Pasteurella multocida detection by 5' *Taq* nuclease assay: A new tool for use in diagnosing fowl cholera

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Abstract

A 5' *Taq* nuclease assay utilising minor groove binder technology and targeting the 16S rRNA gene was designed to detect *Pasteurella multocida* (the causative agent of fowl cholera) in swabs collected from poultry. The assay was first evaluated using pure cultures. The assay correctly identified four *P. multocida* taxonomic type strains, 18 *P. multocida* serovar reference strains and 40 Australian field isolates (17 from poultry, 11 from pigs and 12 from cattle). Representatives of nine other *Pasteurella* species, 26 other bacterial species (18 being members of the family *Pasteurellaceae*) and four poultry virus isolates did not react in the assay. The assay detected a minimum of approximately 10 cfu of *P. multocida* per reaction. Of 79 poultry swabs submitted to the laboratory for routine bacteriological culture, 17 were positive in the 5' *Taq* nuclease assay, but only 10 were positive by culture. The other 62 swabs were negative for *P. multocida* by both 5' *Taq* nuclease assay and culture. The assay is suitable for use in diagnosing fowl cholera, is more rapid than bacteriological culture, and may also have application in diagnosing *P. multocida* infections in cattle and pigs.

Keywords: Pasteurella multocida; Fowl cholera; PCR; 5' *Taq* nuclease assay; Minor groove binder.

The Gram negative bacterium *Pasteurella multocida* infects a wide range of animal species, causing diseases such as fowl cholera in poultry (Glisson et al., 2003), haemorrhagic septicaemia and shipping fever in cattle (Carter and De Alwis, 1989; Frank, 1989) and atrophic rhinitis in pigs (Chanter and Rutter, 1989). *P. multocida* infection is generally diagnosed by isolation and identification of the organism from infected tissues (Glisson et al., 2003). This process takes several days and *P. multocida* can remain undetected on culture plates in the presence of heavy loads of other bacteria (Townsend et al., 2000).

The polymerase chain reaction (PCR) is an alternative to bacteriological culture, and has the potential to detect low numbers of a target organism in heavily contaminated samples. Several PCR tests have been described for detecting or identifying the species *P. multocida* (Kasten et al., 1997; Liu et al., 2004; Miflin and Blackall, 2001; Townsend et al., 1998). These PCRs utilise agarose gels to detect specific amplicons. The PCR of Kasten et al. (1997) cross reacted with *Haemophilus influenzae* and used overnight cultures derived from field samples. The species-specific PCR of Townsend et al. (1998) cross reacted with *P. canis* biovar 2, and use on samples from experimentally infected chickens and slaughtered pigs required some form of culture prior to DNA extraction (Lee et al., 2000; Townsend et al., 2000). The PCR of Liu et al. (2004) was validated on a limited number of *P. multocida* isolates, and was tested on pure cultures but not on samples from infected animals. The PCR of Miflin and Blackall (2001) was designed for isolate identification rather than pathogen detection on raw samples, and also cross reacted with *P. canis* biovar 2 and *P. avium* biovar 2. Recently, *P. canis* biovar 2 and *P. avium* biovar 2 were reclassified as *P. multocida* (Christensen et al., 2004). Therefore, the cross reactions reported by Townsend et al. (1998) and Miflin and Blackall (2001) merely reflect the relatedness of these organisms and do not detract from the specificity of these assays.

The 5' *Taq* nuclease assay offers enhanced sensitivity over conventional PCR (Mackay et al., 2002). We report the design and validation of a 5' *Taq* nuclease assay which will detect *P. multocida* strains representing a wide range of genetic diversity and which cross reacts neither with other *Pasteurella* sp. nor with other members of the *Pasteurellaceae*. Furthermore, this assay can be used to detect *P. multocida* in field samples without prior culture. This assay was designed particularly for diagnosing fowl cholera, but would be suitable for diagnosing *P. multocida* infections in other host species with further validation.

The 16S rRNA gene was selected as a potential target for a *P. multocida* species-specific 5' *Taq* nuclease assay. Multiple sequence alignments incorporating 17 16S rRNA gene sequences representing the three recognised subspecies of *P. multocida* (three for subsp. *gallicida,* 13 for subsp. *septica* and one for subsp. *multocida*), one sequence from an atypical *P. multocida* isolate for which the name *P. multocida* subsp. *tigris*

was proposed (Capitini et al., 2002), 11 sequences representing other *Pasteurella* species, three sequences representing *Mannheimia* species, five sequences representing *Avibacterium* species and one representing *Gallibacterium anatis* were constructed using Pileup (Accelerys GCG, San Diego, USA) on the Australian National Genomic Information Service (ANGIS) website (http://angis.org.au). The sequences were obtained from GenBank (Benson et al., 2000). A region of high sequence divergence among the *Pasteurella* species but little or no divergence within *P. multocida* was selected from these alignments for primer and dual-labelled probe design. A 5' *Taq* nuclease assay utilising minor groove binder (MGB) dual-labelled probe technology (Kutyavin et al., 2000) was designed using Primer Express v1.5 (Applied Biosystems, Melbourne, Australia). The primer and probe sequences are as follows:

Forward primer PMA2f, 5'-ATAACTGTGGGAAACTGCAGCTAA-3'

Reverse primer PMA2r, 5'-GGTCCCACCCTTT(A/C)CTCCTC-3'

MGB probe PMA2, 5'-6FAM-CCGCGTA(A/T)TCTCT-MGBNFQ-3'.

Degenerate bases were incorporated in the reverse primer and probe sequences to compensate for minor sequence variations observed among the *P. multocida* 16S sequences. An extract from one of the alignments showing the positions of the primers and probe is presented in Fig. 1, which also illustrates the conserved nature of this region of the 16S rRNA gene in the three recognised *P. multocida* subspecies and in the atypical *P. multocida* subsp. *tigris*, and the divergence of this region across the range of *Pasteurella* and other related species. The two SNPs necessitating the incorporation of degenerate bases in the probe and reverse primer can be seen on this alignment. The resulting amplicon sequence was compared with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). No significant homology with any other sequence was found.

Primer and probe concentrations were optimised by chequerboard titration on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) using default Corbett cycling conditions (15 s at 95 °C, 60 s at 60 °C) for 50 cycles, a threshold value of 0.1 and DNA from an Australian field isolate of *P. multocida* (J2299/5) as template. Reactions were in 25 µl volumes, contained 2 µl of template and RealMasterMix™ Probe mixture (Eppendorf, Hamburg, Germany). A primer concentration of 0.2 µM per primer and a probe concentration of 0.3 µM gave minimum C_T values with maximum normalised fluorescence and were selected for routine use in the 5' *Taq* nuclease assay. All subsequent 5' *Taq* nuclease assays were performed using the same components,

reaction and template volumes and cycling conditions as the titration, and also included a positive control (DNA from J2299/5) and template-free negative control reactions.

The analytical sensitivity of the assay was determined as follows. Growth from an overnight culture of J2299/5 on 5% sheep blood agar was serially diluted in PBS. Colony counts were performed on sheep blood agar. Cells were collected from 1 ml of each dilution by centrifugation for 10 min at 16000 x g and DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Melbourne, Australia) as per the manufacturer's instructions. Each extract was tested in duplicate in the *5' Taq* nuclease assay. The highest dilution yielding a positive result in both duplicates in the 5' *Taq* assay was 10^{-7}, corresponding to 10.7 cfu per reaction. The individual C_T values at this dilution were 36.89 and 35.35, giving a mean C_T of 36.12.

The formal taxonomic type strains for the three *P. multocida* subsp. were used to assess the analytical specificity of the 5' *Taq* nuclease assay – *P. multocida* subsp. *multocida* NCTC 10322, *P. multocida* subsp. *gallicida* NCTC 10204 and *P. multocida* subsp. *septica* CIP A125. As well, reference strains for 16 somatic serovars within the Heddleston typing scheme (Heddleston et al., 1972) were used (strains X73, M1404, P1059, P1662, P1702, P2192, P1997, P1581, P2095, P2100, P908, P1573, P1591, P2225, P2237 and P2723 – serovars 1 to 16 respectively). The reference strains for the Carter capsule typing scheme (Carter, 1955) were also used (P3881, P1235 for capsule types D and E). The reference strains for Heddleston somatic serovars 2 and 3 (M1404 and P1059) also served as the reference strains for Carter capsule serovars B and A respectively. *P. multocida* strain HIM 843, the former reference strain for *P. canis* biovar 2 (Mutters et al., 1985), was also included in this study.

A total of 40 Australian *P. multocida* field isolates were also used: 17 from poultry, 11 from pigs and 12 from cattle. The poultry and porcine isolates were specifically selected to represent the known diversity of *P. multocida* within Australian poultry and pigs based on prior studies using multi-locus enzyme electrophoresis and/or ribotyping (Blackall et al., 1998; Bowles et al., 2000). The bovine isolates were a random selection of Australian field isolates from routine diagnostic submissions.

The following bacteria either taxonomically related to *P. multocida* or likely to be found in field samples submitted for fowl cholera investigations were also used - *Actinobacillus indolicus* (CCUG 39029 ^{T}), *A. minor* (CCUG 38923 ^{T}), *A. pleuropneumoniae* (Shope 4074 ^{T}), *A. porcinus* (CCUG 38924 ^{T}), *A. rossii* (CCUG 12395 ^{T}), *Alcaligenes faecalis* (ATCC 8750 ^{T}), *Avibacterium avium*,(ATCC 29546 ^{T}), *Av. gallinarum* (P287 ^{T}), *Av. paragallinarum* (ATCC 29545^T), *Av. volantium* (NCTC 3438^T), *Av.* species A (HIM 790-5), *Bordetella*

avium (JPDH 591-77^T), *B. bronchiseptica* (ATCC 19395^T), *B. hinzii* (TC 58^T), *Coenonia anatine* (CCUG 46148^T), *Escherichia coli* (Australian avian field isolate), *Gallibacterium anatis* biovar *anatis* (NCTC 11413^T), *Haemophilus parasuis* (CCUG 3712^T), *Histophilus somni* (ATCC 700025^T), *Mannheimia glucosida* (CCUG 38457^T), *M. granulomatis* (CCUG 45422^T), *M. haemolytica* (CCUG 408^T), *M. ruminalis* (CUG 38470^T), *M. varigena* (CCUG 38462^T), *Ornithobacterium rhinotrachealae* (B3263/91, Reference strain for serovar A), *P. aerogenes* (CCUG 9995^T), *P. canis* (NCTC 11621^T), *P. dagmatis* (NCTC 11617^T), *P. langaaensis* (NCTC 11411^T), *P. mairii*,(CCUG 27189 ^{T}), *P. pneumotropica* (BR447), *P. stomatis* (NCTC 11623 ^{T}), *P. trehalosi* (CCUG 27190 ^{T}), *Pasteurella* species B (CCUG 19794) and *Riemerella anatipestifer* (CCUG 21370^T).

Most bacteria were incubated aerobically for one day on sheep blood agar plates at 37 $^{\circ}$C. The exceptions were *Actinobacillus indolicus, A. minor, A. pleuropneumoniae, A. porcinus, Avibacterium paragallinarum, Av. avium, Av. volantium, Avibacterium* species A and *Haemophilus parasuis* which were incubated on BA/SN agar for one day at 37 $^{\circ}$C. Briefly, BA/SN consists of blood agar base (BBL # 211037, Bacto Laboratories, Liverpool, Australia) which is supplemented immediately before pouring with the following sterile additives: 0.0025% NADH, 0.05% thiamine, 1% heat-inactivated horse serum and 5% oleic acid bovine serum albumin complex consisting of 4.75% bovine serum albumin (fraction V) in normal saline (with the normal saline containing 0.06% oleic acid and 5% 0.05N NaOH). The *Avibacterium* species were grown in a candle jar while all other cultures were grown aerobically.

Four avian viruses were also used - *Gallid herpesvirus 1* (SA2, Fort Dodge Animal Health, Sydney, Australia), two Australian field isolates of Avian adenovirus, and one of *Fowlpox virus*. Avian adenoviruses and *Fowlpox virus* were grown on chicken kidney (CK) cells using standard virological techniques (Schat and Purchase, 1989), and cultures were harvested by three cycles of freeze-thawing. *Fowlpox virus* was titrated on CK cells and the titre was calculated as 50% tissue culture infectious doses ($TCID_{50}$) per µl (Reed and Muench, 1938). Adenoviruses were not titrated but the presence of Adenovirus in the virus preparations was confirmed by electron microscopy. *Gallid herpesvirus 1* was used as supplied by Fort Dodge Animal Health (Sydney, Australia) after titration on CK cells.

Bacterial cultures were prepared for DNA extraction as follows. Bacterial growth from solid media was suspended in 1 ml of PBS (pH 7.4) to a concentration approximating a McFarland Equivalence Turbidity Standard No. 5 (Remel, Lenexa, USA) for DNA extraction. The cells were collected by centrifugation for 10 min at 5000 x g. DNA was extracted from the cell pellets and the avian viruses using a QIAamp DNA Mini Kit

as per the manufacturer's instructions. The DNA extracts were tested in duplicate without dilution in the 5' *Taq* nuclease assay. *Gallid herpesvirus 1* DNA corresponding to approximately 1.0 x 10^4 $TCID_{50}$ of virus, and *Fowlpox virus* DNA corresponding to 1.3 x 10^3 $TCID_{50}$ of virus were tested in the 5' *Taq* nuclease assay.

The 5' *Taq* nuclease assay detected all *P. multocida* type and reference strains. Furthermore, all *P. multocida* isolates, regardless of origin - poultry, porcine and bovine - were detected in the assay. All of these strains and isolates gave C_Ts of < 20. None of the 27 other taxa within the family *Pasteurellaceae* nor any of the other bacteria or viruses reacted in the assay. Although *P. multocida* subsp. *tigris* was not tested, the sequence alignments suggest that this organism would react in the 5' *Taq* nuclease assay. Christensen et al. (2005) proposed that this and similar isolates associated with large-cat bite wounds should be reclassified as a new taxon distinct from *P. multocida*. Should this taxon (taxon 45 of Bisgaard) be granted official standing in nomenclature, this organism's theoretical reactivity in the 5' *Taq* nuclease assay will have little bearing on the assay's usefulness as a diagnostic test for fowl cholera or *P. multocida* infections in production animals, as it would not be expected to occur in these animal species. These results demonstrate that the assay has sufficient analytical specificity for use in diagnosing fowl cholera and also its potential for diagnosing *P. multocida* infections in certain nonavian species.

Seventy-nine bacteriological swabs of avian origin (mostly from poultry) were also tested in duplicate in the 5' *Taq* nuclease assay. The swabs were submitted for routine bacteriological culture. The swabs were vortexed for 15 s in 2.5 ml of sterile PBS, and DNA was extracted from 1 ml of the resulting suspension as described for the determination of analytical sensitivity. Seventeen swabs were positive for *P. multocida* in the 5' *Taq* nuclease assay. *P. multocida* was also isolated from 10 of the positive swabs whereas the other seven swabs yielded mixed cultures in which *P. multocida* was not detected. The C_Ts for the 10 culture-positive swabs were in the range 15.56 - 37.9. The C_Ts for the seven culture-negative swabs were 24.54 - 36.09, suggesting the presence of *P. multocida*, which was probably present on the culture plates but obscured by the other bacterial growth and therefore not detected (Townsend et al., 2000). Two of these swabs came from the tracheas of layer hens that had presented with respiratory signs and reduced egg production, one of which was from a flock where fowl cholera had been diagnosed. The others were from the larynxes of 5 poultry which were diagnosed as having infectious laryngotracheitis, suggesting that these birds may have harboured both *P. multocida* and *Gallid herpesvirus 1*. The remaining 62 swabs were negative in the 5' *Taq* nuclease assay and in culture.

The fact that all 10 culture-positive swabs were also positive in the 5' *Taq* nuclease assay suggests that the 5' *Taq* nuclease assay is at least as sensitive as standard bacteriological culture when applied directly to field

samples. The other published PCRs for *P. multocida* species detection or identification either require a culture step (Kasten et al., 1997; Townsend et al., 1998) or have not been validated for direct application to field material (Miflin and Blackall, 2001; Liu et al., 2004).

The highest dilution of *P. multocida* culture that reacted in both of the duplicate 5' *Taq* nuclease reactions produced a mean C_T of 36.12. The field samples either yielded C_Ts of < 38 or did not react. For routine diagnostic use, the assay could be shortened from 50 to 40 cycles without any adverse effect of sensitivity or on discrimination between positive and negative reactions. Samples giving C_Ts ≤ 38 would be considered positive, whereas samples giving higher C_Ts would be considered suspect or negative.

The 5' *Taq* nuclease assay can be completed in less than one day, making it more rapid than bacteriological culture, and facilitates rapid diagnosis of fowl cholera. The recommended response to fowl cholera outbreaks is to quarantine the premises, dispose of the flock and clean and disinfect the premises before restocking (Glisson et al., 2003). The relative speed of the 5' *Taq* nuclease assay would expedite such a response.

In conclusion, the 5' *Taq* nuclease assay is at least as sensitive as and more rapid than standard bacteriological culture, and can be applied directly to field samples for diagnosing fowl cholera.

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Fig. 1.

Alignment of six partial 16S rRNA gene sequences representing the three subspecies of *P. multocida*, one sequence from *P. multocida* subsp. *tigris*, and 20 sequences representing other related bacterial species. GenBank Accession Numbers and species names are listed to the left of the alignment. Base positions are numbered from the start of GenBank Accession #M75051. Bases which differ from the reference sequence (GenBank Accession #AF294410) are shaded. A "period" denotes the absence of a base at any position. Positions of the primers and probe are indicated on the *P. multocida* sequences by boxes. The sequence shown for *P. multocida* subsp. *tigris* (GenBank Accession #AYO57994) is the reverse complement of the sequence as found in GenBank.


141 151 161 171 181 191 201 211 221
AF294410 Pasteurella multocida subsp. multocida
AF294411 P. multocida subsp. septica
AF326325 P. multocida subsp. septica
AF294412 P. multocida subsp. gallicida
AF326323 P. multocida subsp. gallicida
AF326324 P. multocida subsp. gallicida
AY057994 P. multocida subsp. tigris
PMA2f
PMA2 probe
PMA2r
L06089 P. mairii
M75050 P. stomatis
M75049 P. canis
M75051 P. dagmatis
U57073 P. trehalosi
L06088 P. bettyae
M75083 P. pneumotropica
M75053 P. langaaensis
AJ243202 P. skyensis
L06090 P. testudinis
M75058 Avibacterium avium
M75058 Av. avium
M75059 Av. gallinarum
AF487729 Av. gallinarum
M75070 Av. volantium
M75060 Av. volantium
M75054 Gallibacterium anatis
AF053888 Mannheimia glucosida
M75080 M. haemolytica
AF053887 Mannheimia sp.