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SESSION VI: DIAGNOSTIC METHODS & MICROBIOLOGY

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DIAGNOSTIC METHODS & MICROBIOLOGY

IS SEQUENCING THE SOLUTION?

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Routine identification and even routine culture of anaerobes appears to be on a steady decline among modern clinical laboratories. As capacity for basic anaerobic microbiology wanes, anaerobic isolates are frequently described in limited terms such as "mixed anaerobes," and these isolates often receive no further study or characterization. The incomplete identification and characterization of these organisms has potential to significantly limit our knowledge and understanding of anaerobic pathogens by limiting our understanding of the most common causes of infection, if and when infections might be linked to a common source (outbreaks), whether there are changes in the pattern or frequency of specific organisms, and the appropriate treatment of infections. Moreover, this practice facilitates erosion of primary capacity in the clinical microbiology laboratory. Many clinical laboratories have no capacity to adequately identify anaerobic bacteria. According to recent CAP surveys from 2004 through 2009, an average of 8% of participants (3% of referee) laboratories refer both culture and identification of anaerobes to an external laboratory, and 12% of participants (8% of referees) perform only the initial culture and isolation. On average, only 40% of laboratories (55% of referees) were able to identify commonly-isolated challenge anaerobes to the species level.

The CDC anaerobe reference laboratory receives isolates from across the United States and abroad. Over the past decade, our laboratory has received an average of 85 isolates per year for reference identification. The most commonly received isolates are *Actinomyces* sp. and *Clostridium* sp. (both approx 20%). *Bifidobacterium* sp. and *Propionibacterium* sp. are the next most common isolates and represent approximately 5% each. Prior to introducing 16S sequencing into our routine algorithms for anaerobe identification, as many as 24% of isolates were not identifiable at CDC on the basis of traditional phenotypic and biochemical testing (Gram stain, morphology, biochemical reactions, and GLC). However, since 16S was implemented, the number of unidentified isolates has dropped to nearly zero.

Sequence analysis provides an unambiguous and reproducible data set that can be easily shared between investigators, queried against multiple databases, and archived for future use. Limitations include the high reagent costs and specialized equipment associated with sequencing, as well as the difficulty in selecting the best molecular targets and most appropriate databases. Although the reagent and device costs associated with routine sequence analysis are high, the improvements in turnaround time and definitive ID have been well worth the added expense at our institution.

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IDENTIFICATION OF GRAM-POSITIVE ANAEROBIC COCCI BY MATRIX ASSISTED LASER DESORPTION AND IONIZATION TIME OF-FLIGHT MASS SPECTROMETRY

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Gram-positive anaerobic cocci (GPAC) are part of the commensal microbiota of humans and account for about one-third of the anaerobic bacteria recovered from clinical materials. The phenotypic identification of GPAC is not always straightforward and some species cannot be easily differentiated from each other, e.g. *Peptoniphilus harei* and *Peptoniphilus asaccharolyticus* share the same biochemical features. Nowadays, molecular methods are available to identify GPAC, e.g. fluorescent *in situ* hybridisation (FISH) and 16S rDNA sequencing. More recently, Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has become available for the identification of bacteria. In this study a database of mass spectral patterns of GPAC was compiled using 89 sequenced clinical isolates and reference strains. To test the potential for rapid identification of GPAC by MALDI-TOF MS, 107 blinded clinical isolates were identified using this database. The results were compared to identification results obtained by 16S rDNA-sequencing or FISH.

For all strains, mass spectra of good quality could be obtained. Strains belonging to the same species largely grouped together by cluster analysis of mass fingerprints. For example strains of *Parvimonas micra* and *Anaerococcus vaginalis* were grouped in single clusters. However, a high intraspecific variability for some species was also revealed. For example, strains of which the closest relative was *Peptoniphilus ivorii* were grouped in three distant clusters (<50% similarity of mass fingerprints). Only one strain showed similarity to the type strain DSM 10022. This can be taken as evidence that this species is in fact polyphyletic. A similar result, although less pronounced, was obtained by 16S rDNA sequence analysis. Identification of the blinded clinical isolates was correct for 96 out of 107 strains (90%). Of the 11 strains that could not be identified with MALDI-TOF MS 6 showed less than 98% sequence similarity to the closest type strain, which means that those strains are atypical or probably belong to another (new) species. Two strains were not present in the database at the date of analyses. For the remaining 3 strains it is yet unknown why no identification could be obtained.

MALDI-TOF MS is a very rapid and promising tool for the characterization and identification of anaerobes, even when they are as phylogenetically heterogeneous as GPAC.

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TESTS TO DETECT *CLOSTRIDIUM DIFFICILE:* THE NEXT GENERATION

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Clostridium difficile (*C difficile*) causes 25-30% of cases of antibiotic associated diarrhea and most cases of pseudomembranous colitis. Patients presenting with diarrhea after hospitalization for 3 or more days should be tested for *C difficile*. There are many options available for testing, each of which has inherent advantages and disadvantages. Most laboratories perform toxin testing using an enzyme immunoassay method. In general these tests have sensitivities ranging from 60-70% and specificities of 98%. When using these methods, symptomatic patients with negative tests should be tested by another more sensitive method. Cell culture cytotoxicity neutralization assays (CCNAs) were considered the gold standard in the U.S. until recently. A two step algorithm using an EIA for glutamate dehydrogenase detection followed by testing positives using CCNA, offered an improved alternative until the availability of molecular assays. Although early studies that compared the GDH assay to CCNA demonstrated high sensitivity and negative predictive values, more recent comparisons to toxigenic culture and PCR have shown the sensitivity to be in the mid to high 80's. When testing using a sensitive assay, repeat testing is not cost-effective. Because of the recent increase in a toxin variant epidemic strain, many laboratories have resurrected bacterial culture on selective media to provide isolates for epidemiological characterization. Toxigenic culture has emerged as the new gold standard against which newer assays should be compared. However, there is no agreed upon standard method for culture performance. At least 3 FDA cleared real time PCR assays are available to clinical laboratories and all have been well evaluated in the literature. Several other molecular assays are in development. Because these assays detect a gene that encodes toxin and not the toxin itself it is important that laboratories follow IDSA guidelines and test only patients with diarrhea (defined as 3 loose or unformed stools within a 24 h period). These molecular assays have been shown to be superior to EIAs, CCNA and 2-step algorithms, but not to toxigenic culture. More studies are needed to assess the impact of molecular tests on treatment and nosocomial spread.

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BACTEROIDES FRAGILIS FIBRINOGEN INTERACTIONS

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Bacteroides fragilis is a bacterium that resides in the normal human gastro-intestinal tract. It is also the most commonly isolated Gram negative obligate anaerobe from human clinical infections, such as intra-abdominal abscesses as well as the most common cause of anaerobic bacteraemia. Abscess formation is important in bacterial containment, limiting dissemination of infection and bacteraemia. Human fibrinogen is a 340kDa glycoprotein dimer present in plasma. Each monomer consists of three polypeptide chains; alpha, beta and gamma. Although the primary function of fibrinogen is in blood clotting, it also plays a key role in fibrin abscess formation. We have shown that *B. fragilis* NCTC9343 binds human fibrinogen. A putative ***B****acteroides* ***f****ragilis*-**f**ibrinogen-**b**inding **p**rotein, designated BF-FBP, identified in the genome sequence of NCTC9343, was cloned and expressed in *E. coli*. The purified recombinant BF-FBP binds primarily to the human fibrinogen Bβ-chain. In addition we have identified fibrinogenolytic activity in *B. fragilis* exponential phase culture supernatants. All nine clinical isolates of *B. fragilis* examined to date degraded human fibrinogen. Our data raise the possibility that the fibrinogen-binding protein of *B. fragilis*, along with a variety of fibrinogenolytic proteases, may be important virulence factors that facilitate dissemination of infection via reduction or inhibition of abscess formation.

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COLLAGEN ADHESINS AND PROTEASES OF SOUTH AFRICAN CLINICAL STRAINS OF *BACTEROIDES FRAGILIS*

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Adhesion to, and degradation of, components of the extracellular matrix, such as collagen, is critical in bacterial colonisation and host cell invasion. *Bacteroides fragilis,* an opportunistic human pathogen, binds to collagen and degrades a number of protein substrates, but the proteins responsible for these functions are not known. In this study, South African clinical strains (N=23) of *B. fragilis* were analysed with respect to collagen binding and collagenase activity. The most active strain, *B. fragilis* GSH18, was studied further. Zymography of the outer membrane fraction (OMP), using co-polymerised collagen SDS-PAGE, showed that the bacterium produced two collagenases (45 and 37 kDa). Collagen Far Western analysis of the OMP detected three collagen adhesins (45, 34 and 33 kDa). The 34 kDa adhesin was purified by collagen affinity chromatography and identified by mass spectroscopy and BLAST analysis. The protein was identical to the hypothetical protein, of previously unknown function, encoded by the *B. fragilis* 9343 ORF BF0586. Functionality of the protein was confirmed by targeted insertional mutagenesis of the BF0586 gene and demonstrating the loss of the 34 kDa adhesin in the OMP fraction. This study is the first to report the identity of a collagen adhesin protein in *B. fragilis* and has significance in the context of the pathogenicity of this organism.

Keywords: *Bacteroides fragilis*, adhesins, proteases, collagen

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COMPARISON OF THE BD GENEOHM™ CDIFF ASSAY TO A THREE-STEP ALGORITHM TO DETECT TOXIGENIC *CLOSTRIDIUM DIFFICILE* IN FECAL SAMPLES

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Purpose: The diagnosis of *Clostridium difficile* (Cdiff) infections continues to pose significant challenges in healthcare facilities. The purpose of this study was to examine the current performance of the now commercially available BD GeneOhm™ Cdiff (BD Diagnostics, San Diego, CA) real-time Polymerase Chain Reaction (PCR) assay as a diagnostic test for the detection of toxigenic Cdiff strains from fecal specimens. PCR was compared to a testing algorithm that included an enzyme immunoassay (EIA) assay for the "common antigen" glutamate dehydrogenase (GDH), a toxin A/B EIA, and cytotoxin neutralization/toxigenic culture. The BD GeneOhm™ Cdiff assay is a rapid, qualitative test performed on the Cepheid SmartCycler® (Cepheid, Sunnyvale, CA). The assay uses PCR for the amplification and detection of the toxin B gene (tcdB).
Methods: Briefly, liquid to soft stools received in the clinical laboratory for Cdiff testing were first screened with the TechLab Cdiff chek™-60 EIA for detection of GDH, and positive results were confirmed with the Tox A/B assay (Remel). If the Tox A/B was negative, a cytotoxin neutralization assay (Techlab) and culture for toxigenic Cdiff were performed. Concurrently, the BD GeneOhm™ PCR assay was performed on each stool specimen. Stools were collected, processed, and tested according to the institution's standard of care and each assay was performed according to manufacturer's package insert.
Results: A total of 300 specimens were included in the study: 234 (78%) were negative with both PCR and GDH while 30 (10%) were positive for both. Of 17 initially discrepant PCR/GDH results, 11 resolved with additional testing and chart reviews. Compared to the cytotoxin neutralization assay as the reference standard, the resolved sensitivity, specificity, negative and positive predictive values for the PCR were 94.4%, 95%, 99.2% and 72.3%, respectively. Compared to toxigenic culture as the gold standard, the PCR sensitivity and specificity were 95% and 93.8%. The sensitivity and specificity of the GDH assay were 95% and 88%.
Conclusions: The diagnosis of toxigenic *C. difficile* using combinations of EIA, culture and stool cytotoxin tests can be labor intensive, time-consuming or lack sensitivity or specificity. The current sensitivity of the GDH assay was improved, compared to what we observed two years ago (79%-81%). The BD GeneOhm™ Cdiff assay, performed directly on stool specimens, currently offers performance that is comparable to the multi-test algorithm and produces results that can be considered final in less than 3 hours. In contrast to GDH screening results, the PCR method specifically detected toxigenic *C. difficile* without the need for additional confirmatory testing.

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EXTENSIVE COMPARISON OF THE ESWAB (LIQUID AMIES TRANSPORT SYSTEM) WITH THE PORT-A-CUL SWAB TRANSPORT SYSTEM FOR MAINTAINING CLINICALLY IMPORTANT ANAEROBES AT ROOM TEMPERATURE AND REFRIGERATION TEMPERATURE

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Background: The ESwab (Copan, Murietta, CA) transport system incorporates a flocked nylon swab for sample collection and liquid Amies transport medium. The system potentially collects more material and releases a higher percentage of microorganisms than spun fiber swabs with agar transport media. Data for this system are currently lacking relative to anaerobes. The purpose of this study was to perform a quantitative evaluation of the ESwab for maintaining viability of selected clinically important anaerobic bacteria. For comparison, the BBL Port-A-Cul (PAC; Becton Dickinson, Baltimore, MD) agar tube transport system with polyester swabs was tested.
Methods: Anaerobic bacteria selected for this study were 10 ATCC strains and 18 recent clinical isolates. Quantitative viability studies were performed in triplicate at both controlled room temperature (RT) and refrigerator temperature (4C) at the same time. For each organism/swab device combination, viable counts were performed at zero (0) time, 24 h and 48 h according to the CLSI-40A quantitative elution method.
Results: Initial 0-time counts for most anaerobes studied were nearly 1 log (base 10) higher for the ESwab than for the PAC. After swabs were held 24 h at 4C compared to 0-time, organism recovery from the ESwab was 66%, vs. 46% for the PAC (based on average CFU/ml for 9 organisms). After 48 h at 4C, recovery from ESwab was 50% and recovery from the PAC was 28% compared to 0-time. At room temperature compared to 0-time, the overall recovery of anaerobes from ESwabs at 24 h and 48 h was 41% and 22%, respectively, compared to 41% and 34% for the PAC, respectively. At refrigerator temperature, poor or no survival was noted for *Clostridium difficile* (2 of 3 strains) and *C. clostridioforme* in both systems. *Prevotella bivia* and *Porphyromonas assacharolytica* also were problematic for both systems. A clinical isolate of *Fusobacterium nucleatum* showed much better recovery after it had been held in the refrigerator in both systems compared to room temperature for both systems. At room temperature, recoveries were better for 5/6 strains of anaerobic cocci tested with ESwab compared to PAC at 24 h, though the yields in both systems at 48 h tended to decrease. Also at room temperature, *Porphyromonas levii* survived better in ESwab for 24 h than in PAC, but its recovery in both systems at 48 h was poor.
Conclusions: The results suggest the nylon flocked ESwab system with liquid Amies transport medium is suitable for maintaining viability of clinically important anaerobic bacteria during transport and storage either at 4C or RT. However, the ESwab performed better at 4C than it did at RT, though it maintained viability of most anaerobes tested at RT for 48 h.

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COMPARISON OF REAL TIME PCR AND CONVENTIONAL METHODS FOR DETECTION OF *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile is present in the stool of approximately 20% of hospitalized patients. As an opportunistic pathogen *Clostridium difficile* causes 300,000 to 3,000,000 cases of diarrhea and colitis per year. The infection is often associated with antibiotic use or other factors that alter or shift the normal bowel flora allowing *C. difficile* to flourish and produce toxin. Antibiotic use likely contributes to 25% of cases in hospitalized patients. Prevention of healthcare associated infections is a high priority. Rapid detection of the presence of toxin-producing strains would help hospitals accomplish this goal.

This study compared three methods of toxin B detection in 117 unformed fresh stools from hospitalized patients. The methods included anaerobic toxigenic culture, cell culture cytotoxin neutralization assay, and a laboratory-developed real time PCR for the detection of toxin B gene. For this study, the reference method was defined as a positive result in any two of the methods tested. There were two samples positive by toxigenic culture and negative in the other two assays. A total of 45 samples were positive and 72 samples were negative by the definition of reference. The sensitivity and specificity for cell culture cytotoxin neutralization assay was 86.7% and 98.6% respectively. The sensitivity and specificity for real time PCR was 91.1% and 98.6% respectively vs. reference method and 86.7% and 95.8% vs. toxigenic culture. The sensitivity and specificity for the toxigenic culture method was 95.6% and 97.2% respectively.

This study confirms that our in-house real time PCR is a sensitive method that compares favorably to anaerobic culture with toxin confirmation, the "gold standard." The cell culture cytotoxin assay compared more favorably to PCR than reported in some other published studies. Although there was no statistical difference between the sensitivity of the real time PCR and cell culture cytotoxin assay, the slightly higher sensitivity for real time PCR may represent a clinically significant difference. Real time PCR is faster than culture by 4-5 days and therefore allows patient care decisions to be made and implemented in a timelier and clinically relevant timeframe. Decreasing turn-around time for results may help improve clinical outcome, reduce secondary transmission, reduce length of stay, and decrease medical costs.

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EFFECT OF STORAGE CONDITIONS ON STABILITY OF FREE AND ENCAPSULATED IN PLAIN- OR CYSTEINE-SUPPLEMENTED ALGINATE, *BIFIDOBACTERIUM ANIMALIS* BB-12®

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The main objective of this research work was to study the viability of *Bifidobacterium animalis* BB-12® as free and calcium alginate-encapsulated cells, with or without cysteine, throughout storage, at four different temperatures. Extrusion by aerodynamically assisted flow was used to produce alginate and calcium alginate supplemented with L-cysteine·HCl microcapsules, containing *B. animalis* BB-12®. The microcapsules were suspended in Ringer solution in a 1:9 (g/mL) ratio, and stored at 21, 4, -20 and -80 ºC throughout six months, respectively. In parallel, the viability of free cells in cell suspension, was subjected to the same storage conditions and the corresponding viability assessed. Results showed that at 21, 4 and -20 ºC, the encapsulation did not have a protective effect—free cells maintained their viability throughout longer periods than encapsulated counterparts. At -80 ºC, encapsulation protected *B. animalis* BB-12® in comparison to the behavior of free cells. However, this effect was only observed in calcium alginate microcapsules supplemented with L-cysteine.HCl. After 180 days storage at -80 ºC, a 2 log cycle difference, in viable cells was observed between microcapsules with or without cysteine. The viable numbers of *B. animalis* BB-12® in microcapsules without cysteine was similar to that of free cells. In conclusion, alginate encapsulation revealed a protective effect on viability of *B. animalis* BB-12® stored at -80 ºC when supplemented with L-cysteine.HCl.

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BROTH PRE-AMPLIFICATION ENHANCES SENSITIVITY OF REAL TIME PCR FOR DETECTION OF *CLOSTRIDIUM DIFFICILE* FROM PERI-RECTAL SWABS AND STOOL SPECIMENS

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Purpose: The sensitivity of most real-time PCR assays for detection of toxigenic *C. difficile* is poor compared to the gold standard of anaerobic culture and toxigenicity assay. We describe a two-step method that combines the sensitivity of toxigenic culture with the speed of real-time PCR.
Methods: A total of 96 VRE surveillance peri-rectal swabs (BBL™ CultureSwab™ Plus) were cultured in cefoxitin cycloserine mannitol broth with taurocholate and lysozyme (CCMB-TAL, Anaerobe Systems). In addition, 8 swabs were obtained from *C. difficile* toxin-positive stools. CCMB-TAL cultures were incubated at 37°C for 48 hours. Cultures positive for *C. difficile* growth (alkaline media) were subcultured to pre-reduced 5% sheep blood agar within an anaerobic chamber (Coy). *C. difficile* was identified by colony morphology and PRO disc (Remel). Isolates were confirmed as toxigenic by cytotoxicity assay of cell free supernatants (Diagnostic Hybrids). DNA was extracted from 1 ml 48 hour broth cultures by NucliSENS® easyMag® (bioMérieux). Real-time PCR detection of *tcdB* was performed as previously described (Peterson et al.) on an AB 7500 FAST. Specimens that generated triplicate Ct values ≤ 40 with Tms of 76.0 ± 0.9 were considered toxin positive. Specimens that generated triplicate reactions with Ct > 40 were defined as negative. All other results were considered indeterminate.
Results: Of the 8 toxin positive stools, 100% were positive by PCR and 88% (7/8) were positive by toxigenic culture. Of the 96 peri-rectal swabs, 33% (32/96) were positive by PCR and toxigenic culture, 52% (50/96) were negative by PCR and toxigenic culture and 5% (5/96) were positive by PCR but negative by toxigenic culture. There were 9 peri-rectal swabs that were indeterminate by PCR and negative by toxigenic culture. Based on these data, the sensitivity of this method is 100% 32/32 and the specificity is 90.9% (50/55)) with 9/96 indeterminate results.
Conclusions: CCMB-TAL broth pre-amplification of *C. difficile* from clinical specimens improves the sensitivity of *tcdB* detection by real-time PCR. A second *C. difficile*-specific PCR target or TaqMan-based assay may help resolve PCR positive/culture negatives and indeterminate PCR results.

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THE TRANSCRIPTIONAL REGULATOR *OXYR* AND CATALASE AFFECTS *BACTEROIDES FRAGILIS* AND OTHER *BACTEROIDES* SSP SURVIVAL WITHIN PERITONEAL MACROPHAGES

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To survive oxidative stress, *B. fragilis* must protect itself against the damage caused by reactive oxygen species (ROS). ROS activate a coordinated mechanism of transcription regulation by OxyR. Thus, the aim of this study was to evaluate if *OxyR* and the production of catalase affects the survival of *B. fragilis* (wild type 638R, Δ*kat*B, and Δ*oxy*R) and others *Bacteroides* species (*B. vulgatus* and *B. thetaiotaomicron*) under aerobic stress. In *in vitro* assays strains were grown anaerobically to mid-log phase and exposed to atmospheric O_2. Aliquots were taken at different time points for viable cell counts. *B. fragilis* wild type did not show a decrease in cell viability after exposure to O_2 for 5h, but after 24h it decreased 86.1% in cell viability. The *B. thetaiotaomicron* strain showed a similar behavior with 81.5% decrease in viability. In contrast, the *oxy*R mutant and the *B. vulgatus* strain both had a decrease of more than 99.9% in viable cell counts. The Δ*kat*B mutant had a decrease in viability of only 53.5%. To evaluate the survival of these strains within peritoneal macrophages an interaction assay was performed. Briefly, the bacterial strains were incubated with macrophages with a MOI of 100. Free bacteria were washed with DMEM and the macrophages were lysed after 1, 5 and 24h for bacterial cell count. The Δ*oxy*R mutant and *B. vulgatus* strains showed a greater susceptibility to macrophage killing after 24h of interaction and had a survival rate of 0.8% and 0.54%. The wild type *B. fragilis* was less sensitive and had a survival rate of 35.5% whereas the Δ*kat*B strain and *B. thetaiotaomicron* had a survival rate of 5.4% and 2.9%, respectively. Formation of ROS in the macrophages was accessed by adding nitroblue tetrazolium (NBT) for the incubation period in the macrophage interaction assay. After 24h the samples were washed, fixed and analyzed by differential interference contrast microscopy. It was observed that 638R strain had a higher production of formazan granules when compared to the Δ*oxy*R and Δ*kat*B strains. In conclusion, the Δ*kat*B, Δ*oxy*R mutants are more sensitive to aerobic stress when compared to the parental strain and so is *B. vulgatus* which does not have any of these two genes. *B. thetaiotaomicron* shows a resistance profile similar to the wild type *B. fragilis.* Taken together, these results show that the *B. fragilis* resistance to oxidative stress *in vitro* and in cultured macrophages is dependent on OxyR and to a lesser extent KatB.

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CLOSTRIDIALES BACTERIUM CD3:22—AN ANAEROBIC SPORE-FORMING BACTERIUM ISOLATED FROM SMALL INTESTINE IN A CELIAC DISEASE PATIENT

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Background: The bacterial flora in proximal small intestine of children with celiac disease (CD) and control patients was investigated by 16S rDNA sequencing and culturing. Among other findings it was shown that the presence of unidentified clostridiales bacteria was more common in CD patients than in controls. One strain of a strictly anaerobic Gram-positive spore forming bacilli was isolated from a small intestinal biopsy of a child with CD, born during the time of the Swedish CD epidemic. The strain was subjected to a range of phenotypic and genotypic tests.
Methods: The bacterium was originally isolated from a blood-agar plate cultured in anaerobic atmosphere at 37°C for 48-72 h. The cell morphology was examined by Gram-staining and dark-field microscopy. Biochemical activity was investigated by rapid ID32A, API 20A (BioMerieux) and gas-liquid chromatography (GLC) of metabolic end products. The strain was subjected to sequencing of the 16S rRNA gene.
Results: On blood-agar (48 h at 37°C) the colonies had a diameter of 1-1.5 mm and were flat with irregular edges. A speckled rainbow-colored texture could be observed by stereoscopic microscopy resembling the appearance of *Fusobacterium nucleatum* subsp. *polymorphum.* The cells were Gram-positive spore forming rods, 2×10-20 µm in size. They were slightly decolorized when Gram-stained. The biochemical profile obtained by rapid ID32A pointed to *Clostridium clostridioforme* (94% ID) or *Clostridium perfringens* (50.5% ID). However, the strain showed no similarity in cell or colony morphology to any of these *Clostridium* species. By API 20A no recognizable profile was obtained. The bacterium was saccharolytic and non-proteolytic. The metabolic end products detected by GLC were acetate and butyrate. Optimal growth temperature and pH were 37°C and pH 7.0, respectively. Full-length 16S rRNA gene sequencing showed the strain to be a new species most closely related to *Eubacterium saburreum* (AB525414) with 97.6 % similarity. Strain CD3:22 exhibited higher sequence similarities (98.5-98.6) with strains isolated from the oral microbiome and hemocultures.
Conclusion and scientific importance: The isolate CD3:22 represents a new species with the tentatively proposed name *Anaerobacterium umeaense* (CCUG 58757^T). The bacterium may be involved in the development of CD.

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DIAGNOSTIC METHODS & MICROBIOLOGY

THE USE OF STABLE ISOTOPE TECHNOLOGY TO DETERMINE A BIOREMEDIATION STRATEGY FOR A DECOMMISSIONED CHEMICAL MANUFACTURING FACILITY

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This project examines the benefits of using stable-isotope probing technology to discern an effective and efficient bioremediation strategy for a decommissioned chemical facility contaminated by benzene, nitrobenzene, aniline, and diphenylamine. As early as the 1880's, the site employed a wide variety of chemical manufacturing processes. The production of the above, four target compounds began in 1917, and manufacturing activities continued through 1986. In 2009, a strategy was developed to investigate and enhance the natural, *in situ* groundwater attenuation of these target compounds. In order to determine the best method for increasing indigenous anaerobic microbial bioremediation activities, a thorough evaluation of the degrading microbial community was necessary. Specifically, it was important to determine which members of the microbial community were responsible for the degradation of each compound and how effective this degradation was under *in situ* conditions. Because of the complex anaerobic biogeochemistry of the site, it was determined that the use of stable-isotope probes would be the most efficient method of discerning the degrading community profile. A series of twelve Bio-Trap microbial samplers were produced, with each sampler containing a single, isotopically-labeled target compound. These samplers work because as indigenous microbes consume the labeled compound, the isotopic tag is converted to labeled biomass (PLFA molecules) that can then be isolated for further analysis. In addition, residual labeled compounds can be monitored for the isotopic ratio shifts that occur as a result of biological degradation, as determined by $\delta^{13}C$ measurements, providing further quantifiable metrics for determining biodegradation efficiency. Twelve Bio-Traps were deployed into three representative monitoring wells, such that each well contained one Bio-Trap for each target compound. After six weeks of *in situ* incubation, the Bio-Traps were retrieved and analyzed for the following: total microbial community size and profile, the degree of isotopic label incorporation into biomass (as a quantifiable confirmation of target compound biodegradation), a comparison of isotopic incorporation into biomass versus mineralization, the amount and rate of target compound degradation, the presence of any PLFA indicators to suggest microbial nutritional stress or toxicity, and a $\delta^{13}C$ stable isotope ratio shift in any remaining compound. The results of these analyses demonstrated the presence of an active degrading community under *in situ* conditions and provided site-specific guidance regarding the most effective ways to increase *in situ* bioremediation. As a result, a custom-designed remediation strategy was designed and successfully implemented.

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DIAGNOSTIC METHODS & MICROBIOLOGY

COMPARATIVE STUDY OF ENZYME IMMUNOASSAYS: TECHLAB TOXINS A & B, C DIFF QUIK CHECK FOR GLUTAMATE DEHYDROGENASE (GDH); PCR-BD GENEOHM® AND CYTOTOXIN ASSAY (CTA) FOR THE DETECTION OF *CLOSTRIDIUM DIFFICILE* TOXIN IN ADULT STOOL SPECIMENS

Kafka, J.E.;*[3] Yamamura, D.L.;[1,3] Jissam, A.;[3] McCaffery, W.;[3] Ahmed, B.;[2] Main, C.;[1,3] Lee, C.H.[1,3]
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Introduction: *Clostridium difficile* associated disease (CDAD) requires rapid and reliable testing methods for patient and infection control management. In a multi-site study, toxin AB assay, GDH antigen, PCR testing and cytotoxin assays were performed to determine the performance characteristics of each.
Methods: A total of 471 consecutive stool samples collected from 4 acute care hospitals in Hamilton, Ontario, Canada were tested for *C. difficile* toxin. Toxin AB testing was performed using TOX A/B II™ EIA kit (TechLab®:Blacksburg VA) toxin assay kit. Detection of glutamate dehydrogenase was done using C. Diff Quik Check (Inverness, Ottawa, ON). Both the EIA and the GDH were performed at Hamilton Health Sciences (HHS). BD GenOhm® (BD Franklin Lakes, NJ) PCR testing and Cytotoxin assays (CTA) for *C. difficile* were done according to standard laboratory protocol (used for research) at St. Josephs Healthcare (SJH) centre. For stool samples with any discrepant results Cycloserine, Cefoxitin Fructose (CCFA) medium was used to culture for *C. difficile*. For positive cultures toxin testing was performed to determine toxigenicity. For this study, gold standard was defined as follows: 100% concordance between tests and for discrepant results, positive culture showing toxigenic strain.
Results: EIA toxin A/B – Overall positives 25, true positives 21, false positives 4. Overall negatives 446, true negatives 434, false negatives 12. Giving a sensitivity of 63.6% and specificity of 99%, positive predictive value of 84% and negative predictive value of 97.3%.
GDH antigen – Overall positives 58, true positives 30, false positives 28. Overall negatives 413, true negatives 410, false negatives 3. Giving a sensitivity of 90.9% and specificity of 92.6%, positive predictive value of 51.7% and negative predictive value of 99.3%.
BD PCR – Overall positives 30, true positives 24, false positives 6. Overall negatives 441, true negatives 432, false negatives 9. Giving a sensitivity of 72.7% and specificity of 98.6%, positive predictive value of 80% and negative predictive value of 97.9%.
CTA – Overall positives 25, true positives 25, false positives 2. Overall negatives 446, true negatives 436, false negatives 8. Giving a sensitivity of 75.7% and specificity of 99.5%, positive predictive value of 92.6% and negative predictive value of 98.2%.
Conclusion: For this study the GDH testing showed a better sensitivity because you are testing for the *C. difficile* antigen giving a 99.3% negative predictive value. Therefore the GDH test would be a reliable screening test for negative stool samples. However, further testing on the GDH positive specimens would be required to determine if these positive antigen stools are toxin producers. BD GeneOhm PCR testing showed similar sensitivity and specificity as the cytotoxin assay in approximately 2 hours and may be a suitable confirmatory test.

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PARTIAL SEQUENCE COMPARISON OF THE 16S RRNA, *RPOB*, AND *CPN60* GENES OF HUMAN *CAMPYLOBACTER SHOWAE* ISOLATES

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Campylobacter showae is a gram-negative, anaerobic/microaerophillic, rod-shaped bacterium isolated from human gingival crevices and uses multiple unipolar flagella for motility. The bacterium has been associated with human periodontitis. *C. showae* is known to be catalase-positive, whereas *Campylobacter rectus* is catalase-negative. Species identification and genetic relationships of nine *C. showae* isolates from human saliva, dental plaque, and blood sources were carried out using partial sequence comparison of the 16S rRNA gene (662-696 bp), *rpoB*, encoding the beta subunit of RNA polymerase (456-503 bp), and *cpn60*, encoding the 60 kDa heat-shock protein (432-491 bp). For all of the human isolates tested, the 16S rRNA gene sequencing identified *C. showae* and *C. rectus* species as the first and second choices with ≥99% and 96% sequence identities to the respective reference strain (*C. showae* CCUGs 11641 and 30254 and *C. rectus* CCUGs 11643 and 20446B) sequences. Therefore, all of the nine isolates were genotypically assigned to the species *C. showae* using the 'gold standard'. On the other hand, maximum identities (%) of *rpoB* gene sequences for all the nine human isolates tested were 90-97% and 90-93% when aligned to reference strains C. *showae* CCUGs 11641 and 30254 and *C. rectus* CCUGs 11643 and 20446B, respectively. In addition, the cpn60 gene sequencing identified *C. showae* and *C. rectus* species as the first and second choices with 91-95% and 88-91% sequence identities to the respective reference strain (*C. showae* NEP 16950 and *C. rectus* ATCC 33238) sequences. All the isolates were negative or weakly positive for catalase production. Thus, conventional and biochemical tests of all the isolates will be extensively performed as supplementary methods to obtain their phenotypic characteristics.

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DIAGNOSTIC METHODS & MICROBIOLOGY

MONITORING GENE EXPRESSION WITH FLUORESCENT FLAVIN-BINDING PROTEIN UNDER ANAEROBIC CONDITIONS

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The use of fluorescent protein markers for the detection of gene expression in anaerobic bacteria has been hindered by the requirement of oxygen for the formation of the excited fluorophore. More recently, the development of commercially available flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs) derived from *Bacillus subtilis* and *Pseudomona putida*, (hence called Bs2) whose fluorescence (maximal absorption at 449 nm and emission at 495nm) is independent of the availability of oxygen, makes it an useful tool for the detection of gene expression in anaerobic bacteria. We have used the opportunistic aerotolerant human pathogen *B. fragilis* as a model anaerobic organism to investigate whether the Bs2 gene would be a practical marker for gene expression both *in vivo* and *in vitro* conditions. *ahpC::Bs2* and *dps::Bs2* constructs were used as gene reporters respectively. A maltose/starch inducible promoter (OSU) was also fused to promoterless Bs2 as control for maltose-inducible and glucose-repressed anaerobic gene expression. Anaerobic cultures were grown to mid-log phase and exposed to oxygen, hydrogen peroxide or maltose. Non-induced cultures were also incubated with J774 macrophages monolayer for phagocytosis assay and intra-cellular expression of Bs2 in an anaerobic incubator. Fluorescence was detected using a confocal laser microscope. Bacteria carrying *osu::Bs2* constructs showed inducible fluorescence following addition of maltose anaerobically but not under glucose repressed conditions confirming that the FbFPs are impervious to the absence of oxygen. Both *ahpc::Bs2* and *dsp::Bs2* constructs were fluorescent following induction by oxygen compared to non-fluorescent cells in the anaerobic control cultures. A hydrogen peroxide resistant strain (IB263) in which both *AhpC* and *dps* are constitutively expressed was transformed with the *ahpC::Bs2* and *dps::Bs2* constructs and displayed fluorescence in aerobic and deep anaerobic conditions. Intra-cellular expression of Bs2 was also detected when macrophages were incubated with the *ahpc::Bs2* and *dsp::Bs2* constructs anaerobically. This suggests that *ahpC* and *dps* are expressed *in vivo*. Thus, we show that Bs2 is an alternative tool for the detection of gene expression in anaerobic bacteria in the absence or presence of oxygen in *in vitro* and *in vivo* studies.

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USE OF MULTI-ENZYME PULSED FIELD GEL ELECTRO-PHORESIS (PFGE) TO DIFFERENTIATE WITHIN A SINGLE CLUSTER OF *CLOSTRIDIUM DIFFICILE*

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Purpose: To evaluate the use of multi-enzyme PFGE as a means to resolve clusters of PFGE type NAP7/ PCR-ribotype 078 *Clostridium difficile* isolates from environmental, clinical and animal sources.
Methods and Results: Seventy-five NAP7/ 078 isolates from the CDC collection, including isolates from human clinical (28), livestock (26), food product (18) and environmental (2) cultures, were investigated. Previous characterization by *SmaI* PFGE, toxinotyping, PCR-Ribotyping, and PCR for binary toxin (*cdtB*) and major deletions in the *tcdC* regulator gene demonstrated that all 75 isolates were virtually indistinguishable: all were NAP7, PCR-ribotype 078, toxinotype V, binary toxin positive, with a 39 base-pair deletion in *tcdC*. *SmaI* digestion in NAP7 strains typically produces a suboptimal number of measurable bands (5 to 7), and neither PFGE nor PCR-ribotyping has been shown to adequately distinguish NAP7/ 078 from other closely-related strains, including NAP8 and PCR-ribotype 126. Multi-enzyme PFGE is a common strategy used for cluster confirmation in foodborne outbreaks, particularly among highly clonal bacterial species. In the past, our laboratory has occasionally used *Eag*I as a secondary enzyme for PFGE. For the present study, *Bam*HI, *Bg*lII, *Eag*I, *Eco*RI, *Mlu*I, and *Xho*I were evaluated on the basis of *in silico* modeling. Based on preliminary data, *Mlu*I was chosen and validated across a range of different PFGE types. Restriction of NAP7/ 078 isolates with *Mlu*I resulted in an average of 13 bands, and these could be used to partition study isolates into 9 discrete clusters (Dice/UPGMA; 80% similarity threshold), with a Simpson's index of 67.7 (95%CI: 58.0-77.3). Isolates separated roughly into clusters from human, animal and food sources.
Conclusions: Our findings illustrate that there are subtle genetic differences within the NAP7/ 078 *C. difficile* strain, and that existing strain typing methods may not have adequate resolution for detailed studies, particularly in the context of potential zoonotic and foodborne transmission/ attribution. The addition of a secondary PFGE enzyme is a proven and cost-effective means to improve strain typing resolution among highly clonal species, and may help further our understanding of the molecular epidemiology and transmission dynamics of *C. difficile*.

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DIAGNOSTIC METHODS & MICROBIOLOGY

MULTILOCUS SEQUENCE ANALYSIS OF *BACTEROIDES FRAGILIS* STRAINS

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In the last few years, several studies revealed a remarkable genetic variability in *B. fragilis* species, leading to the proposal of two subdivisions according to the presence or absence of the *cfiA* (metalo-β-lactamase/carbapenase) gene. In a previous study, we identified from a total of 127 strains 16 harboring the *cfi*A gene. The aim of the present study was to identify the presence of these two subgroups in a set of 17 *B. fragilis* strains (seven *cfi*A positive and ten *cfi*A negative) by using sequence data of the genes *gdh* (glutamate dehydrogenase), *pgm* (phosphoglucomutase), *est* (esterase), *rpo*B (RNA polymerase β subunit) and *rrs* (16S rRNA). Analysis of the *est* gene sequences based on phylogenetic trees provided a classification similar to that obtained by *rrs* and *rpo*B typing and revealed the existence of these subgroups in *B. fragilis*. On the other hand, *pgm* and *gdh* sequences did not confirm this division. Multilocus Sequence Analysis (MLSA) allowed the detection of a divergence, and provided highly reproducible and reliable data for *B. fragilis* taxonomy. To test for eventual alterations in virulence within these subgroups, we performed assays for biofilm production and evaluated the virulence in an animal model for one representative strain from each subgroup. No significant alterations in bacterial adherence to an inert surface were detected between the two subgroups. Conversely, the strain representative for subdivision I (*cfi*A-negative) demonstrated a larger capacity to induce intra-peritoneal abscess than the strain from subdivision II (*cfi*A-positive), which was unable to induce abscess. Our results demonstrate the usefulness of the *est* gene sequence to discriminate between the two groups, indicating the potential of this approach as an alternative tool to distinguish between the two subgroups. However, while the two subgroups apparently show no alteration in biofilm production, they differ in abscess formation.

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CHARACTERIZATION OF *LEPTOTRICHIA* ISOLATES FROM HUMAN CLINICAL SPECIMENS AND DETECTION OF 3 POSSIBLY NOVEL TAXON GROUPS

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Introduction: *Leptotrichia* species are Gram-negative, fusiform, non-sporulating, non-motile, strictly anaerobic or aerotolerant rods. The genus *Leptotrichia* currently has 6 species: *L. buccalis*, *L. trevisanii, L. shahii*, *L. hofstadii, L. wadei* and *L. goodfellowii.* Existing literature also refers to leptotrichia-like *Sneathia sanguinegens* and 'Leptotrichia amnionii', which was described but not validly named. Here, 23 leptotrichia like isolates received at the Canadian federal reference centre (National Microbiology Laboratory (NML, Winnipeg MB)) between 1982 to 2009, including 16S rRNA gene sequencing, were reviewed. We describe 15 isolates which could not be assigned to existing species but clustered into 3 novel Leptotrichia-like groups. We report on characteristics of the 23 *Leptotricha* isolates and their phylogenetic relationships.
Method: Polyphasic identification methods (phenotypic, chemotaxonomic, phylogenetic) were used. 16SrRNA gene sequences were BLASTed and compared with Genbank data. Nearly full 16SrRNA gene sequences from wild strains were aligned with Genbank sequences derived from type strains and relationships were inferred using Neighbour-Joining software, found in MEGA4.
Results: No NML isolates were identified as *L. shahii*, *L. wadei*, *L. hofstadii* or "L. amnionii". Strains phenotypically and genetically consistent with (no., source if known) *L .trevisanii* (5, all blood culture), *L. buccalis* (2, blood, tissue) or *L. goodfellowii* (1, unknown), were observed. Three additional taxon groups were found: **Taxon 1:** (6, all blood cultures) were most like the fastidious isolate described by Patel (1999 J. Clin. Microbiol. 37:2064-67); these could be readily distinguished from (also fastidious) *S. sanguinegens* by 16SrRNA gene sequencing. **Taxon 2** (2, 1 blood, 1 bronchial wash) 100% to each other, were otherwise closest to (97-98.1%) *L.wadei.* **Taxon 3** (8, 1 sputum, 1 blood, 6 unknown) had 99-100% identity with each other, but ≤ 97% identity with other named species. Isolates were somewhat aerotolerant. It was difficult to discern among many of these strains by phenotypic means alone.
Conclusions: Diversity was observed here among Leptotrichia-like bacteria. Two previously unrecognized *Leptotrichia* species as well as additional strains consistent with the 'Patel' organism, were detected. The novel taxon groups could not be easily differentiated phenotypically from other *Leptotrichia* species but were well separated by 16S rRNA sequencing. Most isolates were recovered from sterile body fluids.

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DIAGNOSTIC METHODS & MICROBIOLOGY

DEVELOPMENT OF A LIGHTCYCLER REAL-TIME PCR ASSAY FOR DETECTION OF TOXIGENIC *CLOSTRIDIUM DIFFICILE* IN ANIMAL FECAL SAMPLES

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Methods for detection of toxigenic *Clostridium difficile* are cytotoxicity assay, culture, and EIA, which are usually used in a combination. These methods are either labor intensive and time-consuming or have poor sensitivity and specificity. In this study, we developed and evaluated new real-time PCR targeting genes for toxins A, B, and binary toxin. The method was compared with enrichment culture.

Rectal swabs from the piglets (n=247) and stool samples from adult pigs (n=18) and calves (n=51) were obtained in duplicates. Samples were cultured using selective enrichment broth. DNA extraction was preformed using QIAamp DNA Stool Mini Kit (Qiagen, Germany). We developed and validated a real-time PCR targeting *tcd*A, *tcd*B and *cdt*B genes by using hybridization probes with the LightCycler instrument (Roche, Germany). LightCycler Control Kit DNA (Roche, Germany) was used as internal control with *tcd*A and *tcd*B assays.

Real-time PCR (LC) of 28 non-*C. difficile* isolates showed a 100% specificity of primers and probes and furthermore detected all ten toxinotypes of *C. difficile*. In case of toxinotypes VIII ($A^-B^+CDT^-$) and XIa ($A^-B^-CDT^+$) the assay targeting *tcd*A was positive, while these toxinotypes have gene for toxin A, but it is not expressed. The detection limit of the LC for all three genes was found to be 4400 copies of *C. difficile* DNA per g of feces. When using the internal control in duplex assay with PCR for *tcd*A or *tcd*B gene, the detection limit was 44000 copies of DNA. However, despite detection of *C. difficile* in samples with less copies of DNA, the reproducibility was not satisfactory. 189 LC negative and 52 LC positive samples were in complete concordance with cultivation method (76% of all samples). PCR inhibition was observed in 12% culture negative—LC negative samples. Furthermore, LC was negative in 46 culture positive samples (in 10 samples PCR inhibition were observed) and 29 samples were culture negative and LC positive. In comparison with cultivation LC showed 53% sensitivity, 87% specificity, 64% positive predictive value, and 80% negative predictive value.

We compared our assay to enrichment culture, which is not standardized and indicated as a gold standard, therefore culture negative—LC positive samples can not be considered as true ˝false positive˝. Enrichment culture is superior to single use of selective agar CCFA, which is often used in diagnostics. Therefore we can assume that the sensitivity of our assay would be higher in comparison with culture without the enrichment step. This real-time PCR assay offers a rapid method and can shorten the time of detecting toxigenic *C. difficile* in fecal samples, but has to be used in combination with the enrichment step of culture method, due to the low sensitivity of the assay used for a direct detection of *C. difficile* in fecal samples.

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DIAGNOSTIC METHODS & MICROBIOLOGY

IDENTIFICATION OF AEROBIC GRAM POSITIVE RODS USING *HAE* III RESTRICTION ENZYME

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Objective: Phenotypic methods are inadequate for the accurate identification of organisms presumptively identified as aerobic gram positive rods by colony and/or gram stain morphology. Our aim was to assess the feasibility of using *Hae* III restriction enzyme patterns as a method to identify aerobic gram positive rods commonly found in vaginal flora.
Methods: 208 organisms isolated from the genital tract of 137 pregnant women were initially identified as aerobic gram positive rods. *Lactobacillus* and *Gardnerella vaginalis* were ruled out based on their colony and gram stain morphology. The DNA from each isolate was extracted and 16S rRNA direct PCR was performed. PCR products were then digested with *Hae* III restriction enzyme digest. Aliquots were subjected to electrophoresis in 3% high grade agarose gel. 16S rDNA gene sequencing was performed and GenBank BLAST was used to determine the identification. The identification was then compared with corresponding *Hae* III restriction enzyme patterns.
Results: GenBank BLAST identified the following 206 organisms to the species level: *Corynebacterium* (10 species); *Gardnerella vaginalis*; *Actinomyces* (5 species); *Lactobacillus* (6 species); *Brevibacterium* (3 species); *Arthrobacter* (1 species); *Propionibacterium* (2 species); *Actinobaculum* (1 species); *Alloscardovia* (1 species); *Microbacterium* (1 species); *Bacillus* (1 species). *Psuedoclavibacter* and *Actinobacterium* represent 2 of the 208 organisms that were identified only to the genus level. There were 30 unique *Hae* III restriction enzyme patterns that separated 11 of the 13 genera identified by sequencing. Only 2 of 34 patterns were shared by multiple species. One pattern is shared between *Corynebacterium xerosis, C. asperum, C. amycolatum* and *Brevibacterium ravenspurgense*, and the other pattern by *C. kropenstedtii* and *C. psuedogenitalium*. Of the 208 organisms, 81 (38%) were *Lactobacillus* sp. and *G. vaginalis;* having atypical colony and/or gram stain morphology.
Conclusion: This study demonstrates that *Hae* III restriction enzyme can be used to differentiate between most genera and some species. This single restriction enzyme is helpful in separating a large diverse group of organisms that are often placed into "aerobic gram positive rods" based on morphology. Further testing with other enzymes and more species within the list of genera is needed to further develop this library.

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EVALUATION OF GENEXPERT *CLOSTRIDIUM DIFFICILE* ASSAY, RIDASCREEN *C. DIFFICILE* TOXIN A/B ASSAY, TOX A/B II ASSAY, TISSUE CULTURE AND STOOL CULTURE IN THE DIAGNOSIS OF *C. DIFFICILE* INFECTION (CDI)

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Purpose: The aim of this study was to evaluate GeneXpert *C. difficile* Assay (GXCDA), a qualitative automated real–time PCR diagnostic test for rapid identification and differentiation of toxin B and binary toxin of *C. difficile*, along with 4 other methods, for the diagnosis of CDI.
Materials and Methods: Single stool samples from 223 suspected CDI in-patients were collected. Culture and incubation were carried out on the day of receipt according to standard methods. *C. difficile* were identified by API 20 AN. Tissue culture (TC: gold standard) was carried out on monolayer of Vero cells. Two immunoassays, Tox A/B II Assay (TABA) and Ridascreen Toxin A/B Assay (RABA), were performed on the same portion of homogenized stool on the same day. GXCDA was used to detect sequences in gene for Toxin B ($_{tcd}$B), Binary Toxin ($_{cdt}$) and $_{tcd}$C detection nt 117 ($_{tcd}$CΔ117).
Results: Of the 223 specimens 63 (28.3%) tested positive by GXCDA, TC and stool culture, 43 (19.3%) by RABA and 42 (18.8%) by TABA. One sample was positive by RABA but negative with GXCDA, TABA and TC. GXCDA showed 100% sensitivity and 100% specificity unlike TABA and RABA with 66.7% and 66.7% sensitivity, and 100% and 99.4% specificity, respectively. The positive and negative predictive values for GXCDA were each 1. Accuracy of GXCDA, TABA and RABA were 99.5%, 90% and 90%, respectively.
Conclusion: GXCDA is more accurate than the other immunoassays for the direct detection of toxins in stools of CDI patients. It offers sensitivity and specificity for toxin B detection that are comparable to the reference method. With the results available within 1 h, it provides prompt and precise laboratory diagnosis.

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DIAGNOSTIC METHODS & MICROBIOLOGY

BACTEROIDES, PARABACTEROIDES AND CO.: IDENTIFICATION BY MALDI-TOF-MS

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Recent taxonomic changes, which expanded the genus *Bacteroides* to more than 18 members representing well known and new describes species and transferred some former *Bacteroides* species (e.g. *B. distasonis*) in the new genus *Parabacteroides*, are not represented in the databases of commercially available identification systems based on phenotypical tests. Matrix Assisted Laser Desorption/Ionisation (MALDI-TOF-MS) is increasingly used for identification of bacterial isolates. MALDI-TOF-MS identification is based on the comparison of a spectrum of mass signals of an isolate with a variety of spectra stored in a database (DB). In contrast to other identification systems, the MALDI-TOF-MS database allows amendments of additional species regarding to individual requirements. Here we describe the evaluation and amplification of the SARAMIS database (SDB, Anagnostec) for *Bacteroides*, *Parabacteroides* and related species (e.g. *Odoribacter splanchnicus, Bilophila wadsworthia*) by spectra obtained with a MALDI-TOF-MS analyzer (Shimadzu). One of the key features of the SDB is the differentiation of spectra and SuperSpectra (SSP). A SSP contains information of characteristic mass signals of a certain species. The SDB comprises >60.000 spectra and >1600 SSP but less than 10% of both regarding anaerobic species. 350 clinical isolates and 31 reference strains were analysed in parallel to standard identification procedures. In case of discrepant results obtained by standard methods and MTA, additional identification assays or gene sequence analysis have been performed. 10 new spectra and 3 new SSP have been added to the SDB. Especially clearly separated genera and species (e.g. *P. distasonis*, *B. wadsworthia*, *O. splanchnicus*) could easily and accurately be identified by MALDI-TOF-MS and SDB. More closely related species (e.g. *B. dorei* and *B. vulgatus*, *B. ovatus* and *B. xylanisolvens*) could be differentiated by a combination of MTA and phenotypical tests. Interestingly, whereas spectra of *B. fragilis* and *B. thetaiotaomicron* did not showed significant differences, MTA results of *B. caccae* strains seemed to reflect regional differences. Based on MTA as identification method, species as *B. xylanisolvens* and *B. dorei* could frequently been found in clinical material. MALDI-TOF-MS is a promising new tool for a rapid, accurate and cost-effective identification of anaerobic species.

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COMPARISON OF CONVENTIONAL CULTURE, CLONE LIBRARIES, AND PYROSEQUENCING FOR THE ANALYSIS OF BACTERIAL FLORA ASSOCIATED WITH WOUND INFECTION

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Objectives: It has been suggested that only a fraction of the total infecting flora may be recovered by traditional culture-based methods. Molecular methodologies enable researchers to examine the diversity of the bacterial flora associated with infections and to discover new pathogens. We compared conventional culture to clone libraries and pyrosequencing to analyze the bacterial flora associated with wound infections.
Methods: Specimens from 27 patients with surgical or traumatic wound infection were cultured aerobically and anaerobically as outlined in the Wadsworth Anaerobic Bacteriology Manual. Genomic DNA was obtained from all isolates and a region of the 16S rRNA gene amplified. PCR products were sequenced and compared with GenBank. Community DNA was extracted from the clinical specimens using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The DNA was used to construct 16S rRNA clone libraries and to perform bTEFAP (bacterial tag encoded FLX amplicon pyrosequencing).
Results: Altogether, 128 isolates representing 38 genera were isolated by culture, whereas clone libraries produced 172 unique sequences representing 49 genera, and the pyrosequencing 264 sequences representing 57 genera. *Staphylococcus aureus* was isolated from 9 specimens, whereas clone libraries and pyrosequencing detected *S. aureus* 8 and 5 times, respectively. Enterococci, *Enterobacteriaceae*, and *Streptococcus anginosus* group were isolated by culture from 9, 11, and 10 specimens, whereas clone libraries and pyrosequencing detected them from 6, 7, 7, and 5, 7, and 6 specimens, respectively. Culture yielded anaerobes from 15 specimens, clone libraries from 19, and pyrosequencing from 23 specimens.
Conclusions: The results show an obvious bias of the culture to yield only easily cultivable organisms. However, the culture method was consistent in recovering all the putative pathogens, such as *S. aureus*, *Enterococcus* sp. *S. anginosus* group, and *E. coli*. In general, pyrosequencing proved to be a more sensitive method than clone libraries. Both clone libraries and pyrosequencing exhibited PCR-dependent bias. Put together, the results of these different methods provide a comprehensive overall picture of the bacteria found in wound infection.

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DIAGNOSTIC METHODS & MICROBIOLOGY

SURVEY OF BACTERIAL DIVERSITY IN 100 WOUND SAMPLES VIA CULTURE AND REAL-TIME POLYMERASE CHAIN REACTION

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Purpose: Wound infection remains an important problem; to provide rapid and accurate identification of bacterial pathogens will give a better insight into it.
Methods: 100 wound samples were analyzed by culture, 16S rRNA gene sequencing, and real-time PCR (RT-PCR) methods. Primers and probes for RT-PCR detection were designed based on 54 target genes of putative principal pathogens.
Results: Overall, 313 organisms were isolated, including 169 aerobes and 144 anaerobes. *Staphylococcus aureus* was the most common aerobe and the *Bacteroides fragilis* group was the most common anaerobe. 90% of the isolates were identified to species and 94% to genus by 16S rRNA gene sequencing, with ≥ 98% sequence similarity to the GenBank database. RT-PCR generally yielded more bacterial species compared to culture, unless not included in the predicted primer-probe list. Additionally, RT-PCR took only hours to determine the result while culture took 1 to 2 days.
Conclusion: To a certain extent, conventional culture method combined with 16S rRNA gene sequencing provided accuracy in identification and permitted the detection of novel taxa (11 found). RT-PCR was capable of much more rapid identification of "uncultivable" bacteria and bacteria overlooked by culture or affected by antimicrobials.

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PEPTONIPHILUS COXII SP. NOV. AND *PEPTONIPHILUS TYRRELLIAE* SP. NOV. ISOLATED FROM HUMAN SKIN AND SOFT-TISSUE INFECTIONS

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Two groups of previously undescribed anaerobic, Gram-positive cocci, obtained from human skin and soft-tissue infection (SSTI) were characterized using phenotypic and molecular taxonomic methods. Phenotypic and comparative 16S rRNA analysis showed that the strains within these two groups were genotypically homogeneous and that each group was unique within the genus *Peptoniphilus*. The first group is most closely related to *Peptoniphilus ivorii* (94% sequence similarity) and the second group is most closely related to *P. harei* (95% sequence similarity).
Strains: The five novel strains related to *P. ivorii* were cultured from infections of the leg (3), upper back cyst (1) and tonsil biopsy (1). The two novel strains related to *P. harei* were cultured from a leg infection and an infected ischial pressure wound.
Methods: Strains were characterized by conventional phenotypic methods including PRAS biochemicals, API Rapid 32A and Rapid ANAII, as well as, molecular 16S rRNA sequencing.
Results: Both groups of the novel strains phenotypically resemble the genus. Cells are Gram-positive, ~1.0µm in diameter, uniform in size, may occur in pairs or small clusters and are non-motile. At 72hr, colonies are white, entire and ~1.0mm in diameter. Carbohydrates are not fermented. *P. coxii* is indole and catalase negative and on the API Rapid 32A, proline arylamidase is usually the only positive test. *P. tyrrelliae* is indole and catalase positive. On the API Rapid 32A, arginine, histidine and tyrosine arylamidase are positive; alanine, glycine, leucine and phenylalanine arylamidase are weak. Rapid ANA II kits were consistent with the Rapid ANA 32A kits.
Conclusion: Based on these findings we propose two novel species, *Peptoniphilus coxii* sp. nov. and *Peptoniphilus tyrrelliae* sp. nov. The type strain sequences have been deposited in GenBank as *Peptoniphilus coxii* sp. nov., RMA 16757^T (= GU938836) and *Peptoniphilus tyrrelliae* sp. nov., RMA 19911^T (=GU938835).

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DIAGNOSTIC METHODS & MICROBIOLOGY

ANAEROBIC BACTERIA IN DIAGNOSTIC CULTURES—A PRELIMINARY EXPERIENCE

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Background: Infections caused by anaerobic bacteria are common and involve all body systems and sites and these may be serious and life-threatening. Improved isolation techniques and growing resistance of anaerobic bacteria to empiric antimicrobials have underscored the importance of anaerobes in clinical practice.
Purpose of the Study: This study was undertaken over a period of one year from February 2009 to January 2010, to analyze the role played by anaerobic bacteria in clinical infections.
Methods: Tissue, pus, and body fluid samples collected under aseptic precautions were processed for anaerobic culture according to standard laboratory techniques. The anaerobes were identified on the basis of colony morphology, aero tolerance, gram stain, susceptibility to antibiotic identification discs, and biochemical reactions. Beta-lactamase testing, when performed was done using nitrocefin discs.
Results: A total of 74 anaerobic isolates were obtained from 64 specimens during the study period from a total of 628 samples (10.2%) submitted for anaerobic culture. Anaerobes were isolated from a varied spectrum of infections including diabetic foot ulcers, chronic suppurative otitis media, chronic osteomyelitis, subcutaneous abscesses, infected wounds, lung abscess, intra abdominal abscess, pyometra, subdural empyema, otogenic tetanus, antibiotic associated diarrhea and necrotizing fascitis.

40 (62.5%) samples showed mixed aerobic and anaerobic bacteria, while 24 (37.5%) showed only anaerobic bacteria. Gram-negative anaerobes (42, 56.8%) were more commonly isolated compared to gram-positive anaerobes (32, 43.2%). *Bacteroides fragilis (group)* was the most common anaerobe isolated. The anaerobic bacterial profiles of these infections include *Bacteroides fragilis* (30, 40.5%), Anaerobic gram-positive cocci (14, 18.9%), *Peptostreptococcus anaerobius* (6, 8.1%), *Prevotella spp.* (6, 8.1%), *Clostridium spp.* (5, 6.6%), *Clostridium difficile* (3, 4%), *Porphyromonas spp.* (3, 4%) *Fusobacterium nucleatum* (3, 4%), *Clostridium tetani* (2, 2.7%) and *Clostridium perfringens* (2, 2.7%). Beta-lactamase testing performed during the later part of the study revealed beta-lactamase production among 4 (67%) of 6 *Bacteroides fragilis* strains.
Conclusion: Because of their fastidious nature, anaerobes are difficult to isolate from infectious sites and are often overlooked. The key for detection of anaerobes is a high quality specimen taken directly from the infected site followed by appropriate methods of transportation and cultivation of specimens. Even-though a large variety of anaerobes were isolated in this study, further increase in the isolation rates can be achieved by incorporating appropriate specimen transport systems for anaerobic culture.

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TRAM, ENCODED BY THE MOBILE ELEMENT BTF-37, IS ESSENTIAL FOR CONJUGATION WITHIN AND FROM *BACTEROIDES FRAGILIS*

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Introduction: *Bacteroides sp* are reservoirs of antibiotic resistance genes which promiscuously transfer within and from the genus via conjugation. The *B. fragilis* clinical isolate LV23 harbors a 37kb conjugative transposon, BTF-37. Two important conjugal apparatus proteins encoded by BTF-37 are BctA, a predicted coupling protein (CP) that powers DNA translocation, and TraM. Preliminary studies showed that *traM* transcription is up-regulated upon antibiotic exposure, and that TraM localizes to the bacterial inner membrane, and interacts *in vivo* and *in vitro* with both a *B. fragilis* CP and a relaxase: all characteristics of a required and critical conjugal apparatus protein.
Methods and Results: Antisense, quantitative PCR, and mutagenesis approaches were used to further characterize the involvement of TraM in *B. fragilis* conjugation, and to identify specific TraM amino acids required for its interaction with the CP. By introducing different *traM* antisense constructs (AS1, 2 or 3) into *B. fragilis* LV23 to knock-down TraM expression, we determined that AS2 and AS3 impaired *B. fragilis* conjugation frequency to both *B. fragilis* and *E. coli* recipients 100% and 82%, respectively, while AS1 and a control did not. Quantitative PCR confirmed that *traM* transcript levels were downregulated 50% in the presence of AS2, whereas AS3 did not affect *traM* transcription, suggesting that it may block TraM expression at a post-transcriptional or translational level. Mutagenesis studies revealed that two amino acids (F66 and L123) in two predicted coiled-coil domains of TraM were required for TraM interaction with BctA, suggesting that these two amino acids may be essential for TraM function in mediating DNA transfer in *B. fragilis*.
Conclusion: TraM of BTF-37 exhibits characteristics of a critical *Bacteroides sp* conjugal apparatus protein. Antisense studies indicated that TraM is essential for conjugation. This is one of very few studies using antisense technology to knock-down target gene expression in anaerobe, avoiding the difficulty in modifying genes in these genera. Moreover, the amino acids required for TraM interaction with the CP may be important for its function in *B. fragilis* conjugation.

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DIAGNOSTIC METHODS & MICROBIOLOGY

MOLECULAR CHARACTERIZATION OF A PLASMATIC FIBRONECTIN-BINDING PROTEIN IN *BACTEROIDES FRAGILIS*

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Bacteroides fragilis is the Gram-negative strictly anaerobic bacterium most frequently isolated from clinical infections, including intra-abdominal abscesses and bacteremia. The major source of these infections is the normal colonic microbiota where *Bacteroides* spp. outnumber facultatively anaerobic bacteria. A number of factors may contribute to the virulence of these bacteria, including adhesins. Some of them are characterized and can recognize and bind to the Extracellular Matrix Components (ECM), such as, collagens, elastin, laminin and fibronectin. One of the molecules responsible for fibronectin-binding is an outer membrane protein (OMP) previously described by our group, that belongs to the TonB dependent family of protein. Thus, the aim of the present work was to overexpress and further characterize this novel adhesin responsible for the fibronectin recognition. The 1405 strain, isolated from patient presenting with bacteremia, which has high affinity for the fibronectin was used throughout this study. Initially, primers were designed within corporation of restriction sites to amplify a DNA fragment containing the entire TonB dependent ORF deposited in the NCBI database (*http://www.ncbi.nlm.nih.gov*) with the access number BF0466. The pet26b^{+}/Ads-6x his plasmid was transformed into *E. coli* Rosetta cells for expression and induced with 1mM IPTG. The cells were lysated using a sonicator and the protein purified using a Ni^{2+} charged resin (Novagen His-Bind® Kits). All fractions, including the pellet after lysis, and negative control (culture without IPTG) were applied in a SDS-PAGE. The purified eluted fractions were pooled and concentrated with a Pmax Amicon filter. Further experiments are been performed to get a better understanding about the real role of this protein in the pathogenicity of *B fragilis*.

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