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Proteomic analysis of endodontic infections by liquid chromatography–tandem mass spectrometry

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Abstract

Introduction—Endodontic infections are very prevalent and have a polymicrobial etiology characterized by complex interrelationships between endodontic microorganisms and the host defenses. Proteomic analysis of endodontic infections can provide global insights into the invasion, pathogenicity mechanisms, and multifactorial interactions existing between root canal bacteria and the host in the initiation and progression of apical periodontitis. The purpose of this study was to apply proteomic techniques such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the identification of proteins of bacterial origin present in endodontic infections.

Methods—Endodontic specimens were aseptically obtained from seven patients with root canal infections. Protein mixtures were subjected to tryptic in-solution digestion and analysed by reverse-phase nano-LC–MS/MS followed by a database search.

Results—Proteins, mainly of cell wall or membrane origin, from endodontic bacteria especially *Enterococcus faecalis, Enterococcus faecium, Porphyromonas gingivalis, Fusobacterium nucleatum, and Treponema denticola* were identified from all the samples tested. Identified proteins included adhesins, autolysins, proteases, virulence factors, and antibiotic-resistance proteins.

Conclusions—LC–MS/MS offers a sensitive analytical platform to study the disease processes in the root canal environment. The array of proteins expressed in endodontic infections reflects the complex microbial presence and highlights the bacterial species involved in the inflammatory process.

Keywords

bacteria; endodontics; liquid chromatography/mass spectroscopy; proteomics; virulence

Endodontic infections are unique and have a complex polymicrobial etiology often predominated by obligate anaerobes. The exact bacteria that mediate the symptoms or persistence of disease are not known. The initiation and progression of apical periodontitis may involve complex inter-relationships between the root canal microbes and host defense (15). The former may involve virulence factors, toxins, antibiotic resistance proteins, adhesins bacteriocins, and quorum-sensing molecules. The latter might involve host cells, inflammatory mediators, metabolites, neuropeptides or other effector molecules, and immunoglobulins. Both culture-based and molecule-based studies have shown that the most prevalent bacterial species

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present in endodontic infections belong to genera such as *Fusobacterium, Porphyromonas, Prevotella, Bacteroides, Peptostreptococcus* and *Enterococcus*, especially *Enterococcus faecalis*, which is frequently associated with persistent cases (4,6,23,25). The degree of microbial complexity and diversity often present in endodontic infections renders the investigation of individual microorganisms, specific virulence factors, or specific host effector molecules ineffective, or at best insufficient, to fully comprehend the disease process. Conversely, emerging proteomic technologies such as mass-spectrometry-based peptide sequence analysis can provide global insights into the invasion, virulence, pathogenicity mechanisms, and multifactorial interactions existing between the endodontic microorganisms and the host.

Among the proteomic techniques commonly used for analysis of protein expression in biological fluids, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a popular technique for the separation of proteins. However, there are many limitations associated with this technique that have been well outlined (9). More recent efforts have focused on using liquid chromatography/mass spectrometry (LC/MS) with either a top-down (7) or bottom-up (27) approach. The bottom-up or shotgun approach involves the proteolytic digestion of all the proteins in the sample and relies on database analysis of the individual peptides to identify the source proteins in the sample. This method reduces sample handling time and eliminates the need for processing of individual proteins. It allows the direct analysis of extremely complex biological samples and rapidly generates protein profile and sequence information. Tandem mass spectrometry (MS/MS) has become increasingly important and indispensable for identifying complex protein mixtures in high-throughput proteomics experiments (1).

Within the field of oral biology, the use of proteomic methods has largely been confined to the use of 2D-PAGE. Proteomic approaches have been applied to analyse the physiological adaptations or mechanisms of survival, invasion or pathogenicity mainly in *Streptococcus mutans* (8), *Streptococcus oralis* (26), *Fusobacterium nucleatum* (2,30), *Porphyromonas gingivalis* (13,29) and *E. faecalis* (17). Developments in the proteomic analysis of oral pathogens were summarized by Macarthur and Jacques (14). No attempt has been made to identify the proteins that are expressed *in vivo* in the root canal in endodontic infections. In this context, we report for the first time the application of proteomic techniques such as liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) in a shotgun approach for the identification of proteins expressed in the root canal in endodontic infections.

All patient-related procedures used in this study conformed to protocols approved by the Institutional Review Board at the University of Maryland. Patients presenting for endodontic treatment for a tooth with either a primary or a persistent (previously endodontically treated) endodontic infection were included. The teeth involved had a negative pulp test result (for primary cases) and a periradicular lesion at least 3 mm in diameter.

Samples were collected from teeth with endodontic infections, primary ($n = 4$) or persistent ($n = 2$) as per the protocol described previously (4). One case was initially described as a primary infection but upon access it was determined to have undergone incomplete endodontic treatment years earlier and consequently was not assigned a primary/persistent category.

Following isolation with a rubber dam, the field was disinfected with 30% H_2O_2, followed by 5% tincture of iodine and 5.25% sodium hypochlorite. The halides were inactivated with 5% sodium thiosulfate. Following access preparation, microbial specimens were obtained from root canals using one sterile K-file or H-file, which was first used to disrupt canal wall biofilms, and three sterile paper points. The metallic portion of the file and the paper points were placed in sterile, DNA-free and RNA-free vials containing 1.5 ml sterile 10 mM Tris–HCl (pH 8.0) and 0.5 g sterile glass beads (0.71–1.18-mm diameter). The vials were frozen at −70°C until

use. The vials containing paper point specimens were vortexed for 2 min to disperse microbial cellular material into suspension followed by centrifugation at 3144 ***g*** for 10 min. The supernatant was withdrawn and used for proteomic analysis.

The protein mixtures were initially subjected to a buffer exchange with 50 mM ammonium bicarbonate (pH 8.2) and reduced with 10 mM dithiothreitol for 1 h at 56°C. Cysteine residues in the protein samples were alkylated with 40 mM iodoacetamide for 45 min at room temperature with shaking in the dark. Following alkylation, the samples were neutralized by adding 200 mM dithiothreitol and incubated for 1 h. The samples were buffer exchanged with 50 mM ammonium bicarbonate and digested with sequencing-grade modified porcine trypsin (Promega, Madison, WI, USA) overnight at 37°C with a 1 : 50 (weight : weight) trypsin : protein ratio. The peptides were vacuum-dried and reconstituted with 0.1% formic acid before LC–MS/MS analysis.

Mass spectrometric analysis of the extracted peptides was carried out using a nanoscale reverse-phase high-performance liquid chromatography capillary column (10.5 cm × 75 μm inner diameter fused silica reverse-phase C18 column; Pico frit column (New Objective, Woburn, MA, USA). The sample peptides (10 μl injection volume) were separated by a linear gradient of 5%/90% acetonitrile/water mixture, containing 0.1% formic acid in 90 min at a flow rate of 300 nl/min. The gradient was provided by a Surveyor LC pump (Thermo-Finnegan, San Jose, CA, USA). The nanospray voltage was set to 2 kV. Mass spectrometric analysis was carried out on an LCQ Deca XP linear ion trap (Thermo-Finnegan, San Jose, CA, USA) which was operated on positive-ion mode. Peptide ions were detected in a survey scan (five most intense peaks) from 400 to 1800 atomic mass units (amu) (three microscans) followed by five data-dependent MS/MS scans (five microscans each, isolation width 2.5 amu, 35% normalized collision energy). The acquired spectra were further used for a database search.

After the acquisition of MS/MS spectra, they were searched against bacterial protein databases downloaded from the National Center for Biotechnology Information (NCBI), using the software algorithm SEQUEST (28). The SEQUEST criteria used for protein identifications were as follows: strict trypsin enzyme digestion, peptide mass tolerance of <2.5 Da, monoisotopic mass of the precursor and product ions <0.15 Da, possible modifications of methionine oxidation and carboxyamidomethylation. For the positive identification of any individual protein, at least two peptides were required. The threshold of cross-correlation (Xcorr) scores set for peptides were 1.5, 2.0, and 2.5 for +1, +2, and +3 charged fully digested peptides, respectively, and a threshold of 0.08 was required for ΔCn values for individual peptides (16). The same set of MS/MS spectra were searched against *E. faecalis* V583 protein sequences using the SORCERER™ IDA server (SageN, Inc., San Jose, CA, USA). The validation of protein identification was performed with TRANS-PEPTIDE PIPELINE (TPP; open source software: http://www.sourceforge.net) software as specified by the PEPTIDE PROPHET algorithm with a PEPTIDE PROPHET score higher than 0.9.

Preoperative specimen samples taken from seven patients with primary or persistent infections were used for proteomic analysis of endodontic infections. Diversity and prevalence of bacterial phylotypes in these samples were analysed by both broad-based polymerase chain reaction (PCR) cloning and sequencing and by genus-specific PCR and will be reported separately. Application of LC–MS/MS resulted in the identification of a number of bacterial proteins; these are listed in Table 1 and Table 2.

Many of the identified proteins are outer membrane proteins probably involved directly in the pathogenic processes, for example, adhesins, autolysins, proteases, penicillin-binding proteins, and those with a predicted function of virulence, invasion, nutrient binding, and cell envelope biogenesis. A few of the proteins identified were hypothetical with no putative function. The majority of the proteins detected were from phylotypes that had been identified in endodontic

infections such as *E. faecalis, Enterococcus faecium, Treponema denticola, Bacteroides fragilis, Bacteroides vulgatus, F. nucleatum, Parvimonas micra*, and *P. gingivalis*.

Among the proteins of non-enterococcal origin, proteins such as putative outer membrane protein probably involved in nutrient binding, spermidine/putrescine ABC transporter, and penicillin-binding protein 2B were found in more than one patient. Proteins with a putative function in antibiotic resistance, invasion/virulence, and nutrient binding were found in five of the seven patients. Chaperonins and those proteins involved in the stress response were identified in three specimens.

Among the proteins that were identified with *E. faecalis* as the source, proteins such as ABC transporter-ATP-binding protein, cell wall surface anchor family protein, *N*-acetylmuramoyl-L-alanine amidase, penicillin-binding protein, BacB protein, tetM, and VanSG2 sensor protein were very prevalent. Proteins involved in antibiotic resistance, adhesion, membrane transport, virulence, autolysins, bacteriocins, and conjugation were highly predominant and were present in three of the seven patients, highlighting a potentially active role of *E. faecalis* in the infectious processes of endodontic diseases. Other enterococcal species such as *E. faecium* produced proteins involved in the stress response and autolysins along with many uncharacterized proteins.

Even though no purposeful attempt was made to lyse the bacterial cells present in the sample, a few proteins of subcellular origin were also identified. These proteins might have resulted from cell lysis caused by vortexing with glass beads, which was performed to dislodge microbial cells from the paper points, or from autolysed cells.

In most cases, bacterial phylotypes from which the proteins were identified had been detected in the respective patient specimens by clonal analysis (data not shown). In four patients, three with primary disease and one with persistent disease, in whom no enterococci were detected by either broad-range PCR cloning and sequencing or by genus-specific PCR, the proteomic analysis could not detect any proteins of enterococcal origin. In the rest of the specimens where *Enterococcus* spp. were highly prevalent, many proteins of enterococcal origin were detected. Proteins were also identified from other bacteria that are not recognized as strict endodontic pathogens such as *Burkholderia* sp., *Bacillus* sp., and *Lactobacillus* sp. They were either detected in these patient specimens by clonal analysis or were listed in literature as present in periodontal infections, as part of the biofilm communities of dental water lines, or could even be food contaminants (11,22).

P. gingivalis was found to express numerous potential virulence factors such as fimbriae, hemagglutinins, lipopolysaccharides, and various proteases (12). In the samples selected for this study, *P. gingivalis* was not prevalent; it was detected in only one case. Increased expression of chaperonins such as GroEL by *F. nucleatum* under conditions that mimic those in periodontal pockets has been reported (30). The identification of GroEL protein, as well as of other proteins with putative functions as chaperonins, is relevant because they have been reported to be immunodominant bacterial antigens that may play a role in pathogenesis by stimulating the host immune response (24). Proteins involved in cell wall biosynthesis in *S. mutans* (8), ampicillin resistance in *F. nucleatum* (2), and virulence in *P. gingivalis* (29) were also reported by proteomic analysis.

Enterococcal spp., especially *E. faecalis*, are reported to be highly prevalent (12–90%) in endodontic infections and are purported to play an important role in treatment failures (6,10, 19). However, these organisms were also shown to be prevalent in endodontically treated teeth that have no periapical lesions (10,31), thereby raising doubts about their role in the pathogenesis of the lesions in every case. A large number of virulence factors have been recognized among enterococci including gelatinase (21), cytolysin (3), enterococcal surface

proteins (20), and *E. faecalis* collagen adhesin (18). Expression of cytolysin was found to be prevalent by proteomic analysis in enterococcal isolates from endodontic infections (17). The fact that the proteins from the *tet* and *Van* operons were predominant in all the patient cases where enterococci were detected by genomic analysis is important from a treatment perspective because antibiotics are known to be not very effective in treating chronic or localized acute endodontic infections or in preventing flare-ups. *E. faecalis* was highly prevalent in the patient sample in which VanE protein was identified by proteomic analysis. In addition, *E. casseliflavus*, known for its low resistance to vancomycin, was also highly prevalent in the same patient samples where vancomycin-resistance proteins were predominant.

Many studies have outlined the predominant taxa associated with endodontic infections with respect to the clinical manifestations (4,5). However, drawing conclusions on the identity of the most pathogenic or causative organisms from the complex phylogenetic diversity by genomic analysis alone is not feasible. It is evident from proteomic analysis that a majority of the virulence and antibiotic-resistance proteins present in the samples analysed here were secreted by enterococci, stressing their role as potentially serious pathogens among the polymicrobial flora present in endodontic infections.

Proteomic techniques such as LC–MS/ MS offer a very good analytical platform with high sensitivity to study the disease processes in the root canal environment characterized by a complex microbial presence and very low sample concentrations. In addition, proteomic data clearly complement the genomic information gathered on individual specimens in terms of prevalent phylotypes. With all the challenges inherent in proteomic analyses, genomics and proteomics together could provide a better understanding of the etiology and pathogenesis of endodontic diseases. Such global information about both genes and protein expression provides a rational basis for identification of molecular targets that could be used for better diagnoses, prevention, and treatment of endodontic infections.

In conclusion, this study details the first direct identification of bacterial proteins expressed in endodontic infections. It is obvious that the proteins identified in this study represent only a fraction of the major protein components because of the small sample size and the fact that the genomes of many oral bacteria are not yet fully annotated. However, these results provide a new avenue for directly characterizing the virulence-associated proteins commonly present in endodontic infections and thereby for characterizing the pathogenic species among the diverse endodontic taxa that should ultimately lead to better treatment strategies.

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Table 1

Identification of proteins of non-enterococcal origin present in endodontic infections

UniProt acc. no.	Protein	Bacteria	No. of peptides	Biological function
Q39PI2	Lipocalin-like protein (1)[1]	*Burkholderia* sp.	8	Transport, Starvation response
A3I6E1	Microbial collagenase metalloprotease (2)	*Bacillus* sp.	4	Invasion, virulence
Q5LBV7	Hypothetical protein BF2715 (1)	*Bacteroides fragilis*	2	Unknown
Q5LB48	Putative ABC transport system, membrane protein (1)	*B. fragilis*	7	Multidrug transport system
Q5LEF4	Putative outer membrane protein (5)	*Bacteroides vulgatus*	4	Nutrient binding, Transport
A6L076	Penicillin-binding protein (2)	*B. vulgatus*	5	Antibiotic resistance
A5CNU5	Putative beta lactamase/penicillin-binding protein (1)	*Clavibacter michiganensis*	4	Antibiotic resistance
Q8FLL7	Putative virulence-associated protein (1)	*Corynebacterium* sp	2	Virulence
B2QEG9	Fibronectin-binding A domain protein (2)	*Exiguobacterium* sp	5	Adhesin
Q8RGG3	Outer membrane porin F (2)	*Fusobacterium nucleatum*	3	Cell envelope biogenesis
Q8RIE8	Oligopeptide-binding protein oppA (1)	*F. nucleatum*	4	Transport
Q8RI38	Spermidine/putrescine ABC transporter permease (3)	*F. nucleatum*	4	Transport
Q8RHF5	ABC transporter substrate-binding protein (1)	*F. nucleatum*	3	Transport
A5TVG7	*N*-acylneuraminate cytidylyltransferase (1)	*F. nucleatum*	4	Lipopolysaccharide biosynthetic process
Q040U6	Uncharacterized membrane-bound protein (4)	*Lactobacillus gasseri*	3	Unknown
Q9CIL7	Penicillin-binding protein 2B (3)	*Lactococcus lactis*	4	Antibiotic resistance
Q2G7B1	Conserved hypothetical membrane protein (1)	*Novosphingobium aromaticivorans*	12	Unknown
B1DLX6	ABC transporter related (1)	*Paenibacillus* sp.	3	Transport
B1D7U4	Coagulation factor 5/8 type domain protein (2)	*Paenibacillus* sp.	8	Cell adhesion
Q7MWV9	YngK protein (1)	*Porphyromonas gingivalis*	2	Unknown
B2RKT2	Preprotein translocase SecA subunit (1)	*P. gingivalis*	3	Protein export
Q7MUL8	TraG family protein (1)	*P. gingivalis*	3	Conjugation
Q6ABI3	Putative penicillin-binding protein (2)	*Propionibacterium acnes*	4	Antibiotic resistance
Q0S9R0	Possible universal stress protein (2)	*Rhodococcus* sp.	4	Stress response
P95800	groEL protein (1)	*Stenotrophomonas maltophilia*	4	Chaperonins
Q3D4N5	PTS system, IIBC components (1)	*Streptococcus agalactiae*	3	Sugar transport
Q3CY15	Fibrinogen-binding protein (3)	*S. agalactiae*	7	Adhesion
A8AXJ4	Lipoprotein, putative (1)	*Streptococcus gordonii*	5	Adhesion
Q2ZXI8	Binding-protein-dependent transport systems (1)	*Streptococcus suis*	3	Transport
Q73QT1	Bacterial immunoglobulin-like domain protein (1)	*Treponema denticola*	6	Putative adhesin
A3CPZ1	Thioredoxin reductase, putative (1)	*Streptococcus sanguinis*	3	Removal of superoxide radicals, chaperones

[1] Numbers in parenthesis indicate the number of patient samples where the protein was identified.

Table 2

Identification of proteins of enterococcal origin present in endodontic infections

UniProt acc. no.	Protein	No. of peptides	Probability score	Biological function
Q82ZX8	ABC transporter, ATP-binding protein (3)[1]	23	1	Transport
Q837A1	ABC transporter, ATP-binding/permease protein (2)	28	1	Transport
Q82YN1	Aggregation substance PrgB (1)	11	1	Virulence factor
Q833M7	ATP-dependent Clp protease, ATP-binding subunit ClpX (2)	7	1	Chaperone
P37710	Autolysin (2)	5	0.99	Autolysin
Q47779	BacB protein (3)	6	0.99	Bacteriocin, Immunity protein
Q3Y1R6	Cell wall hydrolase/autolysin (1)	7	1	Peptidoglycan catabolic process
Q833P7	Cell wall surface anchor family protein (3)	8	1	Adhesion
Q839K7	Conjugal transfer protein, putative (2)	9	1	Conjugation
Q8KH16	Conserved hypothetical protein (3)	37	1	Unknown
Q835K7	Drug resistance transporter, EmrB/QacA family protein (1)	6	1	Tetracycline transport
Q835S9	Endolysin (1)	3	1	Peptidoglycan catabolic process
Q836Z9	Extracellular protein, putative (1)	4	1	Peptidoglycan turnover
A1YGU1	Extracellular serine proteinase (1)	7	0.99	Proteolysis, Virulence
Q820V6	FtsK/SpoIIIE family protein (3)	7	1	Cell division
Q49SF0	Gls24 (1)	6	1	Stress response
Q832Q0	Glycosyl transferase, group 2 family protein (1)	7	1	Cell envelope biogenesis
Q836W3	Hemolysin A (1)	11	1	Virulence factor
Q838X9	Lipoprotein, putative (2)	8	1	Unknown
Q835M5	Magnesium-translocating P-type ATPase (2)	8	1	Magnesium ion transport
Q832Q4	Membrane protein, putative (3)	13	1	Unknown
Q9RPP2	Membrane-associated zinc metalloprotease, putative (1)	8	1	Pheromone production
Q834G2	Metallo-beta-lactamase, AtsA/ElaC family (1)	4	1	Antibiotic resistance
Q830Y6	*N*-acetylmuramoyl-l-alanine amidase, family 4 (3)	13	1	Peptidoglycan catabolic process
Q79A51	Nickase (1)	7	1	Unidirectional conjugation
Q838B1	Oligoendopeptidase F, putative (1)	5	1	Proteolysis
Q5G3N8	PcfD (1)	5	1	Conjugation
Q9K3C9	Penicillin-binding protein 4 (2)	9	0.99	Antibiotic resistance
Q82ZH7	Peptidase T (1)	3	1	Proteolysis
Q839D6	Peptidase, M20/M25/M40 family (2)	4	0.99	Proteolysis
Q830Q0	Peptidase, M42 family (3)	13	1	Proteolysis
Q834B4	Phosphate ABC transporter, ATP-binding protein (2)	31	1	Phosphate transport
Q82ZM8	Polysaccharide lyase, family 8 (1)	4	1	Cell adhesion
Q82ZY7	Potassium uptake protein (1)	4	1	Potassium ion transport
Q82YM8	PrgE (1)	29	1	Pheromone response
Q837M0	PTS system, IIC component (2)	8	1	Carbohydrate transport
Q8KI44	Putative uncharacterized protein (1)	34	1	Unknown
Q82ZQ8	Rhodanese family protein (2)	13	1	Cyanide detoxification

UniProt acc. no.	Protein	No. of peptides	Probability score	Biological function
Q6WS02	Rlx-like protein (1)	8	0.99	Conjugation
Q82ZJ9	Sortase family protein (2)	8	1	Surface protein anchoring
Q832J2	Sugar ABC transporter, sugar-binding protein (1)	3	1	Transport
Q833W2	Sulfatase domain protein (1)	5	1	Cell envelope biogenesis
Q831L1	Teichoic acid biosynthesis protein, putative (1)	5	1	Teichoic acid biosynthetic process
Q2UXR5	Tetracycline resistance protein (1)	10	0.99	Tetracycline resistance
Q47810	Tetracycline resistance protein tetM (3)	71	1	Tetracycline resistance
Q5GBH8	TetT (1)	4	0.99	Tetracycline resistance
Q82YJ4	Toxin ABC transporter, ATP-binding/permease protein (2)	8	1	Bacteriocin/lantibiotic exporters
Q832B2	TraG family protein (1)	5	1	Unidirectional conjugation
O07108	UDP-*N*-acetylmuramoylalanine-d-glutamate ligase (1)	7	1	Peptidoglycan biosynthetic process
Q3XXB2	Uncharacterized protein conserved in bacteria (1)	7	1	Unknown
Q93A46	VanE (1)	3	1	Peptidoglycan biosynthesis
Q47745	VanSB, Sensor protein (2)	7	1	Response to vancomycin
Q30BF3	VanSG2, Sensor protein (3)	17	1	Signal transduction
Q30BE9	VanTG2 (1)	6	0.99	Response to vancomycin
Q30BF2	VanWG2 (1)	4	1	Unknown
Q836M0	Von Willebrand factor type A domain protein (1)	3	1	Adhesion
P0A4M1	Zeta-toxin (1)	5	1	Programmed cell death of plasmid free cells

Protein-Prophet scores (pp) above 0.9 were accepted; at this cut-off, the rate of false-positive protein identifications is >10%.

[1] Numbers in parenthesis indicate the number of patient samples where the protein was identified.