Undetected *lktA* genes within *Fusobacterium necrophorum*?

In a recent issue of this journal, Ludlam *et al.* (2009) outlined a PCR-based study on the basis of which they claimed that the *lktA* gene was not a universal virulence factor within strains of *Fusobacterium necrophorum* isolated from human and animal clinical cases. The authors also claimed that the detection of *lktA* was unevenly distributed across human and animal cases, between the two subspecies of *F. necrophorum* (subspecies *necrophorum* and subspecies *funduliforme*, hereafter referred to as *Fnn* and *Fnf*, respectively) and between the subspecies in isolates of bovine origin. We consider these unsafe conclusions as we are not convinced that the authors would have been able to detect the wide variety of *lktA* gene sequences that appear to be present within *F. necrophorum* with the PCR conditions they employed.

There are two reasons why we believe this is so, although these reasons are not completely unrelated. When using PCR on novel genetic material, it cannot be assumed that the primers will match the unknown target sequence, as even a single nucleotide mismatch at the 3' end of either primer could completely stop amplification. As a result, the only conclusions that should ever be drawn from such diagnostic PCRs should be framed in terms of amplification or non-amplification, and not sequence presence or absence. To state absolutely that a sequence is not present is unjustifiable. Accordingly in our opinion the title of their paper 'lktA-encoded leukotoxin is not a universal virulence factor in invasive *Fusobacterium necrophorum* infections in animals and man' (Ludlam *et al.*, 2009) overstates the case on the evidence presented. This inability to prove the absence of something is not unique to PCR-based diagnostics, as most diagnostic techniques cannot definitively prove the absence of something. As a result when drawing conclusions from such diagnostic approaches, the wording used needs to reflect what can, and cannot, be proven.

We acknowledge that Ludlam *et al.* (2009) attempted to address the dangers of false

negatives by using a positive control in the form of two geographically distant isolates of *F. necrophorum* that have had their *lktA* genes described (GenBank accession nos AF312861 and DQ672338). However, both of these isolates were from cattle, and recent studies suggest that the host may play a stronger influence on the genetic variation of *lktA* in *F. necrophorum* than geography, with sheep, goats and pigs tending to carry variants of *lktA* that are different to those found in cattle (Zhou *et al.*, 2009a, 2010). Additionally, the *Fnn* *lktA* sequence isolated from cattle in the USA (GenBank accession no. AF312861) and the partial sequence isolated from cattle in New Zealand (Zhou *et al.*, 2009a) are identical.

When we aligned the primer and probe sequences reported by Ludlam *et al.* (2009) with the published *F. necrophorum lktA* sequences (GenBank accession nos

AF312861, AY972049 and FJ230830–FJ230832) in a region where variation has been reported previously (Zhou *et al.*, 2009a), mismatches were found between their sequences and some of the *F. necrophorum lktA* sequences, including three from cloned DNA derived from uncultured strains of *F. necrophorum* (Fig. 1). In particular, there are at least 1, 3 and 1 nucleotide mismatches in the forward, reverse and probe-binding regions, respectively, when compared to *F. necrophorum* variants C and D, the former being the most common *F. necrophorum* variant found in sheep, goats and pigs (Zhou *et al.*, 2009a, 2010). Mismatches in these regions might prevent these variants from being amplified and hence these variants of the *lktA* gene may go undetected. Therefore, it is also possible that the primers and probes reported by Ludlam *et al.* (2009) might not detect all *F.*

(a)	LT2 forward:	ATCGGAGTAGTAGGTTCTGTTGGTGTG
	<i>Fnn</i> :	-----
	<i>Fnf</i> :	-----C-----C-----
	Variant B:	
	Variant C:	-C-----
(b)	LT2 reverse:	GAGTCGGAGTCGCAGCAGCC
	<i>Fnn</i> :	-----
	<i>Fnf</i> :	-----
	Variant B:	---T---T---T
	Variant C:	---T---T---T
(c)	LT2 probe:	AAGCTGAAATTACAGCAGATTCTAAGCAAG
	<i>Fnn</i> :	-----
	<i>Fnf</i> :	-----
	Variant B:	-----
	Variant C:	-----G-----
	Variant D:	-----G-----

Fig. 1. Alignments of the primer and probe sequences reported by Ludlam *et al.* (2009) with *F. necrophorum lktA* sequences: (a) the LT2 forward primer, (b) the LT2 reverse primer and (c) the LT2 probe binding region. Dashes represent nucleotide identical to the top (primer/probe) sequence, and unknown nucleotide sequences are shown by dots. All sequences are presented 5' to 3', and the reverse complementary sequence of LT2 reverse primer is shown. The GenBank accession numbers for the *F. necrophorum lktA* sequences are AF312861 (*Fnn*), AY972049 (*Fnf*), FJ230830 (variant B), FJ230831 (variant C) and FJ230832 (variant D).

necrophorum variants, which may explain why they found only two sequences corresponding to the reported isolates of *Fnn* and *Fnf*.

Interestingly, although we have previously reported a total of four variants of *F. necrophorum* from sheep, goats, cattle and pigs, there was no evidence for the presence of *Fnf* in these animal species (Zhou *et al.*, 2009a, 2010), while in the paper by Ludlam *et al.* (2009) *Fnf* appears to be common in animals, and even dominant over *Fnn* in some animal species such as cattle and sheep.

The issue of substantive variation in *lktA* genes preventing PCR techniques from being utilized to detect fusobacteria has been noted by other authors (Tadepalli *et al.*, 2008). They reported that *Fusobacterium equinum* (a bacterium phenotypically similar to *F. necrophorum*) produces a leukotoxin of equivalent size and potency to that of *F. necrophorum*, and that it is encoded by an *lktA* gene (shown to be present using probes and Southern hybridization), but that no amplicons were produced from the nominal *lktA* gene of *F. equinum* using PCR, probably because of

sequence differences between the *lktA* genes of *F. equinum* and the two *F. necrophorum* subspecies. A portion of the *lktA* gene of *F. equinum* was only recently sequenced (Zhou *et al.*, 2009b) and this confirmed that the gene was substantively different at the DNA sequence level from both *Fnn* and *Fnf*.

While we agree with the conclusion drawn by Ludlam *et al.* (2009) that vaccines against the *lktA* gene of *Fnn* may be unsuitable to be used against all strains, we do not agree with their reasoning and the assumptions that their conclusions are based on. Rather than *lktA* vaccines being likely to fail due to *lktA* not being universally present in all *F. necrophorum* strains, we consider it a possibility that vaccines could fail due to genetic differences observed in the *lktA* genes found in the various *F. necrophorum* strains or other *F. necrophorum*-like bacteria.

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Authors' reply to 'Undetected *lktA* genes within *Fusobacterium necrophorum*?': presence or absence of an amplicon – the cornerstone of molecular diagnostics

Bennett *et al.* (2010) raise some interesting points and present some intriguing sequence data for the leukotoxin gene (*lktA*) of *Fusobacterium necrophorum*, drawn from work recently published by them, indicating three novel variants in the *lktA* gene (Zhou *et al.*, 2009a). Bennett *et al.* (2010) speculate from these data that the conclusions drawn in our previous communication (Ludlam *et al.*, 2009), in which we reported our failure to detect the gene employing PCR in a significant proportion of the two subspecies of *F. necrophorum* (subspecies *necrophorum* and *funduliforme*) recovered from infections in humans and animals (Ludlam *et al.*, 2009), may be unsound.

Whilst we accept that a limitation of PCR-based diagnostics can be the failure to detect a novel variant of a pathogen, we cannot accept the assertion of Bennett *et al.* (2010)

that the only conclusion that should ever be drawn from negative diagnostic PCR results is simply non-amplification of the sequence in question, rather than absence of the gene, or host organism. Following rigorous commercial and in-house test development and validation of PCR-based diagnostics of the sort we reported, diagnosis and management of infectious diseases is now routinely based on both positive and negative PCR results. Indeed, we note that the authors themselves ruled out the presence of *F. necrophorum* subspecies *funduliforme* in material from the lesions of the animal hooves that they examined on precisely this basis; i.e. a negative PCR result for *lktA* (Zhou *et al.*, 2009a).

We were aware of the possible existence of sequence variation and went to great lengths to ensure that putative variants of

the sort described by Zhou *et al.* (2009a) would not pass undetected. Whilst Bennett *et al.* (2010) focus on the LT2 primer set and TaqMan probe that emphasize their novel sequence data, they do not mention the other two primer sets (LT1 and LT3) that we employed, which targeted entirely different regions of the three published entire *lktA* gene sequences (*F. necrophorum* subspecies *necrophorum*, AF312861 and DQ672338; *F. necrophorum* subspecies *funduliforme*, AY972049). Our LT1 primer set is perfectly matched for all three known entire *lktA* gene sequences, while the LT3 forward primer contained just a single mismatch with *F. necrophorum* subspecies *funduliforme* AY972049 at the 5' end of the primer and the LT3 reverse primer was perfectly matched for all three. All three primers sets amplified the *lktA* gene from the two