lktA-encoded leukotoxin is not a universal virulence factor in invasive *Fusobacterium necrophorum* infections in animals and man

Fusobacterium necrophorum is a Gram-negative non-spore-forming anaerobic rod that is an important primary and secondary pathogen in humans and farm animals (Brazier, 2002; Narayanan *et al.*, 2002). *F. necrophorum* is divided into two subspecies, *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme*. The former is more frequently encountered in animal infections and is more virulent in a mouse model of infection than the latter, the main human pathogen (Brazier, 2002; Smith & Thornton, 1993).

The leukotoxin of *F. necrophorum* is considered to be of pre-eminent importance in the pathogenesis of invasive infections in animals and humans (Hagelskjaer Kristensen & Prag, 2000; Saginala *et al.*, 1997). Tan *et al.* (1994) found the toxin in all of 22 invasive bovine isolates and Tadeballi *et al.* (2008) recently reported that all of four human clinical isolates studied carried the leukotoxin-encoding gene.

We report here a larger survey of the distribution of the leukotoxin-encoding gene of *F. necrophorum* in 100 invasive strains recovered from both humans and animals, including non-bovines. We examined consecutive isolates of *F. necrophorum* recovered from invasive infections in 43 humans and 57 animals, received for identification by the Anaerobe Reference Laboratory, Cardiff, UK, and the Veterinary Laboratories Agency, Winchester, UK, respectively. The species of animal, and the number of *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme*, respectively, examined from each were: cow (5, 12); sheep (13, 13); pig (0, 1); alpaca (3, 2); giraffe (0, 2); antelope (2, 0); nyala (1, 0); red deer (0, 1); goat (0, 1); wallaby (0, 1). All 43 human strains were *F. necrophorum* subsp. *funduliforme*.

We investigated the occurrence of the gene employing real-time PCR with primers

specific for the leukotoxin-encoding gene sequence (*lktA*). To exclude the possibility that variability within the leukotoxin-encoding gene may lead to false negatives, two *lktA* gene sequences (displaying 99.75 % nucleotide sequence identity), GenBank database accession numbers AF312861 and DQ672338, from geographically distinct *F. necrophorum* isolates (USA and China), were used to design three PCR primer pairs (LT1, LT2 and LT3), targeting different regions of the coding sequence of the gene. The nucleotide sequences (5'→3') and positions (according to LT gene sequence and accession number AF312861) of the primers were: LT1forward, CGA AAA CTC CAG AAT ATG ATC CGA AAG A (4656–4673); LT1reverse, CTA CCC ACG AAA CAG CTC CTC CCA CAG (5017–4991); LT2forward, ATC GGA GTA GTA GGT TCT GTT GGT GTT G (8689–8716); LT2reverse, GGC TGC TGC GAC TCC GAC TC (8886–8867); LT2probe, FAM–AAG CTG AAA TTA CAG CAG ATT CTA AGC AAG–TAMRA (8822–8851) (FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine); LT3forward, GGA GTA AGA GCA ACT ATG GGA GCA GCT AC (3592–3620); LT3reverse, CCC AAT CCA CCT TTT ACA GCA GCT CG (3977–3952). BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) of the PCR primer/probe sequences revealed a single 100 % identity match with the *F. necrophorum* leukotoxin-encoding gene sequence and no significant matches of concern with other sequences in the database were found. PCR was conducted using the Roche Diagnostics LightCycler, and TaqMan probe for LT2, and FastStart DNA Master SYBR Green I kit for the LT1 and LT3 assays. All study isolates were tested with each pair. Positive (*F. necrophorum* subsp. *necrophorum* NCTC 10576 and ATCC 25286, *F. necrophorum* subsp. *funduliforme* ATCC 51357) and

negative (*Staphylococcus aureus* subsp. *aureus* ATCC 25923) controls, including a no template master-mix control (sterile water), were included in each PCR run. All tests were repeated to confirm reproducibility.

Confirmation that specific amplification had occurred in all cases was obtained by demonstrating that the molecular mass of the PCR products was in agreement with the expected result. Ten randomly selected leukotoxin-positive strains from five humans and five animals (three from cows, one from a sheep and one from a giraffe) were subjected to nucleotide sequence analysis for the region spanning the LT2 primers. Additionally, the LT1- and LT3-derived amplicons from *F. necrophorum* subsp. *necrophorum* NCTC 10576 were also analysed. The sequence of the amplicons was then subjected to a BLAST search analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) to confirm the source as *F. necrophorum*. Fisher's exact probability tests, one-sided *P* value, were performed, employing the computer package StatsDirect (www.statsdirect.com), to assess whether the frequency differences observed between animal and human isolates in terms of the presence or absence of the leukotoxin-encoding gene in the subspecies were statistically significant.

The PCR results for the LT1, LT2 and LT3 primer sets were identical. All three assays were positive for the *F. necrophorum* reference strains and negative for the *S. aureus* control. Nucleotide sequence analysis of the amplified PCR products from the three *F. necrophorum* reference strains were in accord with the anticipated results.

Nucleotide sequence analysis of the five human and five animal isolates revealed a remarkable level of conservation within the LT2 target region, displaying just a single nucleotide change in any one isolate (0.66 % sequence divergence); this was similar to the

two *lktA* gene sequences, which displayed just 24 differences in the entire 9726 bp sequence. In conjunction with the identical results for all isolates with all three LT assays, this indicates that failure to detect the leukotoxin-encoding gene reflected the absence of the gene in these isolates rather than a high degree of genetic variability impairing successful amplification.

Overall, the *lktA* gene was detected in only a minority (47/100) strains studied, but there were marked differences by source and between the two subspecies of *F. necrophorum*. The gene was detected in a minority of human strains (14/43, 33%) but in the majority of animal strains (33/57, 58%, $P=0.01$). The gene was detected in the majority of *F. necrophorum* subsp. *necrophorum* strains (20/24, 83%), but only a minority of *F. necrophorum* subsp. *funduliforme* strains (27/76, 36%, $P<0.0001$). However, the prevalence of the gene in strains of *F. necrophorum* subsp. *funduliforme* of bovine origin was 10-fold higher compared with strains of this subspecies of non-bovine animal origin (11/12, 92%, versus 2/21, 9.5%, $P<0.0001$). In bovines, strains of *F. necrophorum* (combining subspecies) were more commonly leukotoxin-positive than non-bovine animal strains (16/17, 94%, versus 17/40, 42%, $P=0.0002$).

Our study demonstrates that whilst carriage of the *lktA* gene is usual amongst invasive bovine strains of *F. necrophorum*, it is much

less common in non-bovine invasive animal and human strains, and cannot therefore be considered the prime virulence factor in these hosts. A leukotoxin-based *F. necrophorum* vaccine has been shown to provide a protective effect against experimentally induced liver abscesses in cattle, and is licensed in the USA for the prevention of invasive disease in intensively reared cattle (Saginala *et al.*, 1997). Our results indicate that vaccination of non-bovines against leukotoxin may confer less benefit than in bovines. The finding that only a minority of human strains carried the *lktA* gene indicates that it is not an appropriate target for molecular diagnosis of pharyngitis and other invasive infection in humans, for which the *F. necrophorum* *rpoB* gene appears more appropriate (Aliyu *et al.*, 2005).

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