

Typing of *Fusobacterium necrophorum* Strains Using Polymerase Chain Reaction (PCR) Based Methods

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Background: *Fusobacterium necrophorum* as a non-spore-forming Gram-negative anaerobic bacillus is an important human and animal pathogen. It may cause severe systemic infections (Lemierre's syndrome) and some other infections. The aim of this study was to subtype *Fusobacterium necrophorum* by using PCR methods.

Materials and Methods: Twenty five strains of *Fusobacterium necrophorum* subspecies *funduliformis* were used. Extraction of DNA and typing of the strains using REP-PCR, ERIC-PCR and BOX-PCR were done.

Results: Molecular typing of *Fusobacterium necrophorum* using REP1-R-I and REP-2-I primers generated 2 to 5 amplicons ranging in size from 1500bp to 2000bp. GelCompar comparison of banding patterns revealed seven distinct ribotype strains from 25 strains tested of which most were 2 and 4 with 8 and 7 strains respectively. BOX-PCR subtyping generated 2 to 7 comparable amplicons ranging in size from approximately 600bp to more than 2000bp. ERIC-PCR subtyping generated 6 to 11 amplicons ranging in size from approximately 100bp to 1500bp.

Conclusion: *F. necrophorum* strains have genomic variations that suggest they are never truly clonal in nature, or they may have undergone localized genetic variation across worldwide. This study also showed subtypes existing in *Fusobacterium necrophorum* species. We have demonstrated that *Fusobacterium necrophorum* REP-PCR types can be divided into seven, three subtypes by BOX-PCR and six subtypes by ERIC-PCR. BOX-PCR typing proved to be the most discriminatory method, yielding two-seven major bands. The sample size was too small to interpret statistically.

Keywords: Typing, *Fusobacterium necrophorum*, PCR

1. Background

Fusobacterium necrophorum is a non-spore-forming, Gram-negative anaerobic bacillus that may exist as part of the human normal microbial flora. It has been divided into two sub-species called *F. necrophorum* ss. *necrophorum* (biovar A) and *F. necrophorum* ss. *funduliformis* (biovar B). It is an important human and animal pathogen. It may be the causative agent of localized (persistent sore throat syndrome) (1), orbital cellulitis or severe systemic infections. Systemic infections due to *F. necrophorum* are known as Lemierre's syndrome, which is characterized by acute jugular vein septic thrombophlebitis that progresses to sepsis (2); postanginal sepsis or necrobacillosis (3, 4). Septic polyarthrititis is rarely caused in teen-agers following infection with *F. necrophorum* (5). Origin of the infection is unknown but there are evidences that the infection originates from human or animal source (6, 7). This bacterium has a role in upper body infections such as mediastinitis, otitis media, mastoiditis and sinusitis (7). Metastatic abscesses in lungs, liver, kidneys and pyogenic arthritis/osteomyelitis (8, 9), endocarditis (10, 11) and rarely pneumonia and jaundice (12). Untreated or improperly treated cases can be fatal. The most common clinical course of severe infections in humans is a progressive illness from tonsillitis to septicemia in previously healthy young adults, which progresses to fever with rigors and leads to septic shock. It also causes infections in animals; calf diphtheria, labial necrosis in lambs, liver abscess in cattle, foot rot in ungulates, lumpy jaw and necrotic abscesses in wallabies, most of these infections are fatal. *F. necrophorum* possesses important virulence factors such as endotoxin (LPS), leukotoxin, haemolysin and haemagglutinin, which enable it to cause variety of diseases (2).

2. Objectives

The aim of this study was to subtype *Fusobacterium necrophorum* by using PCR methods

3. Materials and Methods

3.1. Bacterial strains

Twenty five strains of *Fusobacterium necrophorum* subspecies *funduliformis* were obtained from the Anaerobic Reference Laboratory, NPHS Microbiology, Cardiff, University Hospital of Wales, UK, which had been isolated from patients with septicemia, tonsillitis and pleuritis.

3.2. DNA extraction and PCR assays

Extraction of DNA and typing of the strains using PCR were done by the method as described previously (13) with some modifications. Briefly, DNA of the strains was extracted using Chelex-100 (Bio Rad, Hemel Hempstead, UK) and was suspended in 2mL of High Performance Liquid Chromatography-grade water (HPLC). It was then vortexed and dispensed in 100µL aliquots in 0.5mL tubes. One micro liter loopful of cells was added to the suspension, heated in a gently boiling water bath for 12 min and centrifuged for 10min at 15000g. The supernatant (40µL) was removed to a fresh tube and used as a crude DNA template.

3.3. REP- BOX- and ERIC PCR assays

For Repetitive Extragenic Palindromic Elements-PCR (REP-PCR), one microliter of extracted genomic DNA was used per reaction. Each 25µL of PCR mixture comprised 20µL of mastermix (including PCR buffer, deoxynucleoside triphosphate, MgCl₂, primers of REP1-R-I: 5'-III CGICGI CATCIGGC-3' and REP-2-I:5'-ICCICTTATCIGGCCTAC-3'

(Pharmacia Amersham) and *taq* DNA polymerase) and 4 μ L of HPLC water. Initial denaturation was 2 min at 95°C and 3s at 94°C. Thirty-one cycles of amplification were performed. Each cycle consisted of 30 s at 92°C, 1 min at 40°C and 8 min at 65°C. The final extension was for 8 min at 65°C. Ten microliters of the PCR product was electrophoresed in a 1% metaphore-ethidium bromide agarose gel. The ladder was super ladder-low 100bp PCR marker (Sigma-Aldrich, Germany). Electrophoresis conditions were a current of 80mA and a voltage of 20volt for 1h. The above method was used for Enterobacterial Repetitive Intergenic Consensus sequences-PCR (ERIC-PCR) and random DNA sequences-PCR (BOX-PCR) with the following exceptions: 0.5 μ L of ERIC1R-1 (5'-ATGTAAGCTCCTGGGG-TGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') primers used in ERIC-PCR, and 1 μ L of BOX AIR (5'-CTACGGCAAGGCGACGCTGACG-3') primer used in BOX-PCR. At the step 4 (annealing temperatures), 50°C was applied instead of 40°C for 1 minute.

The stained banding patterns were acquired using a computer and the GelDoc 2000 image system (Bio Rad). Using GelCompar software package (Applied Maths, Belgium) the bands were compared with library data of *Fusobacterium necrophorum* ribotypes, and ribotypes of the strains were identified. Banding patterns that differed by one or more major bands were assigned as different types.

In each batch, blind-coded repeats were included to test the reproducibility of the methods used, and batches were repeat tests at least twice.

4. Results

Molecular typing of *Fusobacterium necrophorum* using REP1-R-I and REP-2-I primers generated 2 to 5 amplicons ranging in size from 1500bp to 2000bp. GelCompar comparison of banding patterns revealed seven distinct strains from 25 strains tested of which most were 2 and 4 with 8 and 7 strains respectively. Figure 1 shows electrophoresis banding patterns of PCR products in 10 strains. All profiles shared REP-PCR amplicons of approximately 1500 and 1700bp in size. Only one strain belonged to types 5, 6 and 7. The most strains were in type 2 with 8 (32%) strains (Fig. 1).

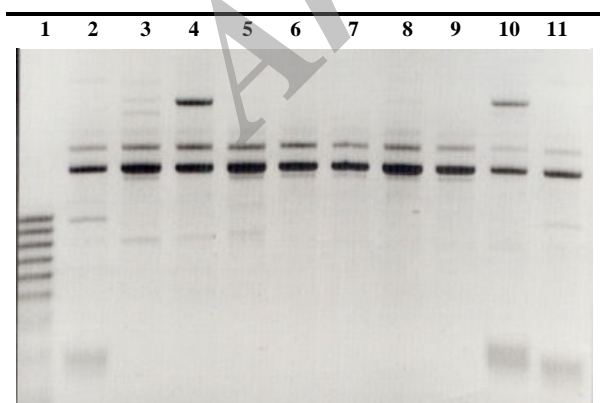


Figure 1. Ten distinct REP-PCR subtypes of *Fusobacterium necrophorum*. Lane 1: 100 bp ladder marker; lanes 2-11: PCR type.

BOX-PCR subtyping generated two to seven comparable amplicons ranging in size from approximately 600 bp to more than 2000 bp. All of the strains shared two bands of 600 and 1000 bp amplicons. According to BOX-PCR, the strains were divided into three groups with the most

groups falling in to group 1 (Fig. 2). ERIC-PCR subtyping generated six to eleven amplicons ranging in size from approximately 100bp to 1500bp. All the strains shared three bands of 300, 1100 and 1500bp amplicons. In this method of PCR, the strains were divided into six groups and the most of the strains were in group 2 (Fig. 3).

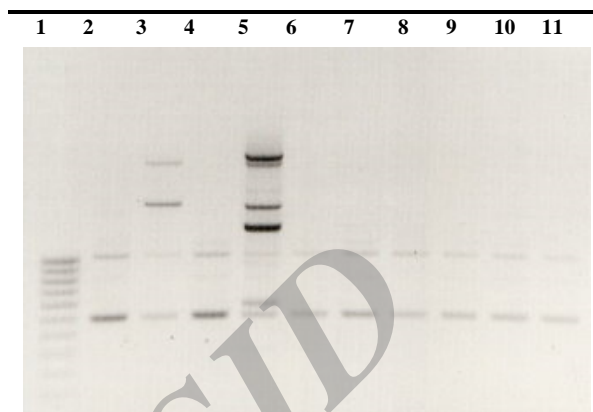


Figure 2. Ten distinct BOX-PCR subtypes of *Fusobacterium necrophorum*. Lane 1: 100bp DNA size marker; lanes 2-11: PCR type

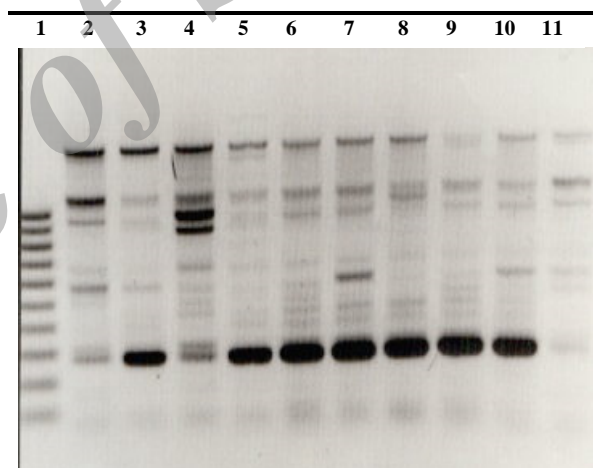


Figure 3. Ten distinct ERIC-PCR subtypes of *Fusobacterium necrophorum*. Lane 1: 100bp ladder marker; lanes 2-11: PCR type

5. Discussion

Fusobacterium necrophorum as an important human and animal pathogen and causative agent of localized (1), orbital cellulitis or severe systemic infections, is identified by using conventional methods. Although using DNA sequencing methods are widely used to type and identify bacterial species in many clinical microbiology laboratories (1, 3, 4), however, subtyping of the bacteria by these methods is needed to establish relationship among the strains isolated from different regions and sources for epidemiological purposes. In this study, there was no meaningful relationship between source of the organism and different subtypes.

Fusobacterium necrophorum strains have genomic variations that suggest they are never truly clonal in nature, or they may have undergone localized genetic variation across worldwide (14). Evidence to support the existence of subtypes of the other species of *Fusobacterium nucleatum* (*F. nucleatum*) has been reported previously (15). This study also showed existence of subtypes in *Fusobacterium necrophorum* species.

We have demonstrated that *Fusobacterium necrophorum* REP-PCR subtypes can be divided into total seven groups, three subtypes by BOX-PCR and six subtypes by ERIC-PCR. From these PCR methods REP-PCR was not suitable for subtyping the strains. Three subtypes from REP and ERIC-PCR were different from other subtypes in having extra bands of 2000bp and 800bp in REP and ERIC-PCR, respectively.

The results obtained from different studies suggest that although BOX-PCR, REP-PCR or ERIC-PCR typing has shown to be sensitive, quick and convenient for the differentiation of some bacterial strains such as *Xanthomonas*, *Pseudomonas* and *E. coli* it does not appear to be as effective for *Fusobacterium necrophorum*. REP-PCR also lacked the power to discriminate between isolates of *F. necrophorum*. Although this method has the capability to differentiate other bacteria (16-18). From these methods, BOX-PCR typing proved to be the most effective method, yielding two-seven major bands.

6. Conclusion

Twenty five strains of *Fusobacterium necrophorum* were isolated from different patients were analyzed by three PCR-based typing methods in order to determine genomic diversity within the strains. The three methods used were repetitive extragenic palindromic elements (REP), conserved repetitive DNA elements (BOX), and enterobacterial repetitive intergenic consensus sequences (ERIC). The performance of each typing method was assessed by comparing powers of discrimination, type ability and reproducibility. All methods had satisfactory levels of type ability and reproducibility as determined by the blind-coded repeats, however, BOX-PCR typing was proved to be the most discriminatory method, yielding two-seven major bands. *F. necrophorum* strains have genomic variations that suggest they are never truly clonal in nature, or they may have undergone localized genetic variation worldwide.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors' Contributions

Ahmad Rahmati performed all the laboratory work and Jon S. Brazier supervised the process.

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