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Development and validation of a multiple locus variable number tandem repeat analysis (MLVA) scheme for Fusobacterium necrophorum

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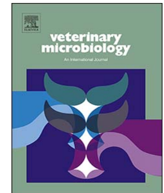
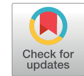


Development and validation of a multiple locus variable number tandem repeat analysis (MLVA) scheme for *Fusobacterium necrophorum*



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ABSTRACT

Fusobacterium necrophorum is associated with various diseases in humans and animals. Reservoirs (sites where the pathogen persists in the absence of disease) of *F. necrophorum* are believed to be present in healthy individuals e.g. tonsillar epithelium, or their environment e.g. soil, but for most diseases the reservoir sites are unknown. Strain typing of *F. necrophorum* would facilitate linking specific reservoirs with a specific disease. The aim of this study was to develop multiple locus variable number tandem repeat analysis (MLVA) as a strain typing technique for *F. necrophorum*, and to test the use of this scheme to analyse both isolates and mixed communities of bacteria. Seventy-three tandem repeat regions were identified in the *F. necrophorum* genome; three of these loci were suitable and developed as a MLVA scheme. The MLVA scheme was sensitive, specific, and discriminatory for both isolates and communities of *F. necrophorum*. The MLVA scheme strain typed 46/52 *F. necrophorum* isolates including isolates of both subspecies and from different countries, host species and sample sites within host. There were 12 unique MLVA strain types that clustered by subspecies. The MLVA scheme characterised the *F. necrophorum* community in DNA from 32/49 foot- and 28/33 mouth swabs from sheep. There were 17 community types in total. In 31/32 foot swabs, single strains of *F. necrophorum* were detected while in the 28 mouth swabs there were up to a maximum of 8 strains of *F. necrophorum* detected. The results demonstrate the potential for this method to elucidate reservoirs of *F. necrophorum*.

1. Introduction

Fusobacterium necrophorum is a Gram-negative, rod-shaped, anaerobic bacterium that is associated with a variety of diseases, termed necrobacilloses, in humans and animals. In humans, *F. necrophorum* causes Lemierre's disease (Kuppalli et al., 2012; Lemierre, 1936; Riordan, 2007) and is associated with pharyngitis (Aliyu et al., 2004; Ludlam et al., 2009), periodontal disease (Enwonwu et al., 1999; Gomes et al., 2004; Jacinto et al., 2008) and appendicitis (Rogers et al., 2016). In animals, *F. necrophorum* causes hepatic abscesses that occur in intensively reared beef cattle (Lechtenberg et al., 1988; Nagaraja and Chengappa, 1998; Narayanan et al., 1997) and it is associated with footrot in sheep (Egerton et al., 1969; Witcomb et al., 2014), foot infections in other ungulates (Clark et al., 1985; Edwards et al., 2001; Handeland et al., 2010), endometritis in cattle (Ruder et al., 1981), calf diphtheria (Panciera et al., 1989), respiratory disease in deer (Brooks et al., 2014) and periodontal disease in wallabies (Antiabong et al., 2013b).

Reservoirs of bacterial pathogens are sites in an organism or the environment where the pathogen lives, and often multiplies (Krämer et al., 2010). *F. necrophorum* is considered to be an opportunistic pathogen (Langworth, 1977; Tan et al., 1996), consequently healthy individuals and/or their environment are assumed to be reservoirs for the bacterium. However, there has been little research on the location of reservoir sites. In cattle, strain typing was used to identify the bovine rumen as the reservoir of *F. necrophorum* that causes hepatic abscesses (Narayanan et al., 1997). In humans, *F. necrophorum* was thought to be part of the throat microflora of healthy individuals (Bartlett and Gorbach, 1976; Lemierre, 1936), however, it has only been detected in people aged 18–39, although Lemierre's disease and other *F. necrophorum* infections can occur at any age (Aliyu et al., 2004; Jensen et al., 2007; Ludlam et al., 2009). In sheep, *F. necrophorum* has been isolated from the gingiva (Bennett et al., 2009; McCourtie et al., 1990) and detected on both healthy and footrot-diseased feet (Calvo-Bado et al., 2011; Frosth et al., 2015; Maboni et al., 2016; Witcomb et al., 2014), but the significance of these sites as reservoirs is unknown. Whilst *F. necrophorum* has been widely assumed to be ubiquitous in sheep faeces and soil (Langworth, 1977; Marsh and Tunnicliff, 1934; Roberts and

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Egerton, 1969; Winter, 2004) this is unsubstantiated.

There are two subspecies of *F. necrophorum*: *necrophorum* and *funduliforme* (Shinjo et al., 1991). These are distinguished by a PCR assay that detects a haemagglutinin-related gene that is present in subsp. *necrophorum* but not *funduliforme* (Narongwanichgarn et al., 2003). To confidently identify reservoirs associated with specific diseases, strain typing of *F. necrophorum* over time is needed, as exemplified by Narayanan et al. (1997). Multiple locus variable number tandem repeat analysis (MLVA) is an objective, repeatable, PCR-based strain typing method that has been used in a variety of epidemiological studies of bacterial pathogens (Eyre et al., 2013; Halkilahti et al., 2013; Mezal et al., 2014; Russell et al., 2013; Vranckx et al., 2011; Wada et al., 2007). MLVA was originally developed to analyse individual isolates but it can also be used to analyse samples that may contain a mixed community of strains within a species (Vranckx et al., 2011). In these cases, MLVA is used to produce a molecular "fingerprint" of the strains present and so identify similarities and differences between communities.

The aim of the current study was to develop an MLVA typing scheme for *F. necrophorum*, and to demonstrate its potential to analyse isolates and community DNA. A selection of *F. necrophorum* isolates from a variety of host species and countries, together with DNA extracted from swab samples from the feet and mouths of sheep, were used to develop and validate the scheme.

2. Materials and methods

2.1. Identification of tandem repeat regions for MLVA analysis

Seventy-three tandem repeat regions (Table S1) were identified from the whole genome shotgun sequence of *F. necrophorum* ATCC 51357 (GenBank Accession number AJSY00000000.1) using the Tandem Repeats Finder software v.4.08 (Benson, 1999). Nine regions were excluded due to insufficient flanking sequence to facilitate PCR primer design for amplification of the target region. There were 34 regions identified using blastn (Altschul et al., 1990), where flanking sequences were present in all of the three published *F. necrophorum* genomes available (accessed March 2014). PCR primers targeting the 3′ and 5′ flanking regions of these 34 repeat regions were designed using BatchPrimer3 v1.0 (You et al., 2008). Eight *F. necrophorum* subsp. *necrophorum* isolates (Table S2) were tested first for amplification of the target region and then for polymorphism at the tandem repeat region. Three loci (Fn13, Fn42 and Fn69; Table S1) showed good amplification and sufficient polymorphism for use in MLVA typing. PCR primers used to amplify the three selected MLVA targets and their tandem repeat sizes are given in Table S3.

2.2. MLVA PCR reactions and cycling conditions

PCR reactions were carried out in a final volume of 25 µl and contained 12.5 µl Bioline MyTaq™ Red Master Mix (2×; Bioline Reagents Ltd., London, UK), 1 µl molecular biology grade bovine serum albumin (BSA; 100 µg ml^{-1}; Sigma-Aldrich Ltd., Gillingham, UK), 1 µl each of forward and reverse primers (10 µM), and 1 µl template DNA. In reactions using mixed DNA, 1 µl betaine (5 M; Sigma-Aldrich Ltd., Gillingham, UK) was also included to improve sensitivity. Cycling conditions were 95 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 55 °C (Fn13 and Fn69) or 62 °C (Fn42) for 30 s, 72 °C for 30 s, followed by final extension at 72 °C for 10 min. All PCR reactions were carried out on an Eppendorf Mastercycler ep gradient machine (Eppendorf, Hamburg, Germany) with DNA extracted from *F. necrophorum* subsp. *necrophorum* DSM 21784 as the positive control and nuclease free H_2O as the reagent blank. PCR products were visualized after ethidium bromide-stained agarose gel electrophoresis and imaged using a Gene Flash imager (Syngene Bio Imaging, Cambridge, UK).

2.3. Validation of the MLVA typing scheme

PCR primer specificity was tested using DNA from a selection of non-target organisms (*Fusobacterium gonidiaformans* [DSM 19810], *Fusobacterium nucleatum* subsp. *polymorphum* [DSM 20482], *Dichelobacter nodosus* [VCS1703A], *Mycobacterium bovis* [BCG], *Escherichia coli, Mannheimia* sp., *Pseudomonas* sp., *Staphylococcus epidermidis, Staphylococcus intermedius,* and *Streptococcus uberis;* all from University of Warwick).

The sensitivity of amplification for each loci was tested using a ten-fold dilution series from 10^6 to 10^1 genome copies µl^{-1} of *F. necrophorum* DSM 21784 DNA added to DNA extracted from *F. necrophorum* negative sheep foot swabs (Witcomb et al., 2014). The number of genome copies in the stock DNA was calculated based on the genome size for *F. necrophorum* subsp. *funduliforme* (2,088,497 bp; Calcutt et al., 2014). A blank containing DNA extracted from *F. necrophorum* negative foot swabs was run alongside the dilution series.

The stability of the MLVA scheme was tested by comparing the MLVA strain type of two *F. necrophorum* isolates before and after ten passages of culture on Fusobacterium Agar, a selective medium based on that used by Brazier et al. (1991) (Wilkins-Chalgren Anaerobe Agar with Gram-negative Anaerobe Selective Supplement (both Oxoid Ltd., Altrincham, UK), 5% defibrinated sheep blood and josamycin (3 µg ml^{-1})).

2.4. Determining PCR amplicon size using fragment analysis

The size, in base pairs, of PCR products was determined using fragment analysis: samples were submitted to DNA Sequencing and Services™ (College of Life Sciences, University of Dundee, UK) and results analysed with Peak Scanner 2 Software (Applied Biosystems, Warrington, UK). Sanger sequencing of the PCR products from each of the three assays for *F. necrophorum* DSM 21784 were used as a reference for the number of repeats to be calculated from the size in base pairs for each sample. A variation in expected size of PCR amplicon of ± 2 bp was tolerated.

2.5. MLVA typing of F. necrophorum isolates

A total of 52 isolates, 43 *F. necrophorum* subsp. *necrophorum* and 9 *F. necrophorum* subsp. *funduliforme,* were used in this study. The country and sites of origin of the isolates are listed in Table 1.

Isolates were cultured on Fusobacterium agar (as above) and then sub-cultured on Wilkins-Chalgren Anaerobe Agar (Oxoid Ltd., Altrincham, UK) with 5% defibrinated sheep blood. All incubations were carried out under anaerobic conditions (Don Whitley MACS-MG-1000 anaerobic workstation; 80% N_2, 10% CO_2 and 10% H_2, Don Whitley Scientific Ltd., Shipley, UK) at 30 °C for 2–5 days. DNA was extracted from cultures using the Qiagen DNeasy Blood and Tissue Kit

Table 1
Country, animal host, site of sample and subspecies of 52 *Fusobacterium necrophorum* isolates tested by MLVA.

Country	Animal	Site	Subspecies	No. of isolates
UK	Sheep	Foot	*necrophorum*	9
		Mouth	*funduliforme*	1
	Cattle	Liver abscess	*necrophorum*	8
			funduliforme	1
USA	Cattle	Liver abscess	*necrophorum*	9
			funduliforme	4
		Footrot	*necrophorum*	6
		Rumen	*necrophorum*	1
			funduliforme	3
	Elk	Footrot	*necrophorum*	4
France	Sheep	Foot	*necrophorum*	1
Spain	Sheep	Foot	*necrophorum*	5

(Qiagen Ltd., Manchester, UK) according to the manufacturer's instructions with a lysis time of 1 h. A *F. necrophorum* specific standard PCR targeting the gyrase B gene (Antiabong et al., 2013a; Jensen et al., 2007) was used to confirm that isolates were *F. necrophorum,* and amplification of the haemagglutinin-related protein gene used to confirm isolates as subspecies *necrophorum* rather than subspecies *funduliforme* (Antiabong et al., 2013a; Narongwanichgarn et al., 2003).

The strain type of *F. necrophorum* isolates was determined by the number of repeats at each of the three loci (Fn13, Fn42 and Fn69) after PCR and fragment analysis. Each strain type was assigned a unique number. The Hunter-Gaston Discriminatory Index (HGDI) for the strain typing scheme was calculated (Hunter and Gaston, 1988) with 95% confidence intervals (Grundmann et al., 2001). Minimum-spanning trees for the isolate strain typing data were created in PHYLOViZ-2.0 (Francisco et al., 2012) using the global optimal eBURST (goeBURST) distance algorithm with Euclidean distance (Francisco et al., 2009). The population was grouped on single locus variants (SLV).

2.6. MLVA typing of F. necrophorum communities from swab samples

Initially a model community was made by combining equal concentrations of DNA from four isolates of *F. necrophorum* which between them contained three variants at both Fn13 and Fn42, and two variants at Fn69. This was then tested to investigate whether, in a mixed community, all the variants at each locus were detected using the MLVA typing scheme.

DNA was extracted from 82 swabs (33 mouth and 49 foot swabs) taken from sheep on six farms (A − F) in England, as described by Purdy (2005). The *rpoB* qPCR described by Witcomb et al. (2014) was used to detect and quantify *F. necrophorum* in these samples, and those confirmed positive for *F. necrophorum* with a load > 10^3 *rpoB* copies swab^{-1} were used for MLVA community analysis (Table 2). On Farm A, samples were collected as part of a longitudinal study: 10 sheep were sampled every 2 weeks for 8 weeks. On Farms B–F, 15 sheep were sampled per farm on one occasion.

For the swab samples, the number of MLVA variants within a locus was determined by fragment analysis. The minimum number of strains in a community was calculated as equal to the greatest number of MLVA variants at one locus. The maximum number of strains detected in a community was calculated by multiplying the number of variants at each locus together (e.g. if a sample contains 1, 2 and 3 variants for the three loci, the minimum number of strains is 3 and the maximum is 6 ($1 \times 2 \times 3$)). Each unique pattern of MLVA variants within these samples was assigned a unique "community type" number. The HGDI and associated confidence interval were calculated based on the frequency of detection of each community type.

Table 2
Detection of *Fusobacterium necrophorum* and success of MLVA analysis by site of swab and farm.

Site and farm	*F. necrophorum*[a]		Community type[b]	
	No.	%	No.	%
Foot swabs				
A	76/152	50	25/37	68
B	2/13	15	0/2	0
C	2/13	15	0/2	0
D	3/14	21	3/3	100
E	3/16	19	2/3	67
F	2/14	14	2/2	100
Mouth swabs				
A	30/38	79	15/16	94
B	7/15	47	6/7	86
C	1/15	7	1/1	100
D	7/15	47	5/7	71
E	1/15	7	1/1	100
F	1/15	7	0/1	0

[a] Number and percentage of samples positive for *F. necrophorum* out of total samples collected.
[b] Number and percentage of samples with an MLVA community type determined out of those analysed using the MLVA scheme.

3. Results

3.1. Validation of PCR amplification of the loci

The PCR assays for the three MLVA loci (Fn13, Fn42 and Fn69) were specific, with no PCR product produced from any of the non-target organisms tested. The detection limit was 10^4 genome copies μl^{-1} of extracted DNA for the Fn13 assay, and 10^3 genome copies μl^{-1} of extracted DNA for the Fn42 and Fn69 assays. The MLVA scheme was stable; the MLVA type of the two isolates matched their original MLVA type after ten culture passages.

3.2. Population diversity of F. necrophorum isolates

The three MLVA loci were characterised in 46/52 (88%) *F. necrophorum* isolates. The 6 isolates that were not fully characterised were excluded from further analysis. In the fully characterised isolates there were three variants at locus Fn13, five at Fn42 and four at Fn69 (Table S4) giving 12 unique MLVA strain types (Table S5), 6 of which were detected only once. The HGDI for the strain typing scheme was 0.85 (95% CI 0.80–0.90), so that two distinct strains would be characterised as different on 85% of occasions.

Strain types varied within subspecies, country, host species and sample site. The goeBURST analysis detected 2 groups (Fig. 1): 11/12 strains were in a major group with strain types 3 (n = 9) and 7 (n = 2) the predicted ancestral strains. Strain type 5 (n = 5) was in an unconnected group by itself. Both subspecies were present in both groups and were clearly clustered within the major group (Fig. 1A); only 1/11 strain types in the major group contained both subspecies. There was no clear clustering of strains by host species (Fig. 1B), country of origin (Fig. 1C), or tissue site (Fig. 1D). The variation in strains indicates that analysis of a greater number of isolates could provide evidence of clustering if it exists.

3.3. Community diversity of F. necrophorum in DNA from swab samples

All expected locus variants were detected in the model community (data not shown) indicating that the MLVA scheme was able to detect strains in mixed communities of *F. necrophorum*. All three MLVA loci were amplified from 28/33 (85%) mouth and 32/49 (65%) foot swab samples (Table 2). There were 17 unique community types (Table S6), 10 of which contained more than 1 strain, these ranged from a minimum of 2 to a maximum of 8 strains. The *F. necrophorum* communities in mouth swabs were more complex than the communities in foot swabs. There were 16 community types in mouth swabs; the overall HGDI was 0.94 (95% CI 0.90 − 0.98). There were only 4 community types in foot swabs; 31/32 (97%) foot swabs had a single strain type (one of strain types 1, 3 and 6 from Fig. 1) consequently the HGDI was not calculated for foot swabs.

The locus variants from the 10 sheep from Farm A are presented in Fig. 2. The same strain was detected on feet over time and 24/25 foot swabs were a single strain (strain type 1 in the isolate analysis (Fig. 1)) rather than a community of *F. necrophorum*. The three locus variants in this strain (13.2, 42.5 and 69.2) were also detected in mouths in sheep 5, 7 and 8, indicating that this strain was potentially present in mouths. There were, however, many more strain types in mouths than feet. In mouths, some locus variants and community types were stable over time for example, the same community type was detected at all four time points in sheep 3 and 7 and in sheep 5 and 10 the community types differed by one additional locus variant present in 50% of the samples. On Farms B-F, as with Farm A (Fig. 2), complex communities (up to 8

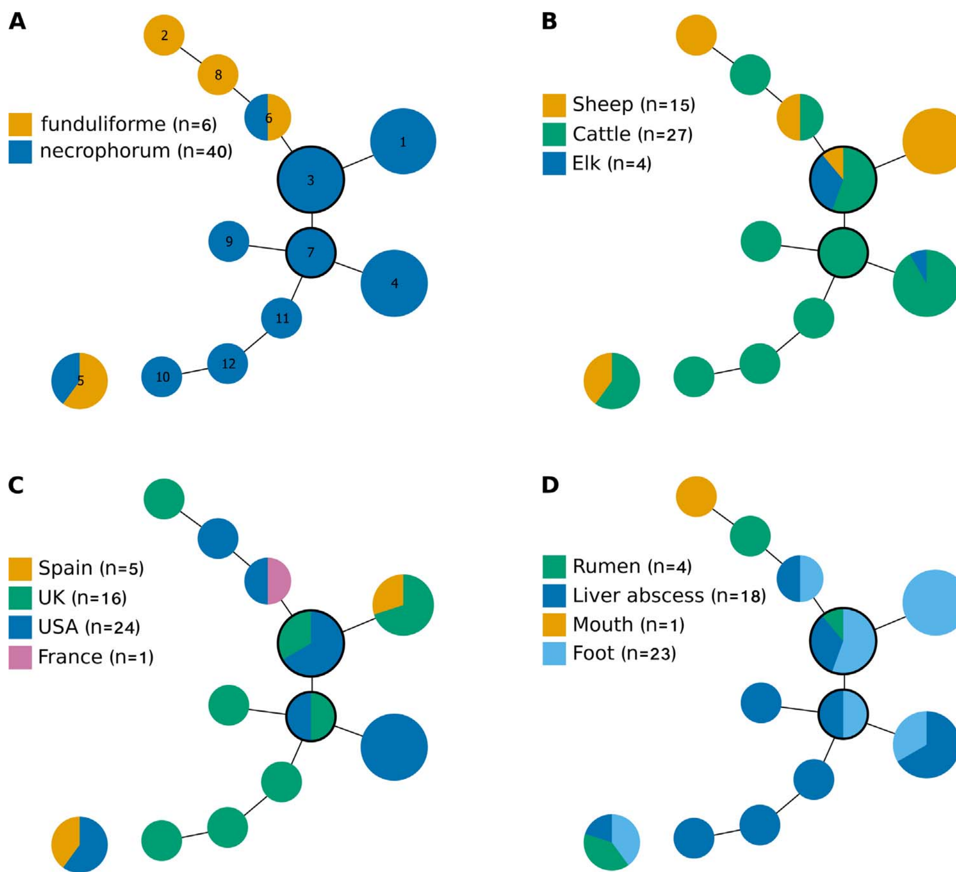

Fig. 1. Analysis of *Fusobacterium necrophorum* MLVA strain type clustering using goeBurst. Single locus variants are connected by solid lines. Numbers indicate MLVA strain type, and size of circle represents number of isolates of each MLVA type. Types 3 and 7 are the suggested founder strain types, indicated by the black border. The shading indicates isolates of (A) different subspecies, (B) different host species, (C) different countries of origin, and (D) different sites of origin. Individual isolates are not always in the same position within a circle between the 4 trees, the coloured sections are placed with the most frequently represented first from the 12 o'clock position.

strains) were present in mouth swabs whereas only single strains of *F. necrophorum* were detected in foot swabs. Community data from Farms B–F is presented in Fig. S1.

4. Discussion

The MLVA typing scheme developed for *F. necrophorum* was specific and sensitive with the potential to strain type isolates and community DNA. Discriminatory ability, stability, epidemiological concordance, typeability and reproducibility are also used to evaluate typing schemes (van Belkum et al., 2007). A HGDI discriminatory value of ≥ 0.95 is recommended for typing schemes (van Belkum et al., 2007). The discriminatory ability of this 3-loci scheme was 0.85 (95% CI 0.80–0.90) for isolates and 0.94 (95% CI 0.90–0.98) for communities of *F. necrophorum*. Ideally, we would have liked the scheme to be more discriminatory, however, there were no further suitable loci. The results from isolates and communities do suggest that the scheme is sufficiently discriminatory for these samples. The identification of the same strain type for two isolates after multiple passages through culture demonstrated the stability of the scheme. Finally, there was good epidemiological concordance for the scheme, for example, single strains were detected on the feet of sheep over time on Farm A, whilst more complex and varied communities were detected in mouths samples over the same time period.

A wide range of *F. necrophorum* isolates from three ruminant hosts and four countries was used to develop the scheme. The MLVA scheme was sufficiently discriminatory to differentiate isolates from the same country, host, site and subspecies. There was no clustering of *F. necrophorum* strain types by country, host or site from the isolates analysed. This might be due to the relatively small number of isolates analysed or because provenance of the samples meant that there were no clusters in the dataset. Clusters might be detectable in a dataset specifically selected to investigate the host disease and its complimentary reservoir, e.g. as reported by Narayanan et al. (1997) for liver abscesses and the rumen reservoir in the same host animal.

This is the first study of communities of *F. necrophorum* in sheep and provides pilot data for further study. The communities in the mouth were more complex than on the feet. There were locus variants in mouths that were never detected on feet from sheep on the same farm, suggesting site-specificity for some strains. In contrast, the strain of *F. necrophorum* detected on feet was potentially (i.e. its 3 loci were present) in the mouths of some sheep on Farm A, suggesting that the mouth could be a reservoir or a spill-over site from feet. With the exception of one sample, only single strains of *F. necrophorum* were detected on feet. The consistency over time and the discriminatory power of the MLVA scheme suggest that this is likely to be a true reflection of the samples analysed. The generalisability of this pattern of very limited diversity on feet is unknown, however, Zhou et al. (2009) also reported the presence of single strains of *F. necrophorum* in 14 DNA samples extracted from foot swabs from sheep.

It is likely that there were loci variants that were not detected in the community DNA samples in the current study because of the limit of detection of the PCR. This may have affected locus Fn13 more because detection of this locus is less sensitive than Fn42 and 69. This limits the use of the scheme for community samples to those with *F. necrophorum* loads of more than $\sim 10^4$ copies per µl of extracted DNA. Improvements in the sensitivity of detection at this locus would enable the analysis of a wider range of samples.

5. Conclusions

A sensitive, specific, stable and discriminatory MLVA typing scheme was developed and validated for both isolates and community DNA samples of *F. necrophorum*. Using samples from sheep, the scheme is epidemiologically plausible and has potential to improve understanding of reservoirs of *F. necrophorum* and their association with

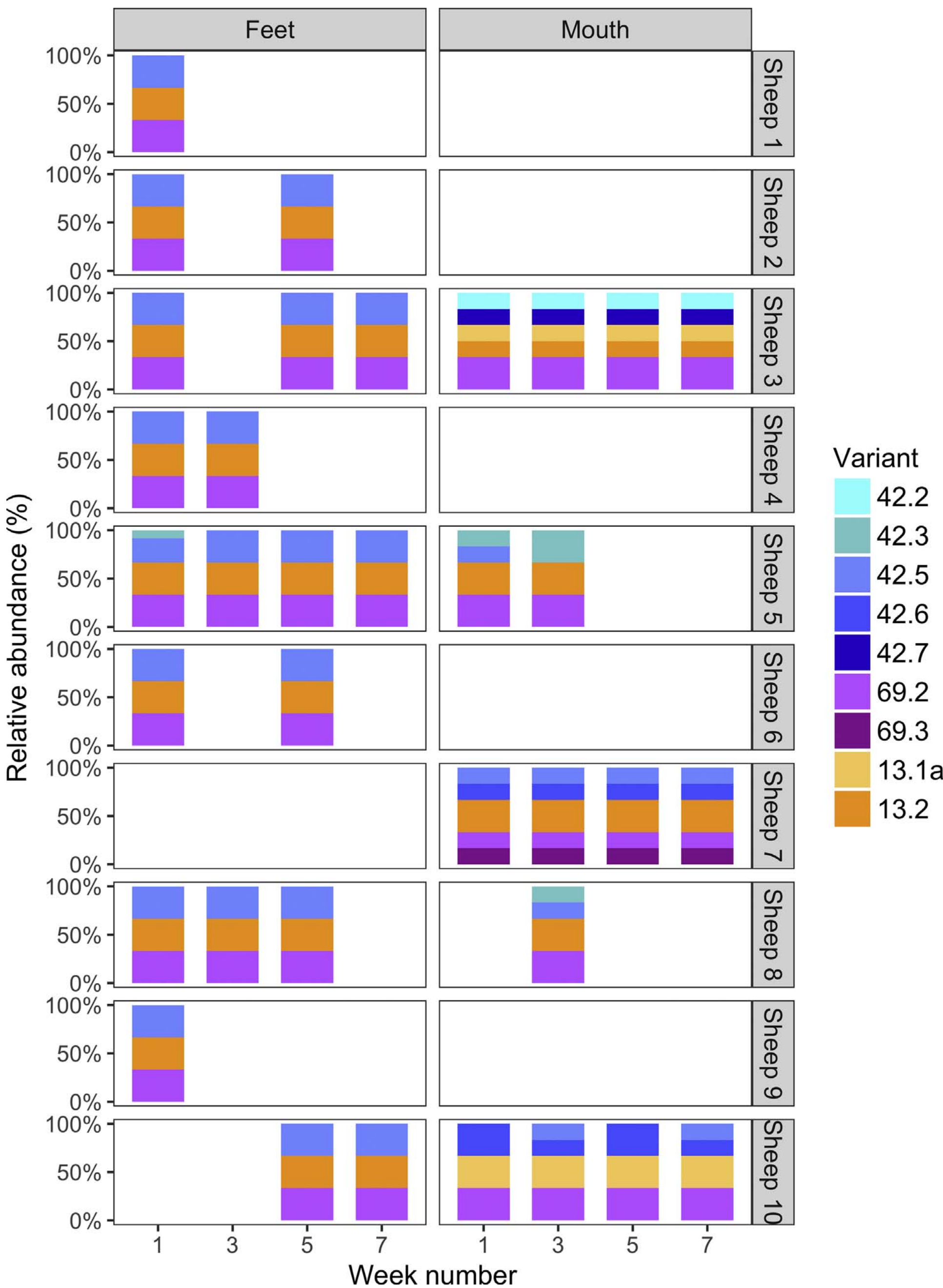

Fig. 2. Relative abundance of locus variants in swab samples from Farm A. The ten sheep from Farm A are listed on the right of the figure. Results from all positive foot swabs from a sheep (sometimes > 1 positive per sheep) are represented in the left-hand panels, and mouths in the right. Note, in all but one of the sheep (sheep 5, week 1) all positive foot swabs contained the same community type, which was represented by a single strain type (strain type 1 in Fig. 1).

necrobacilloses in both non-human and human animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2017.11.017.

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