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1 **Molecular epidemiology of *Pasteurella multocida* in dairy and beef calves**

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16

1 **Abstract**

2 The molecular epidemiology of *Pasteurella multocida* has rarely been studied at the farm
3 level in cattle. The aim of this study was to determine whether single or multiple strains
4 of *P. multocida* tend to exist within farms. Molecular characterisation was carried out on
5 isolates obtained from nasal swabs from 105 calves from 32 randomly selected beef and
6 dairy farms located throughout Scotland, and from 131 calves from 20 farms in the
7 Mayenne region of France, where sampling occurred in response to respiratory disease
8 outbreaks. *P. multocida* isolates were characterised by random-amplified polymorphic
9 DNA (RAPD) typing and pulsed-field gel electrophoresis (PFGE) using restriction
10 enzyme *ApaI*. In addition, isolates representative of each farm/RAPD profile combination
11 were typed by multilocus sequence typing (MLST).

12 Among 105 Scottish isolates, 15 RAPD profiles were distinguished. The majority of
13 farms (27/32) had indistinguishable profiles in all positive animals. Five farms had two
14 profiles. Among 140 French isolates, 23 RAPD profiles were distinguished. More within-
15 farm heterogeneity was observed although 10/20 farms had just one profile (E4) in
16 sampled calves. Profile E4 accounted for 60% (84/140) of French isolates. PFGE was
17 more discriminatory than RAPD but confirmed results with respect to within farm
18 homogeneity or heterogeneity of strains, whereas MLST was not discriminatory enough
19 for farm level epidemiology.

20 As in other host species, either several strains or one dominant strain of *P. multocida* may
21 exist within farms, with evidence for a role of management factors such as movements
22 onto the farm in the number of strains detected.

23

1 **Keywords:** *Pasteurella multocida*, epidemiology, cattle, respiratory, PFGE, RAPD

2

Accepted Manuscript

1 **Introduction**

2 *Pasteurella multocida* may reside in the upper respiratory tract of clinically healthy
3 animals (Dabo et al., 2007; Hotchkiss et al., 2010). It's involvement in the aetiology of
4 bovine respiratory disease (BRD) is increasing, due perhaps to the increased use and
5 efficacy of vaccines against other respiratory pathogens (Autio et al., 2007; Nikunen et
6 al., 2007; Welsh et al., 2004). It is not clear whether disease is due to risk factors that
7 enable resident flora to overwhelm the host immune system or to contagious
8 dissemination of a single virulent clone.

9 Genotypic methods provide tools to investigate the transmission dynamics of *P.*
10 *multocida* at farm and regional levels. Pulsed-field gel electrophoresis (PFGE) and PCR-
11 based methods such as random-amplified polymorphic DNA (RAPD) typing are highly
12 discriminatory and useful for investigations of disease outbreaks (Blackall and Miflin,
13 2000; Dziva et al., 2008). Multilocus sequence typing (MLST) (Subaaharan et al., 2010)
14 has advantages over PFGE and RAPD typing in terms of reproducibility and portability,
15 allowing global analysis of strains. However, the use of housekeeping genes may limit
16 discriminatory power and reduce its utility for investigations of disease outbreaks or in
17 farm level epidemiology.

18 The molecular epidemiology of *P. multocida* at the farm level has rarely been reported in
19 cattle whereas outbreaks of fowl cholera in avian species have been more widely studied
20 (Amonsin et al., 2002; Kardos and Kiss, 2005; Muhairwa et al., 2000; Shivachandra et
21 al., 2005). Outbreaks of pasteurellosis within groups or farms have also been investigated
22 in pigs (Blackall et al., 2000; Marois et al., 2009; Zhao et al., 1992), sheep (Black et al.,
23 1997), buffalo (Biswas et al., 2004) and feedlot calves (Taylor et al., 2010).

1 In this study, we aimed to characterise *P. multocida* isolates present in the upper
2 respiratory tract of calves from a representative selection of Scottish farms and from
3 French calves, sampled as a result of an outbreak of BRD on the farm, to determine
4 whether single or multiple strains of *P. multocida* tend to exist in calves within farms.

6 **Materials and Methods**

7 *Scottish isolates*

8 Nasal swabs were collected from 616 calves less than 10 weeks of age as part of a cross-
9 sectional survey designed to determine carriage of *P. multocida* in 68 randomly selected
10 beef and dairy farms throughout Scotland, February-June 2008 (Hotchkiss et al., 2010).

11 Fresh swab material was plated directly onto selective sheep blood agar (5% citrated
12 sheep blood in agar No.2 base with 10mg/l vancomycin & 10mg/l amphotericin B; E&O
13 Laboratories Ltd). Blood agar plates were incubated overnight at 37°C. Per plate, one
14 colony phenotypically resembling *P. multocida* was subcultured and subjected to PCR for
15 confirmation of species identity (Townsend et al., 1998). All positive isolates were
16 selected for further characterisation.

18 *French isolates*

19 Nasal swabs were collected from a convenience sample of 10 calves (aged 2 weeks – 8
20 months) from each of 20 farms. All farms were suffering from BRD, as characterised by
21 clinical symptoms such as fever, nasal discharge and dyspnoea. Fresh swab material was
22 plated directly onto sheep blood agar, agar containing colimycin and nalidixic acid
23 (Columbia CNA agar with 5% sheep blood; Biomerieux) and Lactose agar with

1 Bromocresol purple (BCP agar; Biomerieux). *P. multocida* was identified on the basis of
2 characteristic colonies on blood agar but no growth on CNA/BCP plates followed by
3 Gram staining, positive catalase and negative urease tests. Only animals with swabs that
4 resulted in abundant *P. multocida* growth were considered positive and only farms with at
5 least 4 out of 10 animals positive for *P. multocida* were considered clinically infected
6 with *P. multocida*. Colonies from infected farms were shipped to Moredun Research
7 Institute (MRI) on swabs in Amies medium (Portagerm; Biomerieux).
8 On arrival at MRI, swabs were vortexed in BHI and cultured overnight, with shaking, at
9 37°C. DNA lysates were prepared by centrifuging 250ul of culture and removing the
10 supernatant, before resuspending the pellet in 200ul of molecular grade water. This was
11 then heated at 95°C for 5 mins and the resulting lysate was used as a template for PCR
12 confirmation of species identity.

13

14 ***Molecular typing - RAPD***

15 An RAPD protocol (Vogel et al., 1999) for Gram-negative organisms was validated for
16 *P. multocida* by ensuring discrimination of archived isolates of distinct sequence types
17 (as determined by MLST) and reproducibility of results. Briefly, 50ng of template,
18 quantified by spectrophotometer, was added to a PCR reaction (total volume 25ul), with
19 GoTaq Green Master Mix (Promega) and 15pmol of primers ERIC-2 (5'-
20 AAGTAAGTGACTGGGGTGAGCG-3') and ERIC-1026 (5'-
21 TACATTCGAGGACCCCTAAGTG-3'). All PCRs were run on the same thermal cycler
22 (G-storm GS1, Gene Technologies Ltd) with cycling conditions of an initial 2 cycles of 5
23 mins at each of 94°C, 35 °C and 72 °C before 31 cycles of 1 min at 94 °C, 1 min at 60 °C

1 and 2 mins at 72 °C, before a final 8 mins at 72 °C. Products were run on 1.5% agarose
2 gels in 1X TAE for 2.5 hours at 100V, with a 100bp molecular weight ladder on either
3 side. For each within-farm comparison, isolates were run in the same PCR reaction and
4 side by side on gels to allow direct comparisons of profiles. Banding patterns were
5 assessed by eye and analysed using the software Bionumerics version 5 (Applied Maths,
6 Sint-Martens-Latem, Belgium). Each grouping of isolates with an indistinguishable
7 banding pattern was assigned a unique RAPD profile number.

8

9 ***Molecular typing – PFGE***

10 PFGE was carried out essentially as described by Lainson et al. (2002). Briefly, agarose
11 plugs were prepared and washed 6 times in TE buffer (10mM Tris-HCl, 1mM EDTA, pH
12 8) before equilibrating in restriction buffer for 1 hour at 30°C. The DNA in the plugs was
13 then digested with 20 units *ApaI* in buffer containing bovine serum albumin (BSA) and
14 incubated at 30°C for 40 hours. The plugs were then loaded onto a 1% agarose gel and
15 sealed. Electrophoresis was carried out at 14°C at 6V/cm with an initial switch time of
16 1.19s increasing to 40s at 23 hours. Gels were stained for 1 hour with SYBR gold
17 (Invitrogen) in TE buffer. They were then rinsed with TE buffer before UV illumination.
18 Relationships between PFGE profiles for all typeable isolates from each population
19 (Scottish or French) were analysed by eye and using Bionumerics. Dendrograms were
20 produced in Bionumerics using Dice coefficient and the unweighted pair group method
21 with arithmetic mean (UPGMA) algorithm. Modified Tenover criteria were used to
22 assign individual restriction profiles (RPs) to main types and subtypes within farms (van
23 Belkum et al., 2007) (isolates with profiles differing by one to four bands should be

1 assigned as subtypes of the same main type). We employed a system of nomenclature
2 whereby subtypes were identified by a suffix to the assigned main type, for example
3 subtypes of main type A45 were A45.1, A45.2 and A45.3.

4

5 ***Molecular typing – MLST***

6 One isolate per RAPD type per farm (n=76, 37 Scottish and 39 French isolates)
7 underwent MLST as per the published protocol (Subaaharan et al., 2010).

8

9 ***Statistical analysis***

10 To compare the discriminatory power of RAPD and PFGE, Simpson's Index of diversity
11 (SID) was calculated (Hunter and Gaston, 1988). The association between numbers of
12 RAPD profiles per farm and both purchase of animals and management system was
13 explored using Fisher's Exact Test.

14

15 **Results**

16 ***Scottish samples***

17 *P. multocida* was confirmed in swabs from 105 animals and 32 farms. An RAPD profile
18 was obtained for all samples analysed (typeability of 100%). Among the 105 isolates (one
19 per animal), 15 RAPD profiles were distinguished. The majority of farms (27/32) had
20 indistinguishable profiles in all positive animals (Figure 1a, Table 1). Five farms had two
21 profiles (Table 1). Most isolates (90/105) belonged to one of five profiles.

22 For PFGE, one isolate could not be recovered from frozen stocks and two isolates proved
23 untypeable by PFGE (typeability of 98%). Therefore, 102 samples were successfully

1 processed by PFGE, producing 36 unique RPs consisting of 30 main types (Figure 2).
2 The finding of homogeneity within farms as indicated by RAPD was supported. One
3 farm which had one RAPD profile showed heterogeneity with PFGE (1/3 isolates was a
4 different main type) (Table 1). As with RAPD, some PFGE profiles (n=5) were shared
5 between farms.

6 Full details of farm type, RAPD, PFGE and MLST profiles can be found in
7 Supplementary Table S1.

8 Simpson's index of diversity (SID) was 81.8% for RAPD and 90.6% for PFGE.

9 There was a significant association between farms that bought in animals and the
10 detection of >1 RAPD profile on the farm (P=0.043). There was also an association
11 between beef farms and the detection of >1 RAPD profile on the farm (P=0.053).

12

13 ***French samples***

14 In total, 165 isolates from 20 farms were subjected to molecular analysis. Of these, 140
15 isolates were confirmed as *P. multocida* and all were typeable by RAPD analysis,
16 producing 23 profiles (typeability 100%). One profile was predominant (E4), accounting
17 for 84 isolates from 15 farms. This profile was also detected on four Scottish farms (13
18 isolates). Two other profiles were shared between farms, whilst the remaining profiles
19 were unique to individual farms. Moderate within-farm heterogeneity was observed
20 (Figure 1b); one profile only was detected on ten farms, two profiles on two farms, 3
21 profiles on 7 farms and 4 profiles on one farm (Table 1).

22 For PFGE, 25/140 isolates were not recovered from frozen stocks and 5/115 proved
23 untypeable despite repeated efforts (typeability of 96%). These missing data included all

1 isolates from one farm, therefore results are available for 19/20 farms. Moderate
2 heterogeneity was found amongst French samples - 71 unique RPs were identified and 54
3 main types (Supplementary Figure S1). However, only four farms had more than three
4 main types in sampled calves (Table 1). Eleven farms had the same number of RAPD and
5 PFGE types (Table 1). In one case, fewer main PFGE types were found than RAPD types
6 (Table 1). In seven farms, more heterogeneity was seen with PFGE (Table 1). Only two
7 PFGE types were found on more than one farm.

8 Full details of farm type, RAPD, PFGE and MLST profiles can be found in
9 Supplementary Table S2.

10 Simpson's index of diversity (SID) was 63.4% for RAPD and 97.6% for PFGE.

11 There was a significant association between farms that bought in animals and the
12 detection of >2 RAPD profiles on the farm ($P=0.019$). The number of beef farms in
13 France was too low for meaningful statistical analysis of an association between farm
14 type and strain heterogeneity.

15

16 *MLST*

17 Five isolates (French) failed to generate an amplicon at 1/7 loci, despite repeated
18 attempts, therefore 20 French RAPD types and all 15 Scottish RAPD types were assigned
19 STs (Supplementary Tables S1 and S2). MLST was less discriminatory than RAPD - 34
20 RAPD types were represented by 13 STs and 22/34 were ST79, ST80 or ST13, all of
21 which are closely related (clonal complex CC13). This clonal complex is highly
22 associated with cattle respiratory isolates (Hotchkiss et al, unpublished data). Ten STs
23 were not associated with CC13 and nine were novel to this study. Absolute concordance

1 occurred between ST123 and RAPD profile E1 (n=7), although definitive relationships
2 between STs and RAPD profiles cannot be determined as only one isolate per RAPD type
3 per farm was subjected to MLST. MLST showed more discrimination than RAPD in one
4 case: RAPD type E16 was represented by both ST125 and ST126. These sequence types
5 are closely related (single locus variants). Due to its low discriminatory power, MLST
6 was considered inappropriate for farm/regional level epidemiology.

8 **Discussion**

9 This study used a PCR fingerprinting protocol (RAPD) and PFGE to examine the
10 diversity at farm level of *P. multocida* collected from the nasal tracts of calves. Diversity
11 at farm level was higher among sampled French than sampled Scottish farms and in both
12 study populations, having multiple profiles was associated with buying in animals. In
13 Scotland, beef farms appeared to harbour more strains of *P. multocida* than dairy farms.
14 However, there was a correlation between beef farms and purchasing animals (P=0.14)
15 and it seems likely that buying in, rather than management system, is the mechanism
16 underlying the observed correlation with strain heterogeneity.

17 ERIC primers (ERIC-1R and ERIC-2) have been used previously for RAPD typing of *P.*
18 *multocida* (Biswas et al., 2004; Kardos and Kiss, 2005; Leotta et al., 2006; Loubinoux et
19 al., 1999; Shivachandra et al., 2005) and the protocol used here typed the isolates with
20 excellent reproducibility and typeability. As described by Leotta et al. (2006), RAPD
21 (using ERIC primers) had higher typeability but lower discriminatory power than PFGE.
22 Assumptions for calculating absolute SID were not met in our study, as samples were not
23 independent, but were clustered into farms (Hunter and Gaston, 1988). However SID was

1 useful as a tool to compare discriminatory power of the two protocols within the study
2 population; no applicability of SID results to the wider cattle population is implied.
3 RAPD analysis is less labour intensive and time consuming to run compared to PFGE.
4 However, relationships between isolates with distinguishable profiles cannot be deduced
5 from RAPD, which is possible to some degree using PFGE and the original criteria of
6 Tenover (Tenover et al., 1995) or modified Tenover criteria (van Belkum et al., 2007).
7 MLST can be used to infer relationships between strains, although where housekeeping
8 genes are selected, the low discriminatory power means it has limited value in disease
9 outbreaks or farm level epidemiology, particularly in clonal organisms such as *P.*
10 *multocida* in cattle, as in the current study.

11 The discriminatory power of methods used should be considered when interpreting
12 results of molecular studies. For example, MLST (and outer membrane protein profiling)
13 detected low levels of diversity in UK BRD cases over many years (Davies et al., 2004),
14 whereas within one feedlot, PCR-fingerprinting techniques detected high levels of
15 diversity in calves that died over the course of one year (Taylor et al., 2010). These
16 results may reflect the characteristics of the typing techniques used as much as the
17 epidemiology of *P. multocida* in cattle.

18 Management factors influence molecular epidemiology at the farm and regional levels.
19 For example, in a duck flock, shown to have a different causative strain of *P. multocida*
20 for each of 4 outbreaks of fowl cholera over a 2 year period (Muhairwa et al., 2000), it
21 was suggested that strains were eliminated by management strategies, such as having
22 animal-free periods within housing, with incursions of novel strains preceding each new
23 disease outbreak. Within a region, strains are sometimes farm specific but may also be

1 shared between owners/premises. Depending on the circumstances, such as distance and
2 relationship between premises, farm to farm transmission has been assigned to wild
3 animals (local transmission between independent farms) or fomite vectors, such as fodder
4 and personnel moving between distant premises within a company (Kardos and Kiss,
5 2005).

6 Movement and introduction of stock appear to be an important factor in the molecular
7 epidemiology of *P. multocida*. In pigs, 4 outbreaks on 4 farms were investigated
8 (Blackall et al., 2000). In two cases, a single strain of *P. multocida* was detected whereas
9 in the other farms, multiple strains were identified. It was hypothesized that this
10 discrepancy was due to higher incidence of animal movements on the units with multiple
11 strains. Heterogeneity of *P. multocida* in disease outbreaks has also been observed in
12 sheep and beef cattle populations that were composed by mixing of animals from
13 different origins, e.g. in sheep, sourced from 20 farms, during transport by sea (Black et
14 al., 1997) and in commercial feedlot cattle that died of fibrinous pleuropneumonia
15 (Taylor et al., 2010). In the latter two papers the authors suggest that disease occurred
16 when a wide range of genetic types in the respiratory tract reacted to risk factors, for
17 example heat stress in the exported sheep. Taylor et al. (2010) also state that their
18 findings may support the hypothesis that *P. multocida* is an opportunistic pathogen. In
19 other words, animals have their own individual strains (which may be very variable, if the
20 animals have come from different sources) which can cause disease in response to risk
21 factors and host stress.

22 In the current study, the diversity seen in the French sample may reflect higher levels of
23 on-farm movements of calves – 8/20 French farms had recently introduced calves

1 compared to 3/32 Scottish farms (Supplementary Table S1). Another difference between
2 the Scottish and French study populations are the ages of calves sampled. The
3 homogeneity found within Scottish farms suggests calf-to-calf (contagious) transmission
4 which is easily understood when considering batches of, almost exclusively, homebred
5 preweaned calves. Conversely, the heterogeneity seen within French farms suggests
6 calves have acquired *P. multocida* from multiple sources. As these animals were older
7 (up to 8 months old in some cases), they would have had more opportunities to encounter
8 multiple sources of infection, through increased mixing and movements.

9 In addition, the clinical status of farms may have influenced the diversity of *P. multocida*
10 identified within calves; further investigations into the pathophysiology of disease and
11 concurrent strain repertoire are being explored within a longitudinal framework.

12 Due to the limited data available, it was not possible to investigate the spatial distribution
13 of strains robustly. However, in Scotland there was some evidence of a cluster of one
14 RAPD profile on Orkney (a group of islands off the North coast of Scotland). Whilst this
15 warrants further investigation, it does suggest farm-to-farm transmission in a
16 geographically discrete population. In addition, the Aberdeenshire region of Scotland
17 appeared to have a high prevalence of unique *P. multocida* RAPD types. This region is
18 known to be an area of high cattle movements - in 2006, 29% of all cattle imported into
19 Scotland from England and Wales were destined for Aberdeenshire (Gubbins et al.,
20 2010).

21

22 **Conclusion**

1 Single or multiple strains of *P. multocida* may exist within groups of calves on beef and
2 dairy farms, which is consistent with findings in poultry and pigs. Farms may have more
3 diverse populations of *P. multocida* if they buy in animals.

4

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10

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- 1 **Table 1** Numbers of Scottish and French farms with specified numbers of profiles of
 2 *Pasteurella multocida* as assigned by random-amplified polymorphic DNA (RAPD)
 3 typing and pulsed-field gel electrophoresis (PFGE)

No. RAPD types	No. PFGE main types	Scottish	French
1	1	26	5
	2	1	1
	3	0	2
	4	0	1
	Incomplete	0	1
2	2	3	2
	Incomplete	2	0
3	2	0	1
	3	0	4
	4	0	1
	7	0	1
4	10	0	1

- 4 Incomplete: farms where some or all isolates were either untypeable or could not be
 5 recovered from frozen stocks

1 **Figure captions**

2 Figure 1a RAPD gel representing *Pasteurella multocida* isolates from four Scottish
3 farms. Lanes 1-3 are farm 11, lanes 4-10 are farm 14, lanes 11 and 12 are farm 29 and
4 lanes 13 and 14 are farm 31. Profile designation is indicated below lane number. M =
5 100bp molecular weight ladder.

6 Figure 1b RAPD gel representing *Pasteurella multocida* isolates from two French farms.
7 Lanes 1-9 are farm 1 and lanes 10-13 are farm 5. Profile designation is indicated below
8 lane number. M = 100bp molecular weight ladder. Ng = negative control lane.

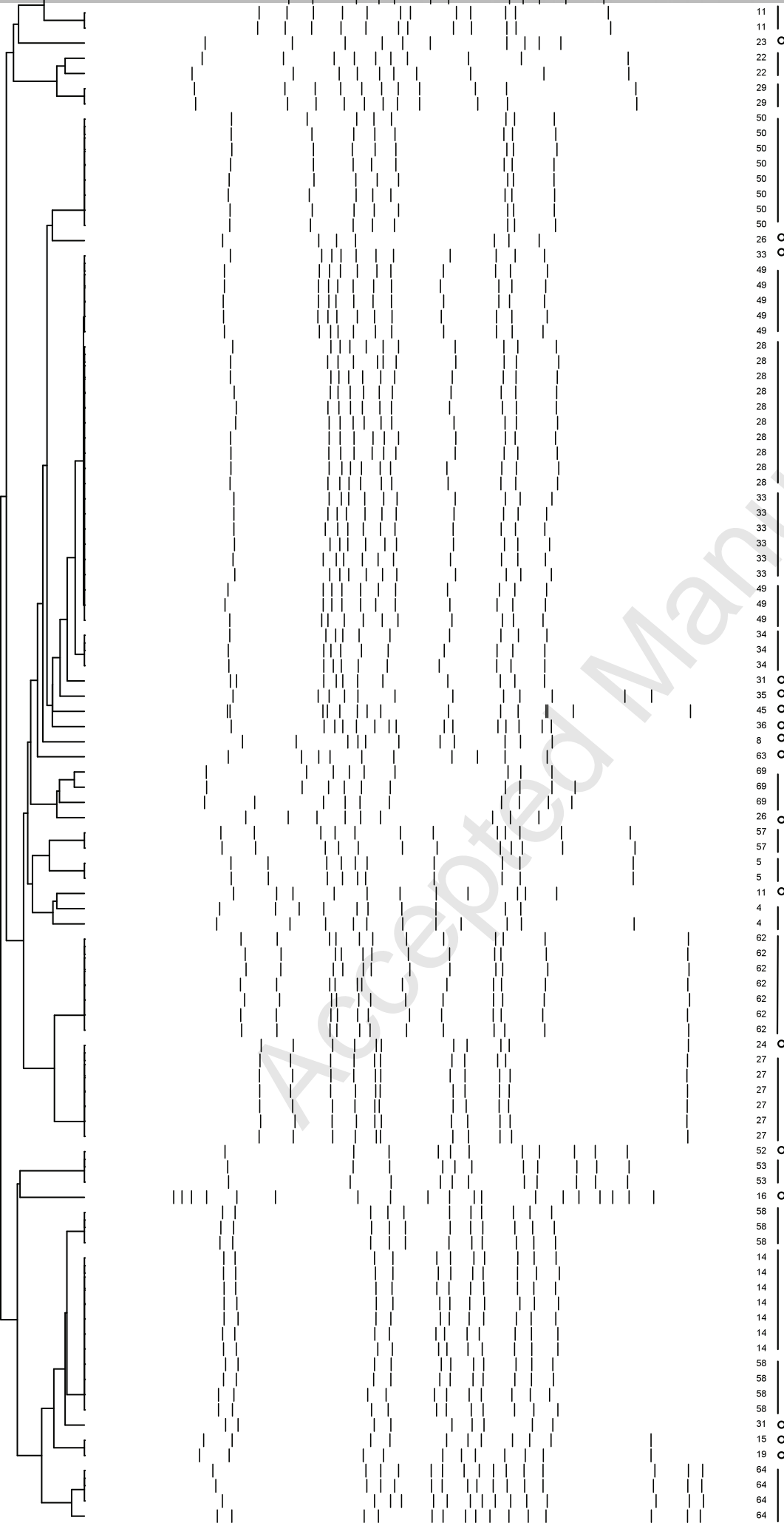
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10 Figure 2 Representation of pulsed-field gel electrophoresis patterns of *Pasteurella*
11 *multocida* obtained after *ApaI* restriction in 102 isolates from cattle from 32 Scottish
12 farms. Above the dendrogram, source farm is indicated and lines demonstrate where
13 profiles are clustered by farm.

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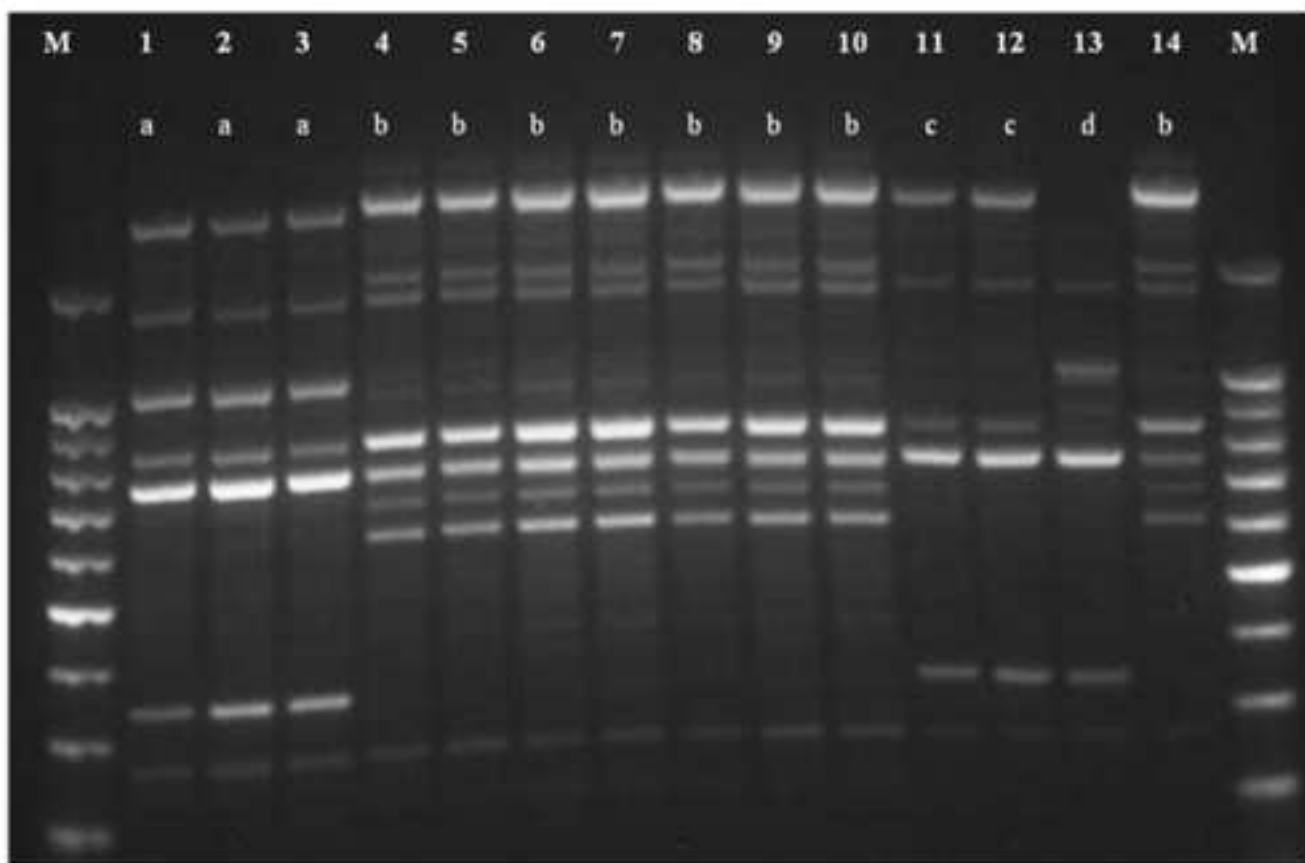
15 Supplementary Figure S1 Representation of pulsed-field gel electrophoresis patterns of
16 *Pasteurella multocida* obtained after *ApaI* restriction in 110 isolates from cattle from 19
17 French farms. The algorithm has split a designated RP into two branches on two
18 occasions; therefore 73 branches are shown where 71 RPs were assigned. Above the
19 dendrogram, source farm is indicated and lines demonstrate where profiles are clustered
20 by farm.

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(a)



(b)

