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

3

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5

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1 **Abstract**

2

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

23

24

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

26

1 1. Introduction

2

3 The genus *Pasteurella* has recently been outlined (Mutters et al., 2005; Anon,
4 2007) and the taxonomical position of *P. multocida* has been defined (Mutters et al.,
5 2005, Christensen and Bisgaard, 2003; Christensen et al., 2005). *P. multocida* is the
6 type species of the genus including the three subspecies; *P. multocida* subsp.
7 *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica*. In addition
8 to *P. multocida*, *Pasteurella sensu stricto* also includes the species; *P. canis*, *P.*
9 *stomatis*, *P. dagmatis* and the unnamed taxon *Pasteurella* species B and two new
10 species-like taxa related to *P. multocida* (Christensen et al., 2005).

11 The pathogenic potential of *P. multocida* in vertebrate animals was recognized
12 over a century ago and infections are broadly termed pasteurelloses. *P. multocida*
13 infects a wide range of animal hosts causing specific infections that manifest
14 differently. Indeed, *P. multocida* has a broad host range, but this peculiar property is
15 poorly understood. Potential virulence factors of *P. multocida* have recently been
16 reviewed (Hunt et al., 2000; Christensen and Bisgaard, 2000; 2003, Harper et al.,
17 2006) but no host-specific factors have been identified as yet.

18 The major diseases of economic significance include porcine progressive
19 atrophic rhinitis (PAR; de Jong 1999), haemorrhagic septicaemia (HS) of cattle and
20 water buffaloes (De Alwis, 1992), fowl cholera of poultry (Christensen and Bisgaard,
21 2000) and snuffles in rabbits (DeLong and Manning, 1994). These infections can vary
22 from slow or latent infections observed with PAR to rapidly developing fatal
23 septicaemias seen with fowl cholera and HS. Additionally, *P. multocida* also plays a
24 significant role in increasing the severity of primary lung lesions in pigs (Pijoan,
25 1999) and ruminants (Frank, 1989) caused by other pathogens, though little is known

1 of the pathogenesis of these infections. Like any other diseases, clinical signs may be
2 suggestive of the aetiology, but obtaining a definitive diagnosis provides essential
3 guidance for effective treatment and for instituting successful control measures. In
4 this respect, diagnostic tests play a pivotal role in confirming clinical cases and in
5 detecting healthy carriers or reservoirs of infection(s). Healthy carriers or latent
6 infections are common to all *P. multocida* infections and play a significant role in the
7 epidemiology of these infections.

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

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

23 Fowl cholera, is another significant septicaemic and worldwide disease of
24 severe economic importance (Christensen & Bisgaard, 2000). Both healthy carrier
25 birds within a flock and infected wild birds can act as sources of infection (Glisson et

1 al., 2003). In peracute or acute cases, chemotherapy may provide limited success,
2 hence rapid detection and subsequent elimination of reservoir birds interrupts the
3 transmission cycle (Glisson et al., 2003).

4 Snuffles, a highly contagious pasteurellosis of rabbits primarily affects the
5 upper respiratory tract with potential fatal consequences (DeLong and Manning,
6 1994). Rabbits often get colonized with *P. multocida* for long durations without
7 clinical signs, and the prevalence of this organism in clinically healthy animals has
8 been estimated to range from 20 to 90% depending on the detection method employed
9 (cited by Sanchez et al., 2004). Infection is often acquired from a carrier dam, and the
10 disease develops when the animals are subjected to some form of stress like
11 transportation. Profound losses in both commercial and research breeders are often
12 inevitable as vaccination (cited by Ruble et al., 1999) and antimicrobial therapy have
13 been found to be largely ineffective (Gaertner, 1991; Mahler et al., 1995). Hence the
14 detection of *P. multocida* in clinically healthy rabbit colonies is important for the
15 control of this disease (Ward, 1973).

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

20 Clearly, diseases caused by *P. multocida* impose a huge economic burden on
21 the livestock industry. This has led to intensive research efforts to understand
22 mechanisms by which this organism invades and causes disease(s). Parallel to this, the
23 desire for rapid diagnostic tests to either complement or substitute traditional methods
24 rose to unprecedented levels. Consequently, a vast amount of literature now exists on
25 diagnostic tests and epidemiology of *P. multocida* and the pathogenetic mechanisms of

1 this organism are slowly being elucidated. Without doubt, very little has been published
2 on the epidemiology and significance of infections caused by *P. multocida* in
3 developing countries. This review aims at generating preconditions for increased
4 knowledge on these infections in developing as well as developed countries by
5 examining potential diagnostic and typing options that may be adapted for their
6 investigations.

7

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

9

10 Though *P. multocida* can grow on basic laboratory media like nutrient agar,
11 blood and chocolate agar are preferentially favourable. In our hands, 5% or 10%
12 bovine or ovine blood agar has consistently yielded reasonable success in isolating
13 *Pasteurella* species (Muhairwa et al., 2000; 2001a; Dziva et al., 2000; 2001). An
14 unquestionable advantage of laboratory isolation is that strains can be collected and
15 archived for further characterisation, confirmation and also for epidemiological
16 studies. Besides, these can also act as vaccine seed strains for the control of respective
17 infections as is the case with autogenous vaccines or bacterins for HS (cited by Verma
18 and Jaiswal, 1998).

19 The source or tissue to be sampled for isolation of *P. multocida* depends on the
20 specific disease. Generally, swabs from the naso-pharynx or tonsillar tissue are most
21 appropriate specimen for isolating *P. multocida* associated with carriage or upper
22 respiratory infections (Lariviere et al., 1993; DeLong and Manning, 1994; de Jong,
23 1999; Jamaludin et al., 2005). For septicaemic conditions like HS and fowl cholera,
24 heart blood or visceral organs of newly dead animals readily yield pure cultures of *P.*
25 *multocida* (De Alwis, 1992; Christensen and Bisgaard, 2000; Glisson et al., 2003).

1 However, if fresh samples from septicaemic diseases cannot be obtained as is often
2 the case in rural areas of developing countries, bone marrow and/or brain may be
3 appropriate for inoculation of blood agar.

4 Primary isolation of *P. multocida* from clinical specimen may be complicated
5 by overgrowth of other host microflora. Selective culture media generally remove this
6 obstacle and numerous of these have been developed for *P. multocida*. A modified
7 Knight's medium was described to be the best method for isolating *P. multocida* from
8 nasal cavities of piglets (Lariviere et al., 1993), but there is no evidence of its wider
9 application. Double selective treatment (*Pasteurella multocida* selective enrichment
10 broth; PMSB and selective agar; PMSA) was claimed to be proficient in isolating *P.*
11 *multocida*, but this tended to reduce the isolation rate of *P. multocida* from both pure
12 and contaminated samples (Moore et al., 1994). Though this selective enrichment
13 procedure provided successful isolation of *P. multocida* from deliberately- infected
14 pond water, testing of PMSA as primary isolation medium for *P. multocida* from
15 suspect avian cholera cases produced a lower detection rate than standard blood agar
16 (Moore et al., 1994). Antimicrobials such as clindamycin, gentamycin, neomycin,
17 amikacin, vancomycin and kanamycin, singly or in combination have been added to
18 agar-based media for isolating *Pasteurella* (Morris, 1958; Smith and Baskerville,
19 1983; Avril et al., 1990) and yielded inconsistent results. Our own experience with
20 selective media revealed variable success in isolating *P. multocida ssp. multocida*
21 from ducks, suggesting that host microflora might influence the selectivity of the
22 media (Muhairwa et al., 2000; 2001a). Comparison of different isolation media by
23 Baldrias et al. (1988) remarkably revealed that conventional sheep blood agar, was by
24 far, the most efficient choice for isolating various species of *Pasteurella* than selective
25 media and mouse inoculation. In addition to blood agar, dextrose starch agar or

1 trypticase soy agar have recently been recommended for primary isolation
2 (Christensen and Bisgaard, 2000).

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

13

14 **3. Phenotypic identification of *P. multocida***

15 *3.1. Colony and biochemical characteristics*

16 Though the detection of *P. multocida* in clinical specimen can be achieved by
17 rapid alternative tests like PCR, standard phenotypic identification techniques have
18 remained trusted in providing a definitive diagnosis. Following isolation, a
19 presumptive identification of *P. multocida* is often made from growth characteristics
20 on blood agar plates, where pure colonies are round, gray in colour, nonhaemolytic,
21 mucoid or non-mucoid with a typical sweetish smell of indole. However, major
22 variations in colony morphology have been observed for *P. multocida*, some of which
23 are host-related. Mucoid colonies are often obtained from pneumonic lesions in cattle,
24 pigs and rabbits while non-mucoid colonies most often are recovered from poultry. It
25 should be remembered that even V-factor dependent isolates have been reported

1 (Krause et al., 1987). A characteristic bipolar staining feature, frequently observed in
2 Gram-stained smears of fresh isolates, is often abolished following serial laboratory
3 subculture. This staining feature is not fully understood, though we can speculate it to
4 be linked to expression of capsule material. It is well-established that serial subculture
5 often results in reduced capsular material (Heddleston et al., 1964) and previously this
6 has been the basis for generating non-capsulated mutants of *P. multocida* (Tsuji and
7 Matsumoto, 1989). By this way, the importance of encapsulation in the virulence of *P.*
8 *multocida* was earlier established. A presumptive diagnosis of *P. multocida* from
9 cases of fowl cholera has been suggested based on observing colonies on dextrose
10 starch agar using a stereomicroscope with an oblique source of light (Heddleston et
11 al., 1964; Bond et al., 1970). Highly encapsulated colonies, often from clinical
12 specimen, assume an iridescent phenotype whereas those from a serial laboratory
13 passage appear blue or take an intermediate range.

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

1

2 *3.2. Semi-automated identification systems*

3 Semi-automated identification systems including the analytical profile index
4 (API) system were developed in the 1980s (Collins et al., 1981; Collins and Swanson,
5 1981; Oberhofer, 1981; Groom et al., 1986), but there appears to be no evidence of
6 their routine use for the identification of *P. multocida*. Though rapid and easy to use,
7 the associated high costs may be prohibiting routine use in most ordinary diagnostic
8 laboratories. Besides, mis-identification of strains appears to be of major concern.
9 Certain biotypes of *Haemophilus influenzae* and *H. parainfluenzae* were identified by
10 the API system as *Pasteurella* species (Hamilton-Miller, 1993). Recently,
11 identification of 40 *Pasteurellaceae* strains to the species level using the API 20NE
12 system was found to be unreliable (Boot et al., 2004). Despite this, there is some
13 evidence on confirmation of *P. multocida* strains by the API 20NE alongside standard
14 sugar fermentation methods (Samuel et al., 2003a).

15

16 **4. Genotype-based detection and identification of *P. multocida***17 *4.1. Species-specific PCR*

18 PCR-based methods employ specific primers targeting a conserved gene
19 within the genome. Development of a species-specific PCR was indeed a significant
20 step in the diagnosis of some *P. multocida* infections. Rapid confirmation of
21 suspected cases of pasteurellosis was facilitated.

22 A recently developed 5' Taq nuclease assay (Corney et al., 2007) promises to
23 be far superior in detecting *P. multocida* in field samples than culture-based methods.
24 Though initially described for detecting *P. multocida* from cases of fowl cholera, the
25 potential for a wider application in diagnosing pasteurellosis in other host species has

1 been reported (Corney et al., 2007). In addition to type and reference strains of *P.*
2 *multocida*, isolates from bovine, porcine and avian sources were all detected in the
3 assay. Specificity was confirmed by negative results obtained with 27 other taxa
4 within the *Pasteurellaceae* family and some selected bacterial species and viruses.

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

18 The PCR of Mifflin and Blackall (2001) amplified a product of 1,432 bp, which
19 was present in avian and porcine strains of *P. multocida*. The authors recommended
20 the test to accurately diagnose fowl cholera and porcine pasteurellosis, but there
21 appears to be no evidence of its wider application in clinical diagnosis. However, this
22 PCR test gave a positive result with biovar 2 variants of *P. canis* and *P. avium*, which
23 together with results from DNA-DNA hybridizations provided the basis of including
24 these variants under *P. multocida* (Christensen et al., 2004).

1 A PCR test based on two putative transcriptional regulators (Pm0762 and
2 Pm1231) has recently been described for the detection of *P. multocida* (Liu et al.,
3 2004). These genes appeared to be unique to *P. multocida* and PCR based on these
4 gave products of 567 bp and 601 bp, respectively (Liu et al., 2004). Confirmation of
5 PCR amplicons was achieved by DNA-DNA hybridization or alternatively by PCR-
6 ELISA (Sanchez et al., 2004). These findings suggest that this could be a species-
7 specific detection test for *P. multocida*, but there is no evidence for further
8 applicability.

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

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

23

24 4.2. Disease specific PCRs

25 4.2.1. PCR methods for PAR

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

14 To cater for large-scale screening, a PCR test adaptable to a microtitre plate
15 format was developed (Kamp et al., 1996). The test employed 2 primer sets derived
16 from the *toxA* gene sequence and was validated to be suitable for large scale screening
17 of nasal and tonsillar swabs from clinically affected animals. The advantage of using
18 2 primers sets was not very obvious. However, it has been suggested that 2 primer sets
19 avoid cross reactions with genes encoding for cytotoxic necrotizing factors which may
20 be found in *E. coli* (cited by Kamp et al., 1996). Consistent with an earlier notion of
21 synergistic contribution of *Bordetella bronchiseptica*, toxinogenic and non-
22 toxinogenic *P. multocida* to PAR in pigs, a multiplex PCR that simultaneously
23 identifies these pathogens has recently been reported (Register and DeJong, 2006).
24 These PCR tests have brought an added advantage for the detection of *P. multocida* in
25 PAR-infected swine herds. A major advantage of these procedures lies in direct

1 detection of the *tox*A gene in nasal swabs, thus shortening the diagnostic process.
2 Today, some control and surveillance programmes rely on these successful, rapid,
3 easy and cheap diagnostic PCR assays (cited by de Jong, 1999).

4

5 4.2.2. HS PCR

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

17

18 4.2.3. Fowl cholera PCR

19 Taking advantage of that hyaluronic acid is the predominant component of the
20 capsule material in capsular type A *P. multocida* strains, a PCR assay based on a
21 section of the hyaluronic acid encoding region (*hyaC-hyaD*) has been reported
22 (Townsend et al., 2001) and this gives a 1044 bp DNA product. Recently, a PCR test
23 targeting a shorter region of the same locus gives a 564 bp amplicon (Gautam et al.,
24 2004). Validation of the specificity and sensitivity of this assay was provided by a
25 successful nested PCR designed alongside this initial PCR (Gautam et al., 2004). This

1 test has been extended to confirm suspected fowl cholera cases in chickens using
2 morbid tissues (Shivachandra et al., 2004). By virtue of targeting a universal
3 component of capsule, these serotype-specific PCR assays are expected to detect all
4 serotype A strains irrespective of the disease condition or host species. However,
5 associations between serotype, diseases and hosts should not be strict since serotype A
6 strains can also cause other diseases in other animals for example snuffles in rabbits
7 and besides, fowl cholera can be caused by capsular types D and F strains that lack
8 this gene.

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

16

17 4.3. DNA-DNA hybridisation

18

19 The first application of this technique for the diagnosis of PAR followed the
20 identification of the *toxA* gene (Petersen and Foged, 1989; Kamps et al., 1990).
21 Several hybridization probes were evaluated by colony hybridization, but only two
22 (an *EcoR1-Xba1* fragment of 2000 bp; a *HindIII* fragment of 1500bp) of the five
23 probes were considered to be of diagnostic value (Kamps et al., 1990). There is lack
24 of evidence in relation to successful field application of these probes. A fluorescein-
25 or biotin-labelled probe based on the amplified region of *toxA* (Nagai et al, 1994) was

1 reported to offer higher sensitivity and specificity (Register et al., 1998). Based on an
2 earlier notion of dual causation of PAR, a two-colour hybridization assay for
3 simultaneous detection of *B. bronchiseptica* and toxinogenic *P. multocida* was
4 developed and subsequently evaluated with 84 primary isolation plates generated
5 from clinical cases of PAR (Register et al., 1998). Direct detection of *P. multocida* on
6 mixed cultures removes the need for purification of colonies which may be time
7 consuming.

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

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

23

24 *4.4. DNA-sequence comparison*

1 The potential of using gene sequence comparison in the identification of *P.*
2 *multocida* has increased with decreased costs of nucleotide sequencing. DNA
3 sequencing mostly has focused on conserved genes aiming for characterization at
4 species and subspecies levels (Kuhnert *et al.*, 2000; Petersen *et al.*, 2001; Davies *et al.*
5 2004; Gautier *et al.*, 2005; Kuhnert and Korczak, 2006). Targeting a higher resolution,
6 MLST methods have been developed based on partial sequencing of seven genes of *P.*
7 *multocida* (*adk*, *aroA*, *deoD*, *gdhA*, *g6pd*, *mdh* and *pgi*) (Davies *et al.* 2004). It
8 remains to be shown if sequence types identified correlate with virulence, type of
9 lesions or host association.

10

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

12

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

21

22 *5.1. Disease specific ELISAs*

23 *5.1.1. PAR*

24

25 As mentioned earlier (4.2.1), differentiation of toxinogenic from
nontoxinogenic strains became crucial following identification of *P. multocida* toxin

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

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

22

23 5.1.2. HS

24 To identify HS-causing organisms, an ELISA test using a live or formalin-
25 inactivated suspension of *P. multocida* was developed (Dawkins et al., 1990).

1 Regardless of the capsular serogroup associated with HS, the assay was reported to
2 have a specificity of 99% and a sensitivity of at least 86%. The authors asserted that
3 this serodiagnostic tool enables assessment of the impact of HS in endemic countries.
4 A similar technique confirmed the passive transfer of antibodies from HS-vaccinated
5 dams to calves (el-Eragi et al., 2001) and antibody responses in buffaloes
6 (Chandrasekaeran et al., 1994) following vaccination against HS. Recently, an outer
7 membrane protein-based ELISA enabled estimation of the levels of maternally and
8 naturally acquired *P. multocida* antibodies in beef calves (Prado et al., 2006).

9

10 5.1.3. Fowl cholera

11 The need to accurately monitor antibody responses to *P. multocida*-derived
12 vaccines in turkeys gave rise to the development of an ELISA test for fowl cholera
13 (Marshall et al., 1981). Using sonicated whole cells of *P. multocida* as the antigen
14 source, ELISA was shown to be far more superior to previously described
15 agglutination tests (Marshall et al., 1981). Recently, an ELISA using purified bacterial
16 cellular constituents prepared by a cell disrupter (French press), was found to be
17 accurate in estimating the prevalence of *P. multocida* in wild birds (Samuel et al.,
18 1999), though subsequent tests showed little association between ELISA-determined
19 antibody levels and carrier status in waterfowls (Samuel et al., 2003a). Today, a
20 commercial ELISA kit (IDEXX FlockChek, Westbrook, Maine) for the detection of
21 *P. multocida* antibodies is available for large-scale screening of poultry sera. The
22 FlockChek *P. multocida* Antibody Test Kits have been employed in serosurvey
23 studies for pathogens of ostriches (Cadman et al., 1994) and backyard chicken flocks
24 (Kelly et al., 1994). In both instances, the authors identified *P. multocida* to be a
25 prevalent pathogen for the respective birds. The availability of this commercial test is

1 a huge asset allowing rapid and accurate detection of fowl-cholera-infected flocks.
2 However, when compared with dot immunobinding assay (DIA) in an unrelated
3 study, the ELISA protocol was found to be less specific (Choi et al., 1990). The
4 authors concluded that DIA offered several distinct advantages over ELISA that
5 included more uniform binding of coating antigen, but these findings have not yet
6 been commercially exploited.

7

8 *5.1.4. Snuffles*

9 Previous work (Marshall et al., 1981) provided the basis for the development
10 of an ELISA test for the detection of *P. multocida* antibodies from infected but
11 clinically healthy rabbits (Klaasen et al., 1985). Despite differences in the nature of
12 the antigen, ELISA-based detection of *P. multocida* in apparently healthy but
13 consistently nasal culture-negative rabbits was confirmed by several independent
14 workers (Holmes et al., 1986; Hwang et al., 1986; Lukas et al., 1987; Zaoutis et al.,
15 1991; Kawamoto et al., 1994). Intriguingly, the majority of these ELISA tests
16 detected antibodies to cross-reacting antigens, thus lacking specificity. Furthermore,
17 some of the assays tended to be serotype-specific, thereby limiting their applicability
18 under field conditions; snuffles is also caused by either serotype A or D (DeLong and
19 Manning, 1994) and potentially serotype F strains (Jaglic et al., 2007). To address
20 these limitations, more sensitive and specific ELISA tests targeting conserved factors
21 among strains associated with snuffles in rabbits have been reported. A 37kDa
22 protein-based capture ELISA (Peterson et al., 1997) and a NanH sialidase-based
23 ELISA (Sanchez et al., 2004) have been commended to be sensitive and specific in
24 detecting infected rabbits, though field applicability remains to be ascertained.

1 Collectively, these findings suggest that an ELISA-based technique could be
2 an extremely valuable serological tool for the diagnosis of pasteurellosis.

3

4 5.2. *In situ* detection using antibodies

5

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

20

21 5.3. *Other antibody detection tests*

22 Potential serological techniques that have not widely been used for diagnosis
23 but typing and epidemiological studies include the; haemagglutination assay (Carter,
24 1955), indirect haemagglutination (Sawada et al., 1982), agar gel diffusion
25 precipitation (Heddleston *et al.*, 1972), mouse protection assay (Carter, 1964) and

1 counterimmunoelectrophoresis (Carter and Chengappa, 1981; Chengappa et al.,
2 1986). Employing passive immunization of mice and by indirect haemagglutination
3 and agglutination test (IHAT), naturally acquired antibodies against *P. multocida*
4 types B and E were successfully detected in the sera of calves (Sawada et al., 1985)
5 suggesting a potential use of this test in diagnosis. However, the reliability of this test
6 could be highly questionable. A 23.5% correlation with positive nasal culture results
7 was obtained with IHAT in naturally infected rabbits and no antibodies were
8 demonstrable in experimentally infected rabbits (Kawamoto et al., 1994). It is most
9 probable that the sensitivity of the serological test is dependent on the *P. multocida*
10 antigen used. IHAT employs a crude capsular extract that is coated on fixed sheep red
11 blood cells and may therefore not detect OMP or LPS antibodies which will be
12 detectable when whole cells are used as an antigen source in tests like ELISA.

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

19 A dot-immunobinding assay using LPS as antigen has also been reported to be
20 efficient in detecting *P. multocida* infection in laboratory rabbits (Manning et al.,
21 1987), but there is lack of its proper assessment.

22

23 5.4. Challenges to serological diagnosis

24 Although serology may be a valuable diagnostic alternative, potential
25 complications arise when the epidemiology of the disease changes. Indeed, in recent

1 years a decrease in the incidence of type E strains of *P. multocida* in southern African
2 countries has been noted (Lane et al., 1987; Voigts et al., 1997; Dziva et al., 2000). To
3 our knowledge, serotype E has never been reported outside Africa and there is lack of
4 evidence that this serogroup still poses a disease threat in domestic animals. This
5 decline in the incidence of serogroup E has been gathered from few reports linking
6 HS with serotype B in regions previously known to harbour serotype E strains.
7 Surprisingly, this apparent change in the prevalent serotype has attracted very little
8 attention. And perhaps the authors can be given some freedom of postulating some
9 probable reasons for this scenario. Firstly, it is known that many African cattle breeds
10 are of Asian origin brought over years of historical trade between the two continents.
11 It is likely that these cattle brought along capsular serotype B carried in their tonsils,
12 which is now encountered in most recent HS outbreaks in Africa. Improved changes
13 in husbandry systems could have promoted reversion to a gene arrangement that
14 encode for type B capsular antigen, thus leading to the disappearance or low incidence
15 of type E strains. Strictly, no major differences exist between these two HS causing
16 serotypes apart from; i) the capsular antigen (Carter, 1955, 1961), ii) that serotype B
17 strains produce hyaluronidase (Carter and Chengappa, 1991), and iii) the
18 electrophoretic position of one major outer membrane protein (Johnson et al., 1991).
19 Intriguingly, a parallel change in prevalent serotypes has also been reported in
20 *Avibacterium [Haemophilus] paragallinarum* strains in South Africa (Bragg et al.,
21 1996).

22 The other hurdle seems to be poor elicitation of systemic immune responses
23 particularly by infections occurring at mucosal surfaces. One such example already
24 discussed is PAR, where it takes up to 3 months for serum detectable levels of
25 antibodies to develop following infection (Levonen et al., 1996). Although sow

1 colostrum provides a concentrated source of antibody, PAR is predominantly a
2 disease of growing piglets that are direct targets for diagnosis in cases of outbreaks
3 rather than sows. However, sow colostrum becomes necessary when establishing
4 PAR-free sow herds. Following intranasal instillation of purified PMT, van Diemen et
5 al. (1994) were able to demonstrate significant differences, but weak humoral
6 responses in piglets, further indicating that serological diagnosis might be
7 inappropriate for this disease.

8

9 **6. Alternative detection techniques**

10

11 Observations that mice and guinea-pigs were extremely susceptible to some
12 serotypes of *P. multocida* provided the first animal models for pasteurellosis (de Jong
13 et al., 1980). Intraperitoneal injection of mice with suspected clinical specimen is
14 often used as a purification procedure for isolating *P. multocida* and for confirmation
15 of the virulence status of some strains. It has been reported that toxinogenic strains of
16 *P. multocida* are often lethal for BALB/c mice whilst non-toxinogenic strains may
17 cause mild disease (Rutter, 1983). Furthermore, toxinogenic strains of *P. multocida*
18 cause skin necrosis when injected intradermally into guinea-pigs (de Jong et al.,
19 1980). Use of live animals has huge implications on animal welfare hence the
20 development of cell-based assays for the detection of toxinogenic *P. multocida*.
21 Various cell lines have been shown to produce results comparable to data generated
22 by other alternative tests; mouse inoculation, guinea pig skin test, ELISA and *toxA*
23 PCR (Rutter and Luther, 1984; Pennings and Storm, 1984; Amigot et al., 1998). The
24 agar overlay method was reported to shorten the identification of toxinogenic strains
25 by 48 hours (Chanter et al., 1986). Radiographic examination of the snout, rhinoscopy

1 and computerized tomography have been described for clinical diagnosis of PAR
2 (cited by de Jong, 1999), but applicability on a wider scale has been hampered by
3 technical difficulties.

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

14

15 **7. Typing methods for *P. multocida***

16 *7.1. Biotyping and macromolecular profiling*

17 Strains from different disease conditions or hosts are often indistinguishable
18 by simple biochemical tests. Biotyping seems to be of little value in epidemiological
19 investigations but remains one of the trusted traditional methods of identifying
20 bacterial species following primary isolation. For *P. multocida*, an extended
21 phenotyping scheme (Bisgaard et al., 1991; Muhairwa et al., 2001a) offers a
22 comprehensive classification technique for this pathogen. In laboratories where
23 resources are limited, five key differentiation sugars described by Biberstein et al.
24 (1991) often yield information that is essential for grouping *P. multocida* into biotypes
25 or subspecies. We have employed these and obtained reasonable success in

1 differentiating isolates from clinical cases (Muhairwa et al., 2001a; Dziva et al., 2001;
2 2004). However, the limiting factor in this scheme is the frequent encounter of
3 unassigned biotypes as previously reported by Fegan et al. (1995). Although biotyping
4 remains one of the key phenotypic typing schemes of *P. multocida*, variation in the
5 utilization of sugars often confounds clear strain differentiation.

6 Outer membrane protein (OMP) profiling offers a relatively quick alternative
7 way to establish relationships between strains. In *P. multocida*, the electrophoretic
8 mobility of 2 outer membrane proteins, designated H and W, provided the basis of
9 typing strains from atrophic rhinitis cases (Lugtenberg et al., 1984). A close
10 association between an OMP profile and pathogenicity as evidenced by the guinea-pig
11 skin test was established (Lugtenberg et al., 1984). Based on the electrophoretic
12 migration of protein H (OmpH), different OMP patterns were demonstrated among
13 capsular serotype strains from various hosts and geographical origins (Vasfi Marandi
14 et al., 1997). OmpH and a heat-modifiable outer membrane protein of *P. multocida*
15 (OmpA) provided another OMP typing scheme. Based on the electrophoretic
16 separation of these 2 major outer membrane proteins and other minor ones, Davies et
17 al. (2003) demonstrated up to 19 OMP types among avian strains of *P. multocida*.
18 However, the variable molecular mass exhibited by OmpA when solubilised at
19 different temperatures (Marandi and Mittal, 1996) suggests that this could not be a
20 consistent typing technique despite a strong correlation between certain capsular types
21 and specific OMP-types (Davies et al., 2003). High resolution OmpA and OmpH
22 profiling of bovine isolates from England and Wales recently revealed no correlation
23 with disease-status and geographic origin (Davies et al., 2004). But electrophoretic
24 protein profiles had previously correlated well with capsular serotype and country of
25 origin (Johnson et al., 1991) suggesting that OMP profiling could still provide a non-

1 serological technique for identifying HS strains of *P. multocida*. Furthermore, minor
2 variations between field and vaccine strains were reported following OMP typing of
3 B:2 isolates (Tomer et al., 2002). Although classifying strains on the basis of
4 electrophoretic mobility of proteins provides a simple typing alternative, the
5 possibility of unrelated proteins migrating at the same rate should be considered as a
6 potential risk.

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

10

11 7.2. Serological typing

12

13 Over a century, researchers have relied primarily on serological typing to
14 classify and/or identify bacterial strains. The first serological classification of *P.*
15 *multocida* was based on agglutination and adsorption tests (Cornelius, cited by
16 Rosenbusch and Merchant, 1939) and Khalifa was the first to correlate serological
17 results with the fermentation of some sugars; xylose, arabinose and mannitol (cited by
18 Rosenbusch and Merchant, 1939). Since then, several schemes have been developed
19 for serological and epidemiological studies of *P. multocida* and among them are;
20 specific agglutination, passive haemagglutination, passive protection of mice and agar
21 gel diffusion precipitin tests (reviewed by Rimler & Rhoades, 1987). The typing
22 scheme developed by Carter (1955; 1961) facilitated the first recognized grouping of
23 *P. multocida* into capsular serotypes (A, B, D and E). Decades later, an improved
24 indirect haemagglutination (IHA) assay that employed glutaraldehyde-fixed sheep red
25 cells was developed (Sawada et al., 1982) and identified a fifth capsular serotype F

1 (Rimler & Rhoades, 1987). In the early days, a trend associating a particular capsular
2 serotype with a specific disease in a distinct host and even geographical location was
3 widely accepted. Typically, serotype B strains were reported to cause HS only in
4 cattle and water buffaloes and restricted to the Asian continent whilst serotype E
5 strains caused HS in Africa (Carter, 1961; Carter and De Alwis, 1989). And despite
6 isolated incidences of acute septicaemic pasteurellosis in pigs due to capsular type B,
7 in India (Murty and Kaushik, 1965) and in Australia (Cameroon et al., 1996), serotype
8 B strains had remained associated with HS in cattle and buffaloes mostly in Asia.
9 However, it is becoming increasingly unsafe to use these associations. Most HS
10 outbreaks in North, Central and Southern Africa are associated with serotype B
11 (Shigidi and Mustafa, 1979; Lane et al., 1991; Martrenchar & Njanpop, 1994; Voigts
12 et al., 1997), previous enclaves for serotype E strains (Carter, 1961; De Alwis, 1992).
13 To further complicate the initial generalizations, capsular serotypes B and D have now
14 been recovered from poultry disease conditions in addition to the usual serotypes A
15 and F (Rhoades and Rimler, 1987; Davies et al., 2003). And in recent years, serotype
16 E strains are hardly isolated from any animal species around the world (Dziva et al.,
17 2000; Ewers et al., 2006). The epidemiology of *P. multocida* is probably changing and
18 in this regard serotyping is not always a good predictor of host-disease relationships.

19 IHA assay enjoyed immense popularity worldwide and became the gold
20 standard for capsular typing (Rhoades and Rimler, 1987; Rimler and Rhoades, 1987)
21 until the advent of PCR-based typing technique (Townsend et al., 2001). Although
22 capsular distribution in various animals could be investigated, some problems existed
23 in the readily dissociation of the isolates that rendered them untypable. Using a tube
24 agglutination test (Namioka and Murata, 1961a) first demonstrated that a single
25 capsular type could have two somatic antigens and that untypable dissociation

1 variants still possessed the same somatic antigen as the parent strain. The somatic
2 antigen typing scheme of Namioka and Murata (1961b) recognized only 6 groups,
3 which appeared to offer very restricted differentiation. Further differentiation of
4 somatic serotypes of *P. multocida* is possible with an agar gel diffusion precipitin test
5 (Heddleston et al., 1972) and identifies 16 somatic antigens (designated 1 to 16). To
6 date, no molecular typing technique has substituted this somatic typing scheme. Due
7 to the laborious nature of the test, researchers more than often rely on capsular typing,
8 which tends to correlate well with some infections.

9 10 7.3. Genotyping methods

11
12 Aiming at tracing outbreak strains or simply sorting of isolates, nucleic acid-
13 based methods are now the cornerstone of typing bacteria. Molecular typing is
14 generally accomplished using; i) restriction enzyme digestion with or without
15 subsequent hybridization with a standard probe (7.3.1) or ii) PCR-based methods
16 (7.3.2), iii) sequencing of multiple loci of predominantly house-keeping genes. The
17 choice of a typing tool depends on available resources. In view of detailed reviews of
18 typing methods for *P. multocida* given elsewhere (Blackall and Mifflin, 2000;
19 Christensen and Bisgaard, 2003), we will only give an overview of a selected few
20 including those we have recently employed in our studies.

21 22 7.3.1. Restriction enzyme digestion with or without subsequent hybridization with a 23 standard probe

24 Restriction endonuclease analysis (REA) is based on specific cleavage of
25 DNA by a restriction enzyme (often derived from different bacterial species)
26 providing a basis for typing. Restriction enzyme digestion of chromosomal DNA
27 produces fragments of different sizes which upon electrophoresis generate a specific

1 pattern that can be visually inspected or computed for analysis. The power of REA
2 was shown when porcine strains *P. multocida* belonging to the same somatic and
3 capsular serotypes were differentiated (Harel et al., 1990), and likewise strains
4 causing atrophic rhinitis in pigs (Gardner et al., 1994). The discriminatory power of
5 REA is dependent upon the restriction enzyme used. In addition to pulse-field gel
6 electrophoresis (PFGE; Boerlin et al., 2000; Liu et al., 2003; Pedersen et al., 2003),
7 REA typing has been one of the most frequently used methods for epidemiological
8 investigations of pasteurellosis (Olson and Wilson, 2001; Samuel et al., 2003b;
9 Weiser et al., 2003; Pedersen et al., 2003).

10 Ribotyping involves an initial digestion of genomic DNA with a restriction
11 enzyme, followed by transfer onto a nitrocellulose membrane that is reacted with a
12 16S or 23S rRNA-based probe. Specific patterns generated are compared among the
13 strains under study for similarity and these are dependent on the restriction enzyme
14 applied. Significantly, information on the genetic diversity and population structure
15 within *P. multocida* has been unraveled using this typing tool (Blackall et al., 1998).
16 We have employed this method in characterizing *P. multocida* strains from Zimbabwe
17 (Dziva et al., 2004), Tanzania (Muhairwa et al., 2001a; 2001b) and Denmark
18 (Petersen et al., 1998). However, when compared to RAPD (see below), we observed
19 that ribotyping offered a limited discrimination capability among *P. multocida* strains
20 from cases of atrophic rhinitis. Typically, strains from Zimbabwe clustered with a
21 reference toxinogenic strain from Denmark. The same strains were shown to be
22 distinct when analyzed by RAPD. Indeed, it has been reported that ribotyping findings
23 should be validated by RAPD in epidemiological surveys of *Pasteurella* from animals
24 (Chaslus-Dancla et al., 1996), hence it should continue to provide a complimentary

1 service to other genotyping methods. The enzymes *HpaII* and *HhaI* have been found
2 most suitable for *P. multocida* (Christensen and Bisgaard, 2000; Rimler, 2000).

3

4 7.3.2. Multiplex PCR typing

5 The multiplex PCR capsular typing scheme of Townsend et al. (2001) has
6 remarkably abolished the labour-intensive traditional indirect haemagglutination
7 (IHA) assays. The IHA test depends on the capsular antigen, but *P. multocida*
8 typically loses much of the capsular material when subcultured on ordinary laboratory
9 media. Consequently, relatively low amounts of capsular antigen are extracted from
10 such strains rendering them untypable. The multiplex PCR has abolished such
11 limitations and provides a fast, simple and cheap capsular serotyping scheme.
12 However, it has recently been reported that this approach could not type 6% of the 48
13 isolates confirmed as *P. multocida* by a species specific PCR (Jamaludin et al., 2005).
14 One should also be aware of slight discrepancies between typing results obtained by
15 the passive haemagglutination test and the PCR test (Townsend et al., 2001).

16

17 7.3.3. Random amplification of polymorphic DNA

18 Random amplification of polymorphic DNA (RAPD) relies on the
19 polymorphic DNA that can be amplified with arbitrary short primers (8-12
20 nucleotides) to generate single or multiple amplicons. By resolving on agarose gel,
21 profiles of DNA fragments from strains under study can be compared. We have
22 shown that RAPD to reliably differentiate *P. multocida* strains where ribotyping is
23 unable to do so (Dziva et al., 2004). RAPD is an easy typing tool which requires
24 minimal molecular biology equipment; a PCR machine and agarose gel
25 electrophoresis. Analysis of resolved fragments can be undertaken by visual

1 inspection, though in some cases it may require a specialized computer programme.
2 Another advantage offered by this protocol is that no prior information of the genome
3 sequence is required to design primers.

4

5 7.3.4. Other genotyping techniques

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

9 Repetitive extragenetic palindromic (REP)-PCR was observed to differentiate
10 *P. multocida* isolates from different outbreaks (Gunawardana et al., 2000) and to sort
11 strains into *P. multocida* subsp. *multocida* and *septica* (Chen et al., 2002).

12 Enterobacterial repetitive intergenic consensus (ERIC) – PCR offers another potential
13 typing tool, though there are indications of low discriminatory power when used in *P.*
14 *multocida* isolates from dogs (Loubinoux et al., 1999).

15

16 **8. Association between population structure of *P. multocida*, hosts, diseases and** 17 **different detection systems**

18

19 With *P. multocida* implicated in a number of diseases that manifest differently
20 in various hosts and also restricted to a geographical region (i.e. capsular type E), it
21 would be sensible to associate population structure, disease, host and possibly
22 detection system. The population structure of *P. multocida* was found to be clonal by
23 multilocus enzyme electrophoresis (MLEE). MLEE and ribotyping showed close
24 relationships between the type-strains of *Past. multocida* subspp. *multocida* and
25 *gallicida*, whereas subsp. *septica* was distantly related to these taxa (Blackall *et al.*,

1 1998). These results were confirmed by DNA sequence comparisons (Kuhnert *et al.*,
2 2000; Petersen *et al.*, 2001; Kuhnert and Korczak, 2006). The population structure of
3 *P. multocida* has not been clearly correlated with specific traits like presence of
4 particular virulence factors, disease patterns or diseases. For example, fowl cholera is
5 normally caused by serotype A of *P. multocida* (Christensen & Bisgaard, 2000),
6 however, one cannot automatically assume that an isolate with capsular type A will
7 cause fowl cholera in poultry. Capsular type A strains can also be recovered from a
8 variety of other hosts and disease conditions. Besides, virulence may vary due to
9 serial subculture on laboratory media (Heddelston *et al.*, 1964).

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

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

24

25 **9. Conclusions and perspectives**

1

2 Considerable progress has been made in the development of diagnostic and
3 typing techniques for *P. multocida* strains associated with diseases of economic
4 importance. These have brought some added advantages to the control and
5 surveillance programmes as evidenced with PAR of pigs (cited by De Jong, 1999). A
6 wide spectrum of these diagnostic tests is now available and those commercially
7 exploited have subsequently proved invaluable in offering rapid diagnosis of
8 pasteurellosis. Despite the unquestionable progress, the majority of these still await
9 successful commercial exploitation. Similarly, availability of typing techniques has
10 contributed to determination of the population structures and to taxonomic revisions
11 of *P. multocida* and its related species. Today, studies for epidemiological tracing and
12 population dynamics of endemic strains are easily undertaken. Whereas these
13 successes have been gathered from a vast amount of studies with strains from the
14 developed world, very little information has emerged from the developing world.
15 Striking differences in the husbandry practices between the two worlds exist, and the
16 contributory role of these to the course of infectious disease and epidemiology has
17 been suggested (Madec and Rose, 2003). In recent years, a noteworthy but
18 unexplained decline in the incidence of serotype E strains in Southern Africa has
19 recently been observed. Whether changes in husbandry practices or shortfalls in
20 diagnostic and typing methods play contributory roles, is solely speculative. Indeed,
21 this low incidence of type E strains has been deduced from very few studies and
22 recent reports linking HS with serotype B in Central and Southern Africa, where type
23 E strains had previously been prevalent. It is clear that in-depth studies are required to
24 confirm this suspected change in epidemiology of HS strains in Africa. For this and as
25 for other investigations, it is important to isolate many isolates from a wide variety of

1 sources in the developing world and to archive them by - 80 °C freezing or
2 lyophilization for further studies. Comparative studies including identification should
3 always include reference strains (Table 1). In this respect, rapid specific diagnostic
4 tests would play an ingenious role in confirming presumptive isolates as *P. multocida*.
5 We have previously suggested that evidence of phenotypic and genotypic divergence
6 call for the further development of PCR tests and DNA sequencing to document
7 doubtful isolates (Christensen et al., 2005). Further development of definitive typing
8 methods involving DNA sequencing might become feasible with the lower price of
9 such analysis to be foreseen in the near future. Whole genome sequencing of more
10 strains involved in major diseases such HS and PAR are seriously needed to
11 supplement the existing information of strain Pm70 probably representing a small
12 population of *P. multocida* associated disease of chicken.

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

19

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23

24 **References**

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

Species	Strain*	Alternative strain number(s)	Reference strain for
<i>P. multocida</i>	CCUG25971	X-73, ATCC11039	Somatic type 1
<i>P. multocida</i>	CCUG25973	P1059, ATCC15742	Somatic type 3
<i>P. multocida</i>	CCUG25974	P1662	Somatic type 4
<i>P. multocida</i>	CCUG25975	P1702	Somatic type 5
<i>P. multocida</i>	CCUG25976	P2192	Somatic type 6
<i>P. multocida</i>	CCUG25977	P1997	Somatic type 7
<i>P. multocida</i>	CCUG25978	P1581	Somatic type 8
<i>P. multocida</i>	CCUG25979	P2095	Somatic type 9
<i>P. multocida</i>	CCUG25980	P2100	Somatic type 10
<i>P. multocida</i>	CCUG25981		Somatic type 11
<i>P. multocida</i>	CCUG25982		Somatic type 12
<i>P. multocida</i>	CCUG25983		Somatic type 13
<i>P. multocida</i>	CCUG25984	P2235	Somatic type 14
<i>P. multocida</i>	CCUG25985	P2237	Somatic type 15
<i>P. multocida</i>	CCUG25986		Somatic type 16
<i>P. multocida</i>	CCUG25987	P1059	Capsular type A
<i>P. multocida</i> subs. <i>multocida</i>	ATCC43017	NCTC10323	Capsular type B
<i>P. multocida</i>	CCUG25988	P3881	Capsular type D
<i>P. multocida</i> subs. <i>multocida</i>	ATCC43019	NCTC10325	Capsular type D
<i>P. multocida</i> subs. <i>multocida</i>	ATCC43020	NCTC10326	Capsular type E
<i>P. multocida</i> subs. <i>multocida</i>	CCUG17976 ^T	W9217	Type strain of species
		NCTC10322 ^T	Capsular type A
<i>P. multocida</i> subs. <i>gallicida</i>	CCUG17977 ^T	NCTC10204 ^T	Type strain of subspecies <i>gallicida</i>
<i>P. multocida</i> subs. <i>septica</i>	CCUG17978 ^T	NCTC11995 ^T	Type strain of subspecies <i>septica</i>
<i>P. multocida</i>	CCUG26990	NCTC12177	toxigenic Capsular type A
<i>P. multocida</i>	CCUG26985	NCTC12178	toxigenic Capsular type D
<i>P. multocida</i>	ATCC6530		HS positive

*CCUG (Culture Collection, University of Göteborg, <http://www.ccug.se>),

ATCC, the American Type Culture Collection, Rockville, U. S. A., [http://www.lgcpromochem-](http://www.lgcpromochem-atcc.com/common/catalog/bacteria/bacteriaIndex.cfm)

[atcc.com/common/catalog/bacteria/bacteriaIndex.cfm](http://www.lgcpromochem-atcc.com/common/catalog/bacteria/bacteriaIndex.cfm)), NCTC (National Collection of Type Cultures, London,

<http://www.hpa.org.uk/nctc/searcher.html>).

Table 2: Genotype- and antibody-based detection methods for *Pasteurella multocida*.

Protocol	Target gene/antigen	Intended target population	Reference	
Genotype				
Conventional PCR	Capsular genes	All capsular types	Townsend et al., 2001	
	<i>toxA</i> gene	Porcine	Lichtensteiger et al., 1996	
	tRNA-intergenic spacer	All	Catry et al., 2004	
	<i>hyaC-hyaD</i>	Avian capsular type A	Gautam et al., 2004	
	23S rRNA	All	Miflin & Blackall, 2001	
	<i>pls</i>	Avian	Kasten et al., 1997	
	Unknown gene	Serotype 1	Rocke et al., 2002	
	<i>toxA</i>	Porcine	Nagai et al., 1994	
	<i>Pm0762</i> and <i>Pm1231</i>	All serotypes	Liu et al., 2004	
	Unknown	All serotypes	Townsend et al., 1998	
	Unknown gene	Capsular type B	Townsend et al., 1998	
	Adenylate cyclase	All	Escande & Crasnier, 1993	
	<i>toxA</i>	All	Kamp et al., 1996	
	16S rRNA-23S rRNA	Serotype B:2	Brickell et al., 1998	
	<i>nanH</i> sialidase	Rabbit	Sanchez et al., 2004	
	Nested PCR	<i>toxA</i>	Porcine	Choi & Chae, 2001
		<i>toxA</i>	Porcine	Kamps et al., 1990
Hybridisation	16S rRNA	All	Mbuthia et al., 2003	
	<i>toxA</i>	Porcine	Register et al., 1998	
5' Taq nuclease assay	16S rRNA	All	Corney et al., 2007	
Sequencing	<i>sodA</i>	All	Gautier et al., 2005	
	16S rRNA,	All and separation of subsp. <i>septica</i>	Kuhnert et al., 2000	
	<i>recN</i>	All and separation of subsp. <i>septica</i>	Kuhnert & Korczak., 2006	
Antibody-based				
ELISA	PMT toxin	Porcine	Foged et al., 1990	
ELISA kit (commercial)	PMT toxin	Porcine	Dako, Glostrup, DK	
	Whole cell lysate	Avian	Samuel et al., 1999	
	Potassium thiocyanate extract	Rabbit	Lukas et al., 1987	
	Boiled cell extract	Rabbit	Klaasen et al., 1985	
	37kDa outer membrane protein	Rabbit	Peterson et al., 1997	
	NanH sialidase	Rabbit	Sanchez et al., 2004	
	Outer membrane proteins	Bovine	Prado et al., 2006	
FlockChek (commercial)	Killed whole cells	HS	Dawkins et al., 1990.	
	Soluble antigen	Avian	IDEXX, Westbrook, Maine	
Indirect haemagglutination	Crude capsular extract	HS strains	Sawada et al., 1985	
	Heat-stable antigen, crude capsular extract, whole cell, formalin extract	Avian	Choi et al., 1990	
Dot immunobinding assay	Heat-stable antigen, crude capsular extract, whole cell, formalin extract	Avian	Choi et al., 1990	
Mouse protection assay	Capsular extract	All serotypes	Carter, 1964.	
<i>In situ</i> detection				

Immunohistochemical staining	Whole cells	Porcine	Thomson et al., 2001
Immunohistochemical staining	Whole cells attached to calf fibrin	Rabbit	Takashima et al., 2001
Peroxidase anti-peroxidase	Whole cells	HS	Horadagoda et al., 1990; 1998
Coagglutination	Soluble antigen	All serotypes	Rimler, 1978

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