

Detection of *Legionella pneumophila*, *Mycoplasma pneumoniae*, and the *Chlamydiaceae* Family from a Single Throat Swab Using the BD ProbeTec™ ET System

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REVISED ABSTRACT

OBJECTIVES:

- (1) To develop a panel of Strand Displacement Amplification assays for the detection of Community Acquired Pneumonia pathogens (*Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydiaceae* Family) from a single dry throat swab (stored without transport medium) using the BD ProbeTec™ ET System.
- (2) To determine analytical sensitivity, specificity, and specimen stability for all three pathogens.
- (3) To evaluate asymptomatic individuals to rule out the potential of a carrier state in otherwise healthy individuals.

METHODS: Analytical sensitivity was determined for each assay by testing serial dilutions of organisms and a cloned target nucleic acid sequence. Specificity was evaluated by challenging the assays with a variety of potential cross-reactants, including genetically related bacteria. To determine whether a carrier state exists for the target organisms, approximately 100 throat swab specimens were collected from asymptomatic volunteers. The stability of dry throat swab specimens was determined by testing a second panel of swabs obtained from asymptomatic individuals and seeded with organisms and stored at various temperatures. All specimens were processed and assayed using a universal buffer system and dried amplification reagents.

RESULTS: The analytical sensitivity of the three assays using cloned target nucleic acid was 160, 131, and 110 copies per test for *L. pneumophila*, *M. pneumoniae*, and *C. pneumoniae* DNA, respectively. The analytical sensitivity using seeded organisms was 61 and 11 organisms per test for *L. pneumophila* and *M. pneumoniae* respectively, and 35 elementary bodies per test for *C. pneumoniae*. None of the potential cross-reactants tested yielded false-positive results with any of the three assays. Dry throat swab specimens were found to be stable for 2 days at 26–30°C, 3 days at 2–8°C and at least 7 days at <-20°C. No false-positive results were observed in testing of throat swab specimens from asymptomatic individuals.

CONCLUSION: The BD ProbeTec ET *L. pneumophila*, *M. pneumoniae*, and *Chlamydiaceae* Family Assays offer the potential for highly sensitive and specific detection of these pathogens. All three assays may be performed from a single swab specimen using a streamlined workflow.

INTRODUCTION

Community acquired pneumonia (CAP) is the sixth leading cause of death in the US.⁽¹⁾ It is diagnosed in approximately 4 million adults each year with 25% of those patients requiring hospitalization.⁽²⁾ Mortality among patients with CAP has risen in recent years due in part to an increasing elderly population. Definitive diagnosis of CAP relies upon culture of the infecting organism and serological analysis, both of which lack adequate specificity and sensitivity. As a result, treatment of CAP is largely empirical and often inappropriate, leading to the emergence of drug-resistant strains. Atypical pneumonia caused by *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*, accounts for 40% of all cases of CAP. These organisms also commonly occur as co-pathogens in mixed infections with a mortality rate as high as 25%.⁽³⁾

We have developed a panel of assays for the detection of all three of these pathogens by homogeneous real-time fluorescent Strand Displacement Amplification (SDA) using the BD ProbeTec ET System. A single dry throat swab specimen can be used to test for all three CAP pathogens using the same streamlined workflow as other assays designed for the BD ProbeTec ET System. Swabs are simply expressed into an SDA buffer that is common to all three analytes and then heated to lyse the organisms and denature the target nucleic acid. Upon cooling, the lysed sample is used to rehydrate amplification reagents that are conveniently dried in color-coded microwells. Each assay incorporates an Internal Amplification Control (IAC) to validate negative results, and the system is designed to provide results for all three analytes for up to 24 samples in less than 2 hours (Figure 1). Here we present data demonstrating analytical sensitivity, specificity, and specimen stability for the three BD ProbeTec ET CAP assays as well as clinical data from analysis of swabs collected from primary health care patients with a persistent cough.

METHODS

DATA ANALYSIS. Data were analyzed using the Passes After Threshold (PAT) algorithm developed for these assays. (Figure 2).

LIMIT OF DETECTION. To determine the analytical sensitivity of the *Chlamydiaceae* Family, *L. pneumophila*, and *M. pneumoniae* assays, SDA was performed on serial dilutions of organisms and dilutions of the cloned target nucleic acid sequences. Sixteen replicates were tested at each target level for the *Chlamydiaceae* Family and *M. pneumoniae* assays, while 32 replicates at each level were tested in the *L. pneumophila* system (Figure 3).

SPECIFICITY. Assay specificity was evaluated using suspensions of known numbers of bacteria in phosphate-buffered saline (Figure 4).

CROSS REACTIVITY. A variety of potential cross-reactants were evaluated at concentrations of $\sim 10^6$ organisms or viral particles/test. These included phylogenetically related species as well as other organisms commonly found in the upper or lower respiratory tract. Three SDA assay replicates were performed with each organism. Detection of the IAC was used to validate negative results (Figure 5A & 5B).

THROAT SWABS FROM ASYMPTOMATIC INDIVIDUALS. To determine whether an asymptomatic carrier state exists for members of the *Chlamydiaceae* Family, *L. pneumophila*, and *M. pneumoniae*, throat swab specimens were collected from healthy volunteers, processed and tested for all three analytes. (Figure 6)

SPECIMEN STABILITY. To determine the stability of samples under different storage conditions, throat swabs were collected from healthy individuals and seeded with organisms at levels near the limit of detection for each of the three CAP assays. Swabs were stored dry at -20°C , $2-8^{\circ}\text{C}$, and ambient temperature ($26^{\circ}\text{C} - 30^{\circ}\text{C}$) for up to 7 days and tested at specified intervals. (Figure 7)

CLINICAL SAMPLES. Throat swab specimens were obtained from Dr. Berndt Claesson (Nova Medical AB, Skövde, Sweden) from primary health care patients with a cough lasting 7–30 days. Two swabs were collected from each patient. One was a Culturette™ EZ swab for BD ProbeTec and the second was a dacron swab for expression in 2-SP media (standard phosphate medium) for ACR testing. These specimens were tested for all three CAP pathogens using the BD ProbeTec ET System. Results for the *Chlamydiaceae* Family and *M. pneumoniae* assays were compared with those from an in-house PCR assay performed at Nova Medical (PCR 1). A sub-set of specimens was tested for *M. pneumoniae* by a second PCR method (PCR 2). No positive samples were discovered when testing these swabs on the BD ProbeTec ET System for *L. pneumophila*. A secondary method was not available for comparison of these results. (Figure 8)

REFERENCES:

- 1 Kanno M, Brown P. Community-Acquired Pneumonia: An Overview. *Curr Infect Dis* 1999;1:49-56
- 2 Battleman DS, Callahan M, Thaler HT. Rapid Delivery and Appropriate Selection to Reduce Hospital Stay of Patients with Community-Acquired Pneumonia: Link Between Quality of Care and Resource Utilization. *Arch Intern Med* 2002 Mar 25;162(6): 682-688
- 3 Gleason P. The Emerging Role of Atypical Pathogens in Community Acquired Pneumonia. *Pharmacother* 2002; 22:2S-11S

Figure 1. BD ProbeTec ET Throat Swab Workflow and Equipment

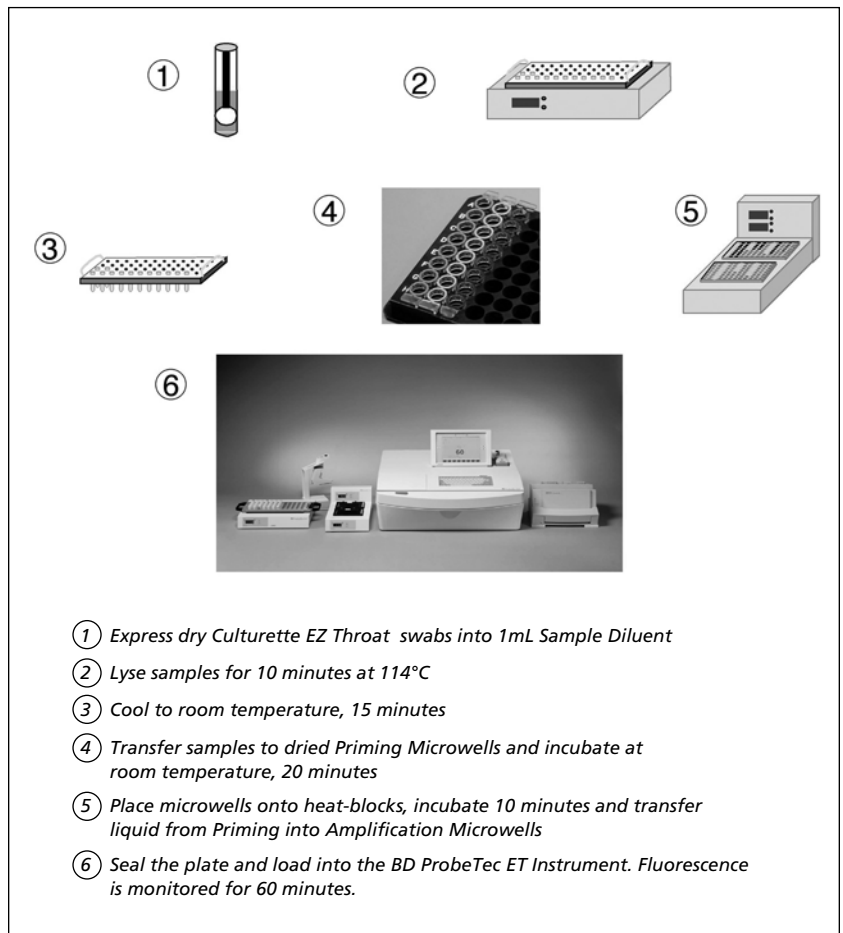


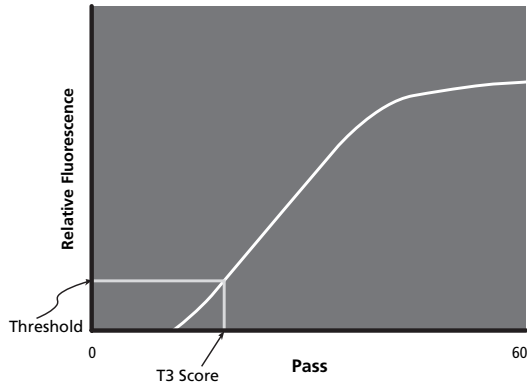
Figure 5A. Organisms Tested as Cross-Reactants in the Three Atypical Pneumonia Assays

Organism	Strain #	Organism	Strain #
<i>Acinetobacter calcoaceticus</i>	ATCC 13339	Influenza virus B (HK/5/72)	BD 4356
<i>Actinomyces israelii</i>	ATCC 10049	<i>Klebsiella pneumoniae</i> ssp. <i>ozaenae</i> type 4	ATCC 11296
Adenovirus-5	ABI 74-070	<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i>	ATCC 13883
<i>Aeromonas hydrophila</i>	ATCC 7966	<i>Lactobacillus acidophilus</i>	ATCC 4356
<i>Blastomyces dermatitidis</i>	ATCC 4292	<i>Legionella micdadei</i>	ATCC 33204
<i>Bordetella bronchiseptica</i>	ATCC 10580	<i>Legionella pneumophila</i>	ATCC 33152
<i>Bordetella parapertussis</i>	ATCC 15311	<i>Moraxella osloensis</i>	ATCC 19976
<i>Bordetella pertussis</i>	ATCC 9797	<i>Mycobacterium tuberculosis</i>	ATCC 27294
<i>Branhamella catarrhalis</i>	ATCC 25285	<i>Neisseria gonorrhoeae</i>	ATCC 19424
<i>Candida albicans</i>	ATCC 44808	<i>Neisseria meningitidis</i>	ATCC 13077
<i>Citrobacter freundii</i>	ATCC 8090	<i>Neisseria mucosa</i>	ATCC 19696
<i>Coccidioides immitis</i>	ATCC 7366	Parainfluenza I virus (Sendai)	BD 951010
<i>Corynebacterium diphtheriae</i>	ATCC 11913	<i>Peptostreptococcus anaerobius</i>	ATCC 27337
<i>Corynebacterium jeikeium</i>	ATCC 43734	<i>Porphyromonas asaccharolytica</i>	ATCC 29743
<i>Cryptococcus neoformans</i>	ATCC 36556	<i>Prevotella oralis</i> (<i>Bacteroides oralis</i>)	ATCC 25260
Cytomegalovirus (AD-169)	ABI AD-169	<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Eikenella corrodens</i>	ATCC 23834	RSV Long strain	ABI 74-093
<i>Enterobacter aerogenes</i>	ATCC 13048	Rhinovirus	ABI 80-015
<i>Enterobacter cloacae</i>	ATCC 13047	<i>Salmonella choleraesuis</i> serotype enteritidis	ATCC 13076
<i>Enterococcus faecalis</i>	ATCC 29212	<i>Salmonella choleraesuis</i> serotype typhi	ATCC 19430
<i>Enterococcus faecium</i>	ATCC 19434	<i>Serratia marcescens</i>	ATCC 8100
Enterovirus (Echovirus-11)	ABI 74-084	<i>Staphylococcus aureus</i> , protein A-producing	ATCC 12598
<i>Escherichia coli</i>	ATCC 11775	<i>Staphylococcus aureus</i> , non-protein A-producing	ATCC 25923
<i>Fusobacterium nucleatum</i>	ATCC 25586	<i>Staphylococcus epidermidis</i>	ATCC E155
group B <i>Streptococcus</i>	ATCC 12386	<i>Stenotrophomonas maltophilia</i>	ATCC 13637
<i>Haemophilus influenzae</i>	ATCC 33533	<i>Streptococcus mutans</i>	ATCC 25175
<i>Haemophilus parainfluenzae</i>	ATCC 7901	<i>Streptococcus pneumoniae</i>	ATCC 6303
Herpesvirus-1	ABI 68-097	<i>Streptococcus pyogenes</i>	ATCC 19615
<i>Histoplasma capsulatum</i>	ATCC 12700	<i>Veillonella parvula</i>	ATCC 10790
Influenza virus A (PR8)	BDAD PR8		

None of the BD ProbeTec ET CAP assays exhibited cross-reaction with any of the organisms tested

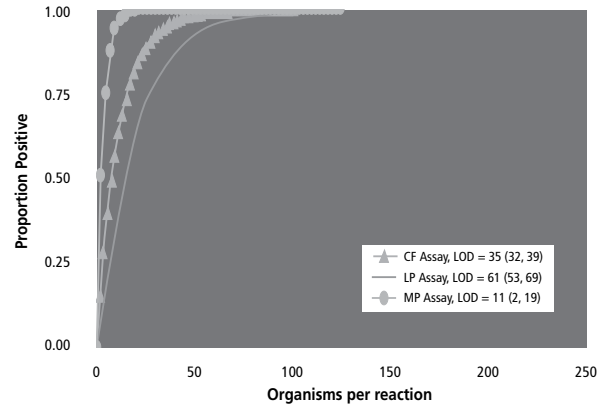
RESULTS

Figure 2. BD ProbeTec ET PAT Algorithm



- Time to Threshold (T3) is the time at which the background corrected signal crosses a pre-determined threshold
- The same threshold is used for every sample
- Passes After Threshold (PAT) score = 60 - T3
- Negative samples have PAT = 0
- Positive samples have PAT >0

Figure 3. LOD Curves for the Atypical Pneumonia Panel on the BD ProbeTec ET System



The limit of detection for all three assays is estimated with 95% confidence limits.

Figure 4. Specificity Panels

Specificity Panels								
Chlamydiaceae Family			Mycoplasma pneumoniae			Legionella pneumophila		
Organism	Strain #	Source	Organism	Strain #	Source	Organism	Serogroup	Source
<i>C. pneumoniae</i>	AR-39	ATCC 53592	<i>M. pneumoniae</i>	M. Eaton strain Mac	ATCC 15492	<i>L. pneumophila</i>	1	ATCC 33152
<i>C. pneumoniae</i>	TW-183	ATCC VR-2282	<i>M. pneumoniae</i>	FH strain	ATCC 15531	<i>L. pneumophila</i>	1	ATCC 33153
<i>C. pneumoniae</i>	CM-1	ATCC VR-1360	<i>M. pneumoniae</i>	M129-B7	ATCC 29342	<i>L. pneumophila</i>	2	ATCC 33154
<i>C. pneumoniae</i>	2043	ATCC VR-1355	<i>M. pneumoniae</i>	M52	ATCC 15293	<i>L. pneumophila</i>	3	ATCC 33155
<i>C. pneumoniae</i>	CDC/CWL-029	ATCC VR-1310	<i>M. pneumoniae</i>	Bru	ATCC 15377	<i>L. pneumophila</i>	4	ATCC 33156
<i>C. pneumoniae</i>	2023	ATCC VR-1356	<i>M. pneumoniae</i>	P1 1428	ATCC 29085	<i>L. pneumophila</i>	5	ATCC 33216
<i>C. trachomatis</i>	A	ATCC VR-571	<i>M. pneumoniae</i>	Mut 22	ATCC 39505	<i>L. pneumophila</i>	6	ATCC 33215
<i>C. trachomatis</i>	B	ATCC VR-573	<i>M. pneumoniae</i>	UT MB-10P	ATCC 49894	<i>L. pneumophila</i>	7	ATCC 33823
<i>C. trachomatis</i>	Ba	ATCC VR-347				<i>L. pneumophila</i>	8	ATCC 35096
<i>C. trachomatis</i>	C	ATCC VR-572				<i>L. pneumophila</i>	9	ATCC 35289
<i>C. trachomatis</i>	D	ATCC VR-885				<i>L. pneumophila</i>	10	ATCC 43283
<i>C. trachomatis</i>	E	ATCC VR-348				<i>L. pneumophila</i>	11	ATCC 43130
<i>C. trachomatis</i>	F	ATCC VR-346				<i>L. pneumophila</i>	12	ATCC 43290
<i>C. trachomatis</i>	G	ATCC VR-878				<i>L. pneumophila</i>	13	ATCC 43703
<i>C. trachomatis</i>	H	ATCC VR-879				<i>L. pneumophila</i>	14	ATCC 43736
<i>C. trachomatis</i>	I	ATCC VR-880				<i>L. pneumophila</i>	5	ATCC 33735
<i>C. trachomatis</i>	J	ATCC VR-886						
<i>C. trachomatis</i>	K	ATCC VR-887						
<i>C. trachomatis</i>	L1	ATCC VR-901B						
<i>C. trachomatis</i>	L2	ATCC VR-902B						
<i>C. trachomatis</i>	L3	ATCC VR-903B						
<i>C. psittaci</i>	Cal-10	BD 86-31306						
<i>C. psittaci</i>	Borg	ABI 4094-032895						

ATCC: American Type Culture Collection
All strains tested were successfully detected by the respective assays

Figure 5B. Organisms Tested as Cross-Reactants in Specific Assays

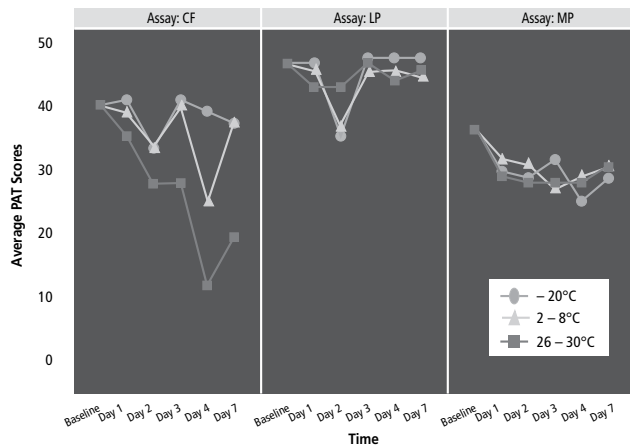
Chlamydiaceae Family				Legionella pneumophila		Mycoplasma pneumoniae	
Organism	Strain #	Organism	Strain #	Organism	Strain #	Organism	Strain #
<i>Acinetobacter lwoffii</i>	ATCC 19001	<i>Mycobacterium intracellulare</i>	ATCC 13950	<i>Legionella anisa</i>	ATCC 35292	<i>Acholeplasma laidlawii</i>	ATCC 23206
<i>Alcaligenes faecalis</i>	ATCC 8750	<i>Mycobacterium smegmatis</i>	ATCC 19420	<i>Legionella bozemanii</i>	ATCC 33217	<i>Alcaligenes faecalis</i>	ATCC 8750
<i>Bacillus subtilis</i>	ATCC 12100	<i>Mycoplasma genitalium</i>	ATCC 33530	<i>Legionella cherrii</i>	ATCC 35252	<i>Chlamydia pneumoniae</i>	ABI AR-39
<i>Bacteroides fragilis</i>	ATCC 25285	<i>Mycoplasma hominis</i>	ATCC 23114	<i>Legionella cincinnatiensis</i>	ATCC 43753	<i>Chlamydia trachomatis</i>	ABI LGV2
<i>Candida glabrata</i>	ATCC 90030	<i>Mycoplasma pneumoniae</i>	ATCC 63-030	<i>Legionella dumoffii</i>	ATCC 33279	<i>Legionella pneumophila</i>	ATCC 33152
<i>Candida tropicalis</i>	ATCC 750	<i>Neisseria cinerea</i>	ATCC 19696	<i>Legionella erythra</i>	ATCC 35303	<i>Mycoplasma arginini</i>	ATCC 23206
<i>Clostridium perfringens</i>	ATCC 13124	<i>Neisseria polysaccharea</i>	ATCC 43768	<i>Legionella fairfieldensis</i>	ATCC 49588	<i>Mycoplasma buccale</i>	ATCC 23636
<i>Corynebacterium renale</i>	ATCC 19412	<i>Peptostreptococcus productus</i>	ATCC 27340	<i>Legionella feeleeii</i>	ATCC 700514	<i>Mycoplasma faucium</i>	ATCC 25293
<i>Edwardsiella tarda</i>	ATCC 15469	<i>Plesiomonas shigelloides</i>	ATCC 14029	<i>Legionella gormanii</i>	ATCC 33297	<i>Mycoplasma fermentans</i>	ATCC 19989
Epstein-Barr Virus Sigma	Simga104H0854	<i>Prevotella melaninogenica</i>	ATCC 25845	<i>Legionella hackeliae</i>	ATCC 35250	<i>Mycoplasma gallinarum</i>	ATCC 15319
<i>Flavobacterium meningosepticum</i>	ATCC 13253	<i>Prevotella oralis</i>	ATCC 33269	<i>Legionella jordanii</i>	ATCC 33623	<i>Mycoplasma gallisepticum</i>	ATCC 19610
<i>Gardnerella vaginalis</i>	ATCC 14018	<i>Propionibacterium acnes</i>	ATCC 6919	<i>Legionella longbeachae</i>	ATCC 33462	<i>Mycoplasma genitalium</i>	ATCC 33530
<i>Gemella haemolysans</i>	ATCC 10379	<i>Proteus mirabilis</i>	ATCC 29906	<i>Legionella maceachernii</i>	ATCC 35300	<i>Mycoplasma hominis</i>	ATCC 23114
Herpes Simplex Virus, type II	ABI 4079-022895	<i>Providencia stuartii</i>	ATCC 35031	<i>Legionella micdadei</i>	ATCC 33204	<i>Mycoplasma hyorhinis</i>	ATCC 17981
HIV-1	ABI 4314-042198	<i>Salmonella minnesota</i>	ATCC 9700	<i>Legionella oakridgensis</i>	ATCC 33761	<i>Mycoplasma orale</i>	ATCC 23714
HPV type 16	ATCC 45113D	<i>Salmonella typhimurium</i>	ATCC 13311	<i>Legionella saintelensis</i>	ATCC 35248	<i>Mycoplasma penetrans</i>	ATCC 55252
HPV type 18	ATCC 45152D	<i>Staphylococcus epidermidis</i>	ATCC E155	<i>Legionella spiritensis</i>	ATCC 35249	<i>Mycoplasma primatum</i>	ATCC 15497
<i>Kingella kingae</i>	ATCC 23330	<i>Stenotrophomonas maltophilia</i>	ATCC 13637	<i>Legionella warseleensis</i>	ATCC 49508	<i>Mycoplasma synoviae</i>	ATCC 25204
<i>Lactobacillus brevis</i>	ATCC 14869	<i>Streptococcus mitis</i>	ATCC 6249	<i>Chlamydia pneumoniae</i>	ABI AR-39	<i>Mycoplasma salivarium</i>	ATCC 23064
<i>Legionella pneumophila</i>	ATCC 33152	<i>Streptomyces griseus</i>	ATCC 10137	<i>Chlamydia trachomatis</i>	ABI LGV2	<i>Ureaplasma urealyticum</i>	ATCC 27618
<i>Listeria monocytogenes</i>	ATCC 7644	<i>Trichomonas vaginalis</i>	ATCC 30001	<i>Mycoplasma pneumoniae</i>	ATCC 23064		
<i>Mobiluncus mulieris</i>	ATCC 35243	<i>Ureaplasma urealyticum</i>	ATCC 27618				
<i>Moraxella lucanata</i>	ATCC 17967	<i>Vibrio parahaemolyticus</i>	ATCC 17802				
<i>Morganella morganii</i>	ATCC 25830	<i>Yersinia enterocolitica</i>	ATCC 27729				
<i>Mycobacterium avium</i>	ATCC 25291						
<i>Mycobacterium goodii</i>	ATCC 14470						

Figure 6. Throat Swabs From Asymptomatic Individuals (n=100)

Assay Type	Positive	Unreportable Results	Inhibition Rate
<i>M. pneumoniae</i>	0	3	3%
<i>L. pneumophila</i>	0	0	0%
<i>Chlamydiaceae</i> Family	0	0	0%

A carrier state did not exist for *M. pneumoniae*, *L. pneumophila* or the *Chlamydiaceae* Family in the asymptomatic population tested

Figure 7. 7 Day Seeded Throat Swab Stability



Seeded swabs were stable for 2 days at 26-30°C, 3 days at 2-8°C, and 7 days at -20°C

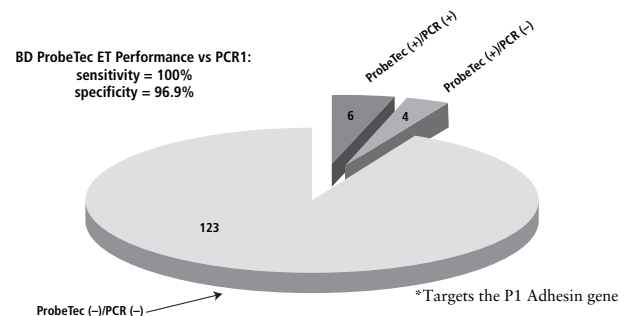
CONCLUSIONS

- The limits of detection (LODs) of the BD ProbeTec ET *Chlamydiaceae* Family, *L. pneumophila*, and *M. pneumoniae* assays were 35, 61, and 11 organisms/test respectively.
- All three assays were shown to be specific and not to cross-react with any of the closely related organisms tested or with other species found in the upper or lower respiratory tract.
- An asymptomatic carrier state does not appear to exist when tested across all 3 assays. The *M. pneumoniae* assay generated 3 non-reportable results (N=100).
- Organisms were shown to be stable on dry seeded throat swabs for 2 days at 26-30°C, 3 days at 2-8°C, and 7 days at -20°C, thereby demonstrating flexibility and convenience in sample collection and storage.
- The BD ProbeTec ET System CAP panel offers the ability to test for the *Chlamydiaceae* Family, *L. pneumophila*, and *M. pneumoniae* organisms from a single throat swab specimen and incorporates the following features:
 - An Internal Amplification Control (IAC) to validate negative results
 - A high degree of analytical sensitivity and specificity
 - A streamlined workflow with rapid time-to-results

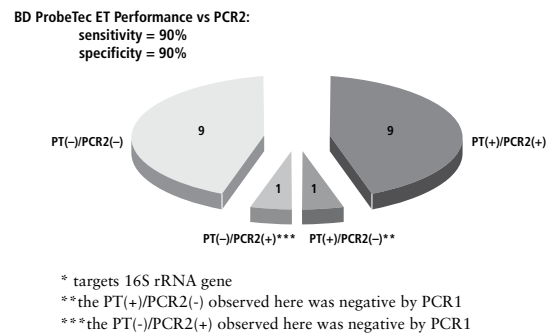
Figure 8. Testing of Clinical Specimens

- BD ProbeTec ET *M. pneumoniae* Assay results (n=133) were compared with results from a PCR method that targets the P1 Adhesin gene (PCR1). A subset (n=20) of these were tested by a second PCR method that targets the 16S rRNA gene (PCR2).
- BD ProbeTec ET *Chlamydiaceae* Family Assay results (n=133) were compared with results from a PCR method that targets the 16S rRNA gene.
- None of the specimens tested for *L. pneumophila* (n=101) yielded positive results in the BD ProbeTec ET *L. pneumophila* assay.

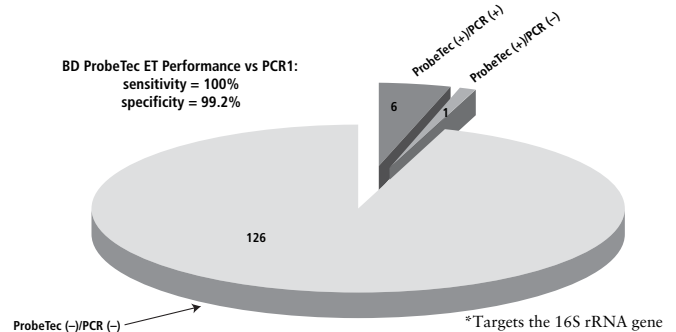
Detection of *M. pneumoniae*: BD ProbeTec ET vs PCR1*



Detection of *M. pneumoniae*: BD ProbeTec ET vs PCR2*



Detection of *Chlamydiaceae* Family: BD ProbeTec ET vs PCR*



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