCR typing}

The multiplex PCR capsular typing scheme of Townsend et al. (2001) has remarkably abolished the labour-intensive traditional indirect haemagglutination (IHA) assays. The IHA test depends on the capsular antigen, but \textit{P. multocida} typically loses much of the capsular material when subcultured on ordinary laboratory media. Consequently, relatively low amounts of capsular antigen are extracted from such strains rendering them untypable. The multiplex PCR has abolished such limitations and provides a fast, simple and cheap capsular serotyping scheme. However, it has recently been reported that this approach could not type 6\% of the 48 isolates confirmed as \textit{P. multocida} by a species specific PCR (Jamaludin et al., 2005).

One should also be aware of slight discrepancies between typing results obtained by the passive haemagglutination test and the PCR test (Townsend et al., 2001).

7.3.3. \textit{Random amplification of polymorphic DNA}

Random amplification of polymorphic DNA (RAPD) relies on the polymorphic DNA that can be amplified with arbitrary short primers (8-12 nucleotides) to generate single or multiple amplicons. By resolving on agarose gel, profiles of DNA fragments from strains under study can be compared. We have shown that RAPD to reliably differentiate \textit{P. multocida} strains where ribotyping is unable to do so (Dziva et al., 2004). RAPD is an easy typing tool which requires minimal molecular biology equipment; a PCR machine and agarose gel electrophoresis. Analysis of resolved fragments can be undertaken by visual
inspection, though in some cases it may require a specialized computer programme.  

Another advantage offered by this protocol is that no prior information of the genome sequence is required to design primers.

7.3.4. Other genotyping techniques

Amplified fragment length polymorphism (AFLP) has been used recently for typing *P. multocida* isolates (Amonsin et al., 2002; Moreno et al., 2003) and was shown to provide better resolution than RAPD (Huber et al., 2002).

Repetitive extragenetic palindromic (REP)-PCR was observed to differentiate *P. multocida* isolates from different outbreaks (Gunawardana et al., 2000) and to sort strains into *P. multocida* subsp. *multocida* and *septica* (Chen et al., 2002). Enterobacterial repetitive intergenic consensus (ERIC) – PCR offers another potential typing tool, though there are indications of low discriminatory power when used in *P. multocida* isolates from dogs (Loubinoux et al., 1999).

8. Association between population structure of *P. multocida*, hosts, diseases and different detection systems

With *P. multocida* implicated in a number of diseases that manifest differently in various hosts and also restricted to a geographical region (i.e. capsular type E), it would be sensible to associate population structure, disease, host and possibly detection system. The population structure of *P. multocida* was found to be clonal by multilocus enzyme electrophoresis (MLEE). MLEE and ribotyping showed close relationships between the type-strains of *Past. multocida* subsp. *multocida* and *gallicida*, whereas subsp. *septica* was distantly related to these taxa (Blackall et al.,
1998). These results were confirmed by DNA sequence comparisons (Kuhnert et al., 2000; Petersen et al., 2001; Kuhnert and Korczak, 2006). The population structure of *P. multocida* has not been clearly correlated with specific traits like presence of particular virulence factors, disease patterns or diseases. For example, fowl cholera is normally caused by serotype A of *P. multocida* (Christensen & Bisgaard, 2000), however, one cannot automatically assume that an isolate with capsular type A will cause fowl cholera in poultry. Capsular type A strains can also be recovered from a variety of other hosts and disease conditions. Besides, virulence may vary due to serial subculture on laboratory media (Heddelston et al., 1964).

In this respect, genotypic methods like REA or RAPD considered to provide high level resolution, have not been able to show correlation between serotypic characteristics and genotype (Al-Haddawi et al., 1999; Olson and Wilson, 2001; El-Tayeb et al., 2004). However, a significant association between serotype and RAPD and AFLP has been reported though serotyping provided a lower resolution (Huber et al., 2002). A closer genetic link between the vaccine strain and isolates from vaccinated birds than those from unvaccinated counterparts was established by RAPD and ALFP, but not by serotyping (Huber et al., 2002).

It has already been discussed that associations of capsular type, disease, host and even geographical region no longer hold true (section 7.2). It also remains unclear whether a particular detection system can be associated with disease, host or population structure. Therefore, the use of phenotypic tests and confirmatory genotypic technique(s) remain crucial in establishing a definitive diagnosis of *P. multocida* infections.

9. Conclusions and perspectives
Considerable progress has been made in the development of diagnostic and typing techniques for *P. multocida* strains associated with diseases of economic importance. These have brought some added advantages to the control and surveillance programmes as evidenced with PAR of pigs (cited by De Jong, 1999). A wide spectrum of these diagnostic tests is now available and those commercially exploited have subsequently proved invaluable in offering rapid diagnosis of pasteurellosis. Despite the unquestionable progress, the majority of these still await successful commercial exploitation. Similarly, availability of typing techniques has contributed to determination of the population structures and to taxonomic revisions of *P. multocida* and its related species. Today, studies for epidemiological tracing and population dynamics of endemic strains are easily undertaken. Whereas these successes have been gathered from a vast amount of studies with strains from the developed world, very little information has emerged from the developing world. Striking differences in the husbandry practices between the two worlds exist, and the contributory role of these to the course of infectious disease and epidemiology has been suggested (Madec and Rose, 2003). In recent years, a noteworthy but unexplained decline in the incidence of serotype E strains in Southern Africa has recently been observed. Whether changes in husbandry practices or shortfalls in diagnostic and typing methods play contributory roles, is solely speculative. Indeed, this low incidence of type E strains has been deduced from very few studies and recent reports linking HS with serotype B in Central and Southern Africa, where type E strains had previously been prevalent. It is clear that in-depth studies are required to confirm this suspected change in epidemiology of HS strains in Africa. For this and as for other investigations, it is important to isolate many isolates from a wide variety of
sources in the developing world and to archive them by -80 °C freezing or lyophilization for further studies. Comparative studies including identification should always include reference strains (Table 1). In this respect, rapid specific diagnostic tests would play an ingenious role in confirming presumptive isolates as *P. multocida*. We have previously suggested that evidence of phenotypic and genotypic divergence call for the further development of PCR tests and DNA sequencing to document doubtful isolates (Christensen et al., 2005). Further development of definitive typing methods involving DNA sequencing might become feasible with the lower price of such analysis to be foreseen in the near future. Whole genome sequencing of more strains involved in major diseases such HS and PAR are seriously needed to supplement the existing information of strain Pm70 probably representing a small population of *P. multocida* associated disease of chicken.

In conclusion, the ever-ending quest for easy, cheap and rapid diagnostic and typing techniques will continue to breed a challenge for evolving diagnostic technologies for *P. multocida* and other infectious organisms in general. And with further public availability of whole genome sequences of more *P. multocida* strains, the design of new probes and more specific PCRs for rapid diagnosis of pasteurellosis is bound to be made easier and faster.

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Table 1. List of reference strains of *Pasteurella multocida* available from culture collections with public access.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain*</th>
<th>Alternative strain number(s)</th>
<th>Reference strain for</th>
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</thead>
<tbody>
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<td>CCUG25971</td>
<td>X-73, ATCC11039</td>
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<td>CCUG17977&lt;sup&gt;T&lt;/sup&gt;</td>
<td>NCTC10322&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Capsular type A</td>
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<td><em>P. multocida</em> subs. <em>gallicida</em></td>
<td>CCUG17978&lt;sup&gt;T&lt;/sup&gt;</td>
<td>NCTC10204&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Type strain of subspecies gallicida</td>
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<tr>
<td><em>P. multocida</em> subs. <em>septica</em></td>
<td>CCUG17978&lt;sup&gt;T&lt;/sup&gt;</td>
<td>NCTC11995&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Type strain of subspecies septica</td>
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<td><em>P. multocida</em></td>
<td>CCUG26990</td>
<td>NCTC12177</td>
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<td><em>P. multocida</em></td>
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<td><em>P. multocida</em></td>
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Table 2: Genotype- and antibody-based detection methods for *Pasteurella multocida*.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Target gene/antigen</th>
<th>Intended target population</th>
<th>Reference</th>
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<tr>
<td><strong>Genotype</strong></td>
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<td>Conventional PCR</td>
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<td>toxA gene</td>
<td>Porcine</td>
<td>Lichtensteiger et al., 1996</td>
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<td></td>
<td>tRNA-intergenic spacer</td>
<td>All</td>
<td>Catry et al., 2004</td>
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<td>hyaC-hyaD</td>
<td>Avian capsular type A</td>
<td>Gautam et al., 2004</td>
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<td></td>
<td>23S rRNA</td>
<td>All</td>
<td>Miflin &amp; Blackall, 2001</td>
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<td>pls</td>
<td>Avian</td>
<td>Kasten et al., 1997</td>
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<td>Unknown gene</td>
<td>Serotype 1</td>
<td>Rocke et al., 2002</td>
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<td>toxA</td>
<td>Porcine</td>
<td>Nagai et al., 1994</td>
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<td>Pm0762 and Pm1231</td>
<td>All serotypes</td>
<td>Liu et al., 2004</td>
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<td>Townsend et al., 1998</td>
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<td></td>
<td>Adenylate cyclase</td>
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<td>Escande &amp; Crasnier, 1993</td>
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<td>toxA</td>
<td>All</td>
<td>Kämpfe et al., 1996</td>
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<td></td>
<td>16S rRNA-23S rRNA</td>
<td>Serotype B:2</td>
<td>Sanchez et al., 2004</td>
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<td>nanH sialidase</td>
<td>Rabbit</td>
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<td>Nested PCR</td>
<td>toxA</td>
<td>Porcine</td>
<td>Choi &amp; Chae, 2001</td>
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<td>Hybridisation</td>
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<td>Porcine</td>
<td>Kämpfe et al., 1990</td>
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<td>Mbüthia et al., 2003</td>
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<td>Porcine</td>
<td>Register et al., 1998</td>
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<td>16S rRNA</td>
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<td>Corney et al., 2007</td>
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<td>5’ Taq nuclease assay</td>
<td>sodA</td>
<td>All</td>
<td>Gautier et al., 2005</td>
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<td>Sequencing</td>
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<td>Kühnert et al., 2000</td>
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<td>recN</td>
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<td>Kühnert &amp; Korczak., 2006</td>
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<tr>
<td><strong>Antibody-based</strong></td>
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<td>ELISA</td>
<td>PMT toxin</td>
<td>Porcine</td>
<td>Foged et al., 1990</td>
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<td>ELISA kit (commercial)</td>
<td>PMT toxin</td>
<td>Porcine</td>
<td>Dako, Glostrup, DK</td>
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<td></td>
<td>Whole cell lysate</td>
<td>Avian</td>
<td>Samuel et al., 1999</td>
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<td>Potassium thiocyanate extract</td>
<td>Rabbit</td>
<td>Lukas et al., 1987</td>
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<td>Boiled cell extract</td>
<td>Rabbit</td>
<td>Klaasen et al., 1985</td>
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<td>37kDa outer membrane protein</td>
<td>Rabbit</td>
<td>Peterson et al., 1997</td>
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<td>NanH sialidase</td>
<td>Rabbit</td>
<td>Sanchez et al., 2004</td>
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<tr>
<td></td>
<td>Outer membrane proteins</td>
<td>Bovine</td>
<td>Prado et al., 2006</td>
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<td></td>
<td>Killed whole cells</td>
<td>HS</td>
<td>Dawkins et al., 1990</td>
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<tr>
<td>FlockChek (commercial)</td>
<td>Soluble antigen</td>
<td>Avian</td>
<td>IDEXX, Westbrook, Maine</td>
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<td>Indirect haemagglutination assay</td>
<td>Crude capsular extract</td>
<td>HS strains</td>
<td>Sawada et al., 1985</td>
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<tr>
<td>Dot immunobinding assay</td>
<td>Heat-stable antigen, crude capsular extract, whole cell, formalin extract</td>
<td>Avian</td>
<td>Choi et al., 1990</td>
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<td>Mouse protection assay</td>
<td>Capsular extract</td>
<td>All serotypes</td>
<td>Carter, 1964</td>
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**In situ detection**
<table>
<thead>
<tr>
<th>Technique</th>
<th>Sample Type</th>
<th>Antigen</th>
<th>Reference</th>
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<tr>
<td>Immunohistochemical staining</td>
<td>Whole cells</td>
<td>Porcine</td>
<td>Thomson et al., 2001</td>
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<td>Whole cells</td>
<td>Rabbit</td>
<td>Takashima et al., 2001</td>
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<td>Peroxidase anti-peroxidase</td>
<td>Whole cells attached to calf fibrin</td>
<td>HS</td>
<td>Horadagoda et al., 1990; 1998</td>
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<td>Coagglutination</td>
<td>Soluble antigen</td>
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<td>Rimler, 1978</td>
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