Chapter 7

# A Real-Time PCR-Based Diagnostic Test for Organisms in Respiratory Tract Infection

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Additional information is available at the end of the chapter

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**Abstract**

Respiratory tract infection, especially pneumonia, is a significant cause of morbidity and mortality worldwide. Although rapid and accurate identification of the pathogens and the corresponding treatment, which is based on the microbiological results, is required in the healthcare setting, the current clinical tests lack high sensitivity and flexibility. As of yet, a comprehensive approach has not been able to work these issues out. Meanwhile, the development of molecular techniques enables the detection of organisms from respiratory specimens speedily as well as precisely and aids the settlement of such issues. With our novel approach that employs relative quantification, we successfully set the cutoff value to discriminate the causative pathogen from colonizing commensal organisms by real-time PCR. In this way, a diagnostic system for respiratory pathogens was devised and validated through clinical sample testing. In this chapter, a real-time PCR-based test capable of differentiating causative pathogens in respiratory specimens is described, and also its principle and the utility of this approach are illustrated.

**Keywords:** PCR, real-time PCR, pneumonia, respiratory tract infection, sputum, HIRA-TAN, commensal organism, foreign organism, non-commensal organism, nucleic acid amplification test, NAAT

## 1. Introduction

Pneumonia is a common disease in healthcare settings and is a significant cause of morbidity and mortality worldwide. Despite there being a mere two-dozen species of pathogens responsible for most cases of pneumonia, the causative pathogen cannot be identified by clinical tests involving smear and culture of sputum, antigen tests, and serological assays in up to 50% of the cases [1–4]. Therefore, the identification of the causative pathogen(s) with

high detection power will allow for the selection of targeted antibiotics. Accordingly, timely identification has been shown to reduce the mortality rate and, on a long-term basis, the emergence of drug-resistant pathogens [5]. To identify such pathogens and thus to obtain the desired benefits, a clinical test is required that is sensitive, rapid, accurate, easily performed, and cost-effective.

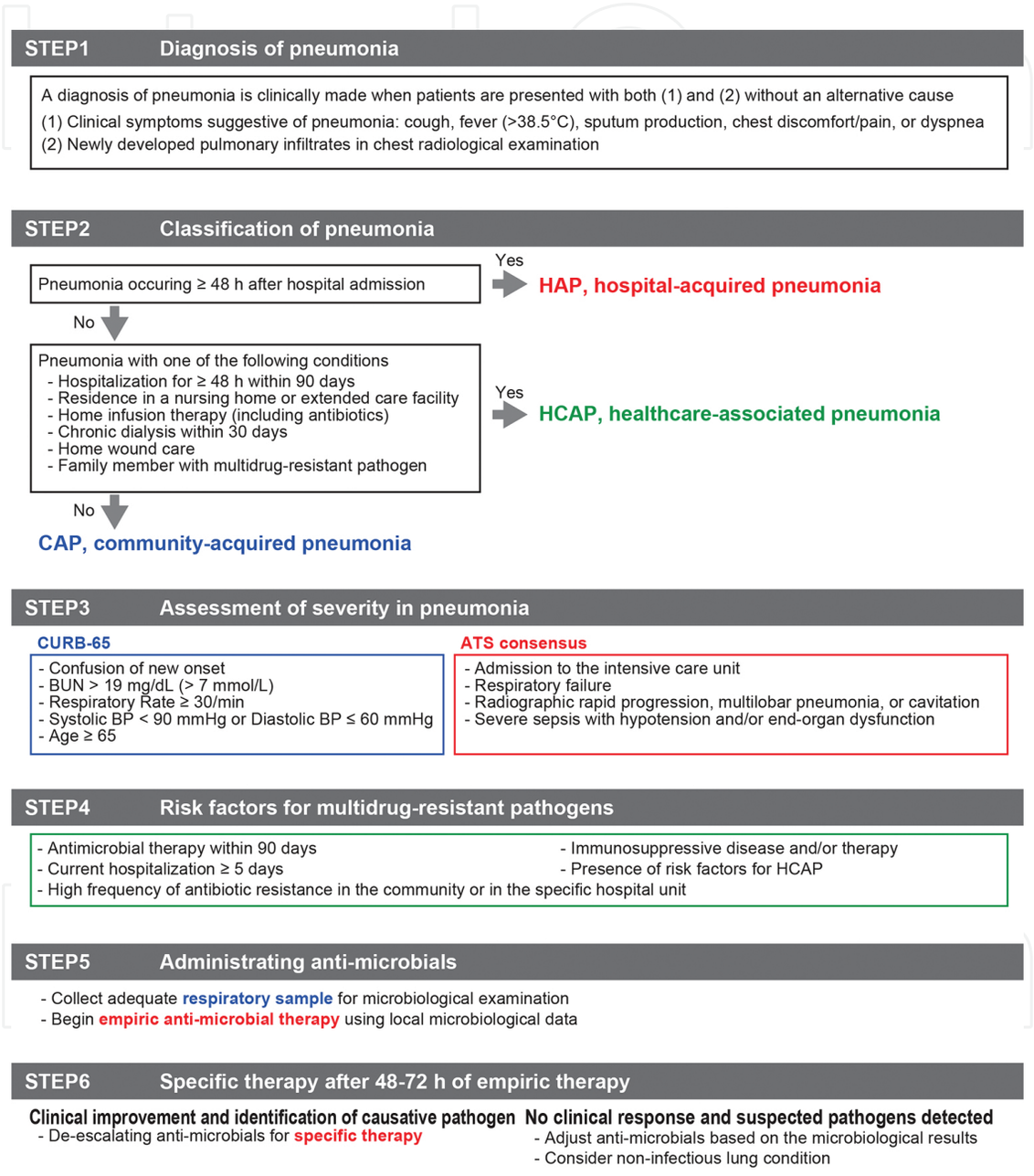


**Figure 1.** The overall strategy and assessment of pneumonia. The current consensus for diagnosis through treatment of pneumonia was summarized by STEP1 through STEP6 [3, 4]. Although examples of assessment, such as CURB-65 for CAP and ATS consensus for HAP, were illustrated in STEP3, an appropriate index ought to be chosen in each situation.

The development and wide usage of molecular techniques, such as polymerase chain reaction (PCR), brought about remarkable advances in clinical medicine. Detection of causative

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pathogens in pneumonia has been optimized in the last few decades, and from that point, PCR has played a principal role in laboratory medicine [6–8]. Currently PCR-based approaches, however, are mainly used to identify foreign organisms (such as *Mycoplasma pneumoniae* or *Legionella pneumophila*) in respiratory specimens [2, 9–11]. Even if such clinical tests have a multiplex detection system, identification of foreign organisms alone shows limited clinical value since the majority of pneumonia is caused by commensal organisms (such as *Streptococcus pneumoniae* or *Haemophilus influenzae*). Thus, distinguishing causative pathogens from among detected commensal organisms in respiratory samples, which contain colonizing organisms, has been challenging due to the highly sensitive detection power of PCR.

In this chapter, a real-time PCR-based test which is capable of differentiating therapeutic targets from detected colonizing commensal organisms in respiratory samples is described, and also its principle and the utility of this approach are illustrated.

## 2. Clinical approach and current strategy for pneumonia

Pneumonias are as of now classified as either the community-acquired pneumonia (CAP) or the hospital-acquired pneumonia (HAP), depending on the place pneumonia is acquired (**Figure 1**). Each has a specific spectrum of causative pathogens and allows medical professionals to speculate on the causative pathogen and initiate empirical antimicrobial therapy covering most of the speculated pathogens (**Figure 2**). Following confirmation of the causative pathogen, the antimicrobial should be switched/de-escalated to more specific and appropriate medication.

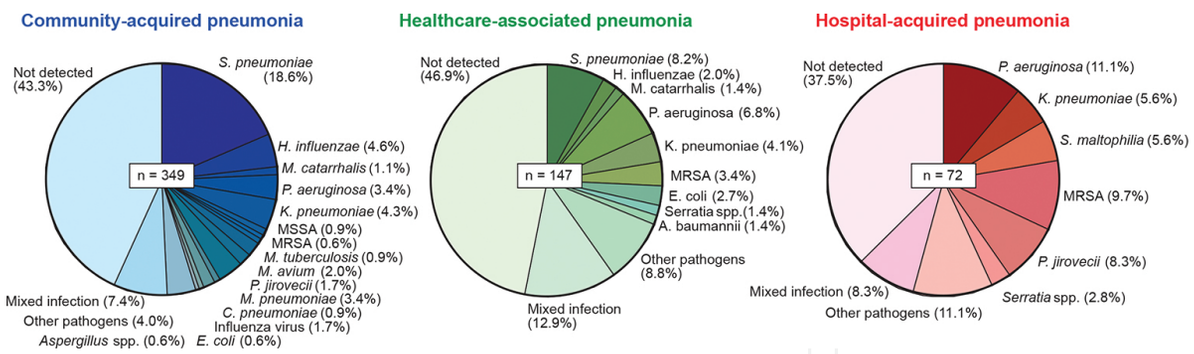


**Figure 2.** Etiology of three types of pneumonia diagnosed by conventional methods in clinical study. Five-hundred and sixty eight patients were enrolled in the prospective study where seven institutions participated [12]. Three-hundred and forty nine, 147, and 72 patients who met the criteria of pneumonia shown in **Figure 1** were categorized as community-acquired pneumonia (CAP), healthcare-associated pneumonia (HCAP), and hospital-acquired pneumonia (HAP), respectively, in which causative pathogen(s) were summarized in pie charts. In this study, all patients tested Gram stain and culture of expectorated sputum, urine antigen for *Streptococcus pneumoniae*, and real-time PCR for *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Coxiella burnetii*, *Legionella pneumophila*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Mycobacterium kansasii*, and *Pneumocystis jiroveci*. Urine antigen was tested for in the case of *L. pneumophila* for admitted cases only and influenza antigen for flu season only. Mixed infection indicates the cases in which two or more causative pathogens were identified at one time.

## 3. Organisms in respiratory tract infection

### 3.1. Conventional diagnostic tools for causative pathogens

A wide variety of laboratory tests including culture-based methods, antigen tests, and serology have been available for diagnosis and treatment of pneumonia (**Table 1**). Nevertheless, despite comprehensive evaluations with a range of different tests, as many as 40% of the causative pathogens causing CAP, HCAP, and HAP remain undiagnosed (**Figure 2**). While defining the pathogenic role of the respiratory organisms during pneumonia is still difficult, a commensal organism and foreign organism are often considered to be a causative pathogen when the below criteria are met. These criteria have provided legitimate results and thus have been used in clinical practice.

### 3.2. Commensal organism

Commensal organisms form part of human normal flora in the respiratory system (e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Pseudomonas aeruginosa*). However, in specific situations as when the host defense is weakened, the number of organisms grows beyond their typical ranges and then causes pneumonia. Since the majority of them reside in the respiratory tract, when they are detected from respiratory sample, there is no unequivocal criterion to discriminate causative pathogen from colonizing one by the sputum examination.

### 3.3. Foreign organism (non-commensal organism)

Foreign organisms account for a small portion of pneumonias (e.g., *Mycoplasma pneumoniae*, *Legionella pneumophila*, or *Mycobacterium tuberculosis*) (**Figure 2**). They do not reside in the respiratory tract or are rarely identified from healthy individuals, and thus their detection indicates that they are the causative pathogens.

### 3.4. Criteria for commensal organisms to be the causative pathogen

A commensal organism that fulfills at least one of the following three criteria is considered to be a causative pathogen when (1) an organism is identified from the normally sterile site (blood or pleural effusion); (2) a morphologically compatible organism, coexisting with abundant neutrophils, is observed through Gram staining and later confirmed by sputum culture; or (3) for *Streptococcus pneumoniae* only, the urine antigen test is positive.

### 3.5. Criteria for foreign organisms to be the causative pathogen

Foreign organisms that fulfill at least one of the following two criteria are considered to be a causative pathogen when (1) an organism is identified by culture, antigen test (involving serum, urine or nasopharyngeal specimen), or PCR test or (2) a paired serological test reveals a significant increase in antibody titer (more than four times).

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| | Rapid test | Confirmed diagnosis |
|---|---|---|
| **Commensal organisms** | | |
| **Gram-positive cocci** | | |
| *Streptococcus pneumoniae* | Gram stain, urine antigen | Culture |
| *Streptococcus milleri* group | | Culture |
| *Staphylococcus aureus* | Gram stain | Culture |
| **Gram-negative cocci** | | |
| *Moraxella catarrhalis* | Gram stain | Culture |
| **Gram-negative rods** | | |
| *Haemophilus influenzae* | Gram stain | Chocolate agar culture |
| (Non-fermenter) | | |
| *Pseudomonas aeruginosa* | Gram stain | Culture |
| *Acinetobacter baumannii* | | Culture |
| *Stenotrophomonas maltophilia* | | Culture |
| *Burkholderia cepacia* | | Selective agar culture |
| (Enterobacteria) | | |
| *Klebsiella pneumoniae* | Gram stain | Culture |
| *Escherichia coli* | | Culture |
| *Serratia marcescens* | | Culture |
| **Anaerobes** | | |
| Anaerobes | Gram stain from sterile sample | Anaerobic culture |
| **Foreign organisms** | | |
| **Atypical organisms** | | |
| *Mycoplasma pneumoniae* | NAAT | Serology CF, PPLO culture |
| *Legionella pneumophila* | NAAT, urine antigen | BCYE culture |
| *Chlamydophila pneumoniae* | NAAT | Serology MIF |
| *Chlamydophila psittaci* | NAAT | Serology MIF |
| *Coxiella burnetii* | NAAT | Serology IIF |
| *Mycobacterium Tuberculosis* | NAAT, AFB smear | Lowenstein-Jensen culture |
| **Opportunistic organisms** | | |
| *Actinomyces israelii* | | Anaerobic culture, microscopy for sulfur granules |
| *Nocardia asteroides* | Gram stain, AFB smear | Culture |
| *Pneumocystis jiroveci* | NAAT, Giemsa stain | |
| **Fungi** | | |
| *Aspergillus fumigatus* | GMS stain, Galactomannan test | Sabouraud agar culture |
| *Cryptococcus neoformans* | Antigen test | Sabouraud agar culture |
| *Histoplasma capsulatum* | Antigen test | Serology CF, Sabouraud agar culture |
| *Coccidioides immitis* | | Serology ID, Sabouraud agar culture |
| **Viruses** | | |
| Influenza virus | NAAT, rapid antigen | |
| Parainfluenza virus | NAAT | Serology EIA |
| RS virus | NAAT, rapid antigen | |
| *Human metapneumovirus* | NAAT | Serology EIA |
| Adenovirus | NAAT, antigen test | |
| *Cytomegalovirus* | pp65 antigen | |
| **Parasites** | | |
| *Paragonimus westermani* | Microscopy for ova | Microscopy for ova or worms |

Representative laboratory tools to diagnose clinically significant pathogens which cause pneumonia are listed [13]. NAAT, nucleic acid amplification test; CF, complement fixation; PPLO, pleuropneumonia-like organism; BCYE, buffered charcoal yeast extract; MIF, micro-immunofluorescence; IIF, indirect immunofluorescence; AFB, acid-fast *Bacillus*; GMS, Gomori methenamine silver; ID, immunodiffusion; EIA, enzyme immunoassay.

**Table 1.** Current laboratory diagnosis of pathogens causing pneumonia.

### 3.6. Issues with applying conventional diagnostic tools for identifying causative pathogens in respiratory samples

Although an organism requires specific agar or a unique detection assay, applying such different kinds of diagnostic tools for all patients with pneumonia is not practical and feasible from the standpoint of labor required. Moreover, the most significant issue is the culture-based method, which is a standard test for respiratory samples containing colonizing organisms or normal flora. It is not capable of discriminating a causative pathogen from isolated commensal organisms.

## 4. A real-time PCR for respiratory samples

### 4.1. Applying molecular techniques to identifying the causative pathogens in respiratory tract infection

Nucleic acid amplification test (NAAT), such as a PCR-based test or reverse transcription-PCR (RT-PCR), for purulent sputum is a logical and beneficial strategy. Firstly, if PCR cannot detect a suspected pathogen, the pathogen is less likely to be the causative pathogen due to the highly sensitive nature of PCR which can amplify even small numbers of pathogens. Secondly, PCR is capable of identifying foreign organisms that cannot grow on the standard culture agar. Thus, their detection conclusively yields the causative pathogens. Thirdly, PCR is a speedy test, and the result can be delivered to medical professionals in an early phase of the treatment. Finally, from the standpoint of NAAT, organisms causing respiratory tract infections can be simply divided into two categories; commensal organism and foreign organism (see Sections 3.2 and 3.3). Therefore, PCR does not require the pathogen-specific agar or growth conditions since all organisms are dealt with at the nucleic acid (DNA or RNA) level in the laboratory.

### 4.2. Issues with applying molecular diagnostic tools for identifying causative pathogens in respiratory samples

A major problem associated with a PCR-based test, similar to conventional methods, is its inability to discriminate a commensal organism causing pneumonia from the same organism colonizing in the airway.

Given the unique aspect of sputum, we assumed that, although setting a cutoff value by the direct quantification of bacterial cell number in respiratory samples would fluctuate and thus provide indistinct discrimination between causative pathogen and colonizing organism, using the relative quantification would be more stable even using the sputum which lacks homogeneity and reproductivity. To overcome this challenge, we proposed the "battlefield hypothesis," in which the ratio of pathogen to human cells in the respiratory samples would be an indicator for the dominant pathogen in the "pneumonia battlefield." The principal of this hypothesis is that the relative number of combatants (i.e., pathogens) causing the current state of pneumonia is considered a major determinant.

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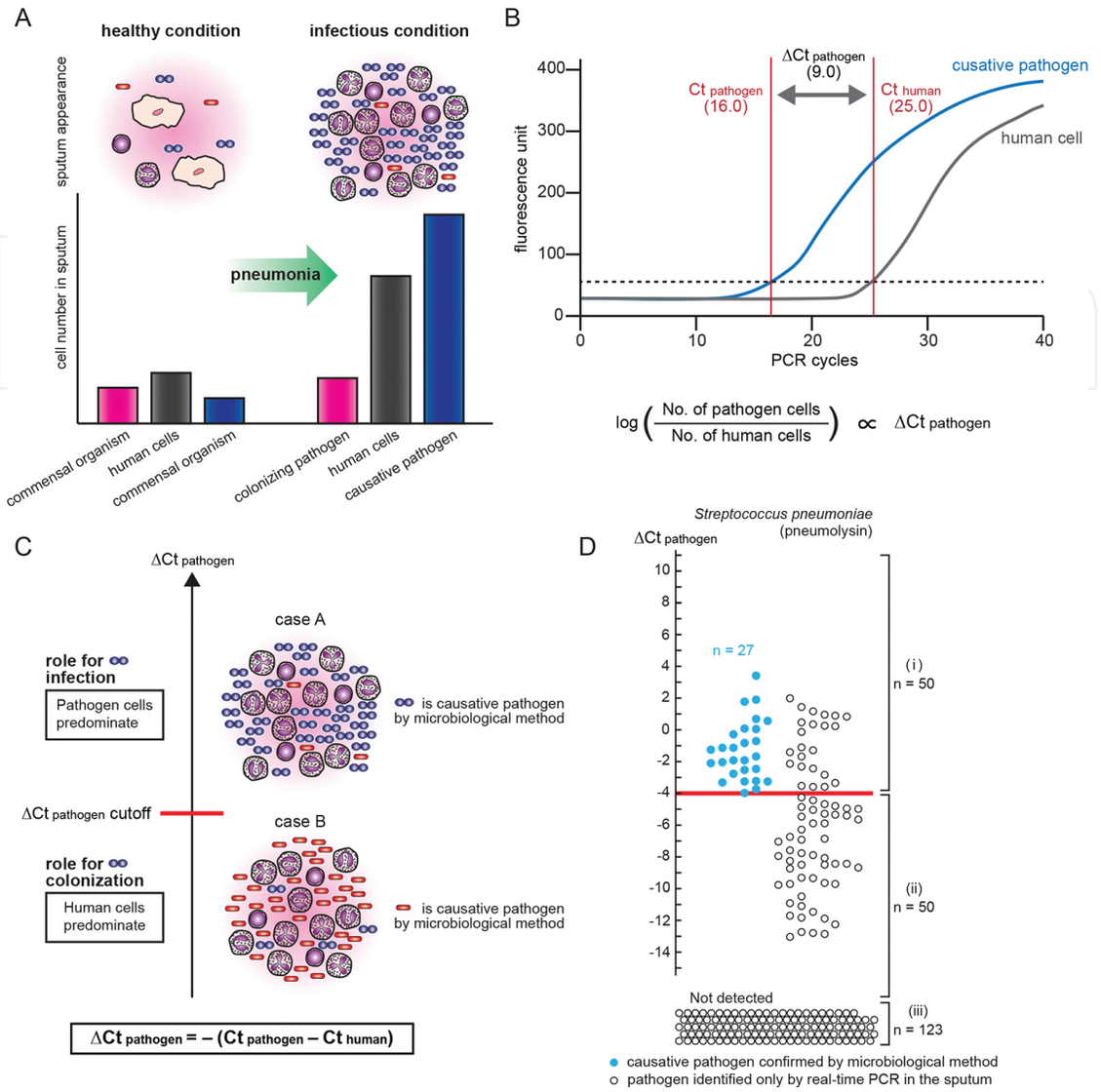


**Figure 3.** Battlefield hypothesis. (A) When pneumonia occurs, the number of human inflammatory cells (gray) increases at the inflammation site where that of the causative pathogen (blue) outnumbers the human cells. Meanwhile, the colonizing pathogens (red) lag behind. Thus, the ratio of causative pathogen cells to human cells was considered as a practical indicator for the discrimination of the causative pathogen from the colonizing organisms. (B) The ratio of cell numbers between two cell types in sputum is measureable by quantitative PCR. The Ct (threshold cycle) is the number of PCR cycles at which the fluorescent signal passes the threshold. $Ct_{human}$ is the Ct for the human-specific gene, $Ct_{pathogen}$ is the Ct for the pathogen-specific gene, and both are log proportional to the number of the cells during PCR cycles, which is accordingly formulated to $\Delta Ct_{pathogen} = -(Ct_{pathogen} - Ct_{human})$. (C) Since $\Delta Ct_{pathogen}$ indicates the ratio of pathogen cells to human cells, the $\Delta Ct_{pathogen}$ cutoff can be determined. Two examples of pneumonia cases were displayed; case A is *Streptococcus pneumoniae* (*S. pneumoniae*) pneumonia (Gram-positive cocci in purple), and case B is *Haemophilus influenzae* (*H. influenzae*) pneumonia (Gram-negative bacilli in red). In case A, the $\Delta Ct_{S.\ pneumoniae}$ value above which a pathogenic role of *S. pneumoniae* in pneumonia was confirmed should show a higher value, while a $\Delta Ct_{S.\ pneumoniae}$ value would demonstrate lower value in case B in which *H. influenzae* dominated human cells and *S. pneumoniae* played a colonizing role. (D) Determination of the $\Delta Ct_{pathogen}$ cutoff in a case of *S. pneumoniae* pneumonia. $\Delta Ct_{pathogen}$ was measured for commensal organisms in a pilot study (n = 223). Respiratory samples from patients with pneumonia in which *S. pneumoniae* was identified using commensal organism criteria (1)–(3) (see Section 3.4) were illustrated as blue circles, and samples in which none of criteria (1)–(3) was met were depicted as white circles. The $\Delta Ct_{S.\ pneumoniae}$ cutoff (a red line) was determined as the smallest $\Delta Ct_{S.\ pneumoniae}$ for the blue circles. Sputum in which *S. pneumoniae* was not detected and $\Delta Ct_{S.\ pneumoniae}$ was not applied is shown at the bottom (shown as "not detected").

### 4.3. Battlefield hypothesis

With the battlefield hypothesis, the ratio of the cell number of a commensal organism to human cell numbers is assumed to be an index of the organism's pathogenic role. When pneumonia occurs, the number of human cells, mostly inflammatory cells, drastically climbs at the site of infection where that of causative pathogen exceeds the human cells (**Figure 3A**). On the other hand, pathogens that merely colonize the affected area do not proliferate. In a real-time PCR-based system specializing in quantification, the specific primers and probe can amplify the target sequence log proportionally, in which the ratio of pathogen to human cells is formulated as $\Delta Ct_{pathogen} = -(Ct_{pathogen} - Ct_{human})$ (**Figure 3B**). As indicated by the battlefield hypothesis, a threshold value that discriminates commensal organisms from organisms colonizing the airway would be set up as $\Delta Ct_{pathogen}$ cutoff (**Figure 3C**).

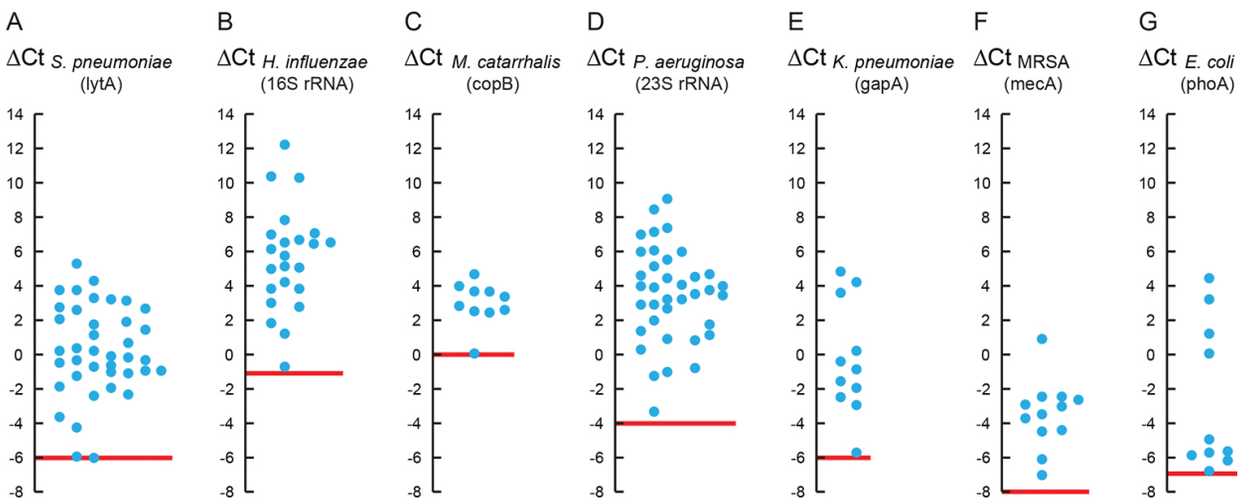


**Figure 4.** Determination of $\Delta Ct_{pathogen}$ cutoff. The $\Delta Ct_{pathogen}$ was measured for seven representative commensal organisms from the past clinical study (n = 533, from May 2007 to January 2009 at the Saitama Medical University Hospital and the participating institutes). Samples from patients with pneumonia in which a causative pathogen was identified using criteria (1)–(3) (see Section 3.4) are shown as blue circles. The $\Delta Ct_{pathogen}$ cutoff (a red line) was defined as the smallest $\Delta Ct_{pathogen}$ for the blue circles. The $\Delta Ct_{pathogen}$ cutoff was defined below: (A) −6 for *S. pneumoniae*, (B) −1 for *H. influenzae*, (C) 0 for *M. catarrhalis*, (D) −4 for *P. aeruginosa*, (E) −6 for *K. pneumoniae*; (F) −8 for MRSA, and (G) −7 for *E. coli*. Brackets indicate the target gene specific for each pathogen.

In order to verify the hypothesis, we first screened *S. pneumoniae* by specific real-time PCR (targeted to the pneumolysin gene) in 223 pneumonia patients (**Figure 3D**). By setting the cutoff as the smallest value of $\Delta Ct_{pathogen}$, the samples were classified into three groups: (i) samples with $\Delta Ct_{pathogen}$ > cutoff (−4), in which case the organism was considered to have a high chance of being a causative pathogen which was called a "therapeutic target"; (ii) samples with $\Delta Ct_{pathogen}$ < cutoff, in which case the detected organism had little chance of being a causative pathogen since the pathogen-to-human ratio has never been shown in a causative pathogen in pneumonia; and (iii) samples lacking organism detection, in which case the pathogen was unlikely to be a causative pathogen.

| | Gene (accession #) PCR product | | Sequence (5'–3') | | Gene (accession #) PCR product | | Sequence (5'–3') |
|---|---|---|---|---|---|---|---|
| **Control** | | | | **Foreign organisms** | | | |
| 1 | *Homo sapiens* | | | 11 | *Mycoplasma pneumoniae* | | |
| | SFTPC | Fw | GCAGTGCCTACGTCTAAGCTG | | 16S rRNA | Fw | AGTAATACTTTAGAGGCGAACGGGTGA |
| | (U02948.1) | Rv | TAGATGTAGTAGAGCGGCACCTC | | (NC_000912.1) | Rv | TCTACTTCTCAGCATAGCTACACGTCA |
| | 130 bp | Dp | CGAGATGCAGGCTCAGCACCCTC | | 227 bp | Dp | ACCAACTAGCTGATATGGCGCA |
| | | | | 12 | *Chlamydophila pneumoniae* | | |
| | | | | | 53KD-antigen | Fw | GCAACCACGGTAGCAACACAAATTA |
| | | | | | (E12535) | Rv | AATTGAGCGACGTTTTGTTGCATCT |
| **Commensal organisms** | | | | | 364 bp | Dp | AGCGGCTGTCAAATCTGGAATAAAAG |
| 2 | *Streptococcus pneumoniae* | | | 13 | *Chlamydophila psittaci* | | |
| | lytA | Fw | ACGCAATCTAGCAGATGAAGCA | | ompA | Fw | GTATGTTCATGCTTAAGGCTGTTTTCAC |
| | (AE005672) | Rv | TCGTGCGTTTTAATTCCAGCT | | (X56980.1) | Rv | TCCCACATAGTGCCATCGATTAATAAAC |
| | 75 bp | Dp | TGCCGAAAACGCTTGATACAGGGAG | | 291 bp | Dp | CCAGAAGAGCAAATTAGAATAGCGAGCA |
| 3 | *Haemophilus influenzae* | | | 14 | *Coxiella burnetii* | | |
| | 16S rRNA | Fw | TTGACATCCTAAGAAGAGCTCAGAGA | | Transposase | Fw | GTCTTAAGGTGGGCTGCGTG |
| | (Z22806.1) | Rv | CTTCCCTCTGTATACGCCATTGTAGC | | (M80806) | Rv | CCCCGAATCTCATTGATCAGC |
| | 267 bp | Dp | ATGGCTGTCGTCAGCTCGTGTT | | 295 bp | Dp | AGCGAACCATTGGTATCGGACGTTTATGG |
| 4 | *Moraxella catarrhalis* | | | 15 | *Legionella* spp. | | |
| | copB | Fw | GACGGGTGAGTAATGCCTAGGA | | 16S rRNA | Fw | AGGCTAATCTTAAAGCGCCAGGCC |
| | (U69982.1) | Rv | CCACTGGTGTTCCTTCCTATATCT | | (FR799709) | Rv | GCATGCTTAACACATGCAAGTCGAAC |
| | 298 bp | Dp | AGTGGGGGATCTTCGGACCTCA | | 198 bp | Dp | CATATTCCTACGCGTTACTCACCCGT |
| 5 | *Pseudomonas aeruginosa* | | | 16 | *Legionella pneumophila* | | |
| | 23S rRNA | Fw | TCCAAGTTTAAGGTGGTAGGCTG | | mip | Fw | TAACCGAACAGCAAATGAAAGACG |
| | (AJ549386) | Rv | ACCACTTCGTCATCTAAAAGACGAC | | (S72442.1) | Rv | AAAACGGTACCATCAATCAGACGA |
| | 94 bp | Dp | AGGTAAATCCGGGGTTTCAAGGCC | | 264 bp | Dp | TGATGGCAAAGCGTACTGCTGAA |
| 6 | *Klebsiella pneumonia* | | | 17 | *Bordetella pertussis* | | |
| | gapA | Fw | TGAAGTATGACTCCACTCACGGT | | BP485 | Fw | CGAGCCACTGTTTCTATTGATTGA |
| | (M66869) | Rv | CTTCAGAAGCGGCTTTGATGGCTT | | (BX640412) | Rv | CGGGCCTCATCTTCGTTCAG |
| | 670 bp | Dp | CCGGTATCTTCCTGACCGACGA | | 118 bp | Dp | TGTGCGTGTTTTCCCCAGAGCCCC |

| | Gene (accession #) PCR product | | Sequence (5'–3') | | Gene (accession #) PCR product | | Sequence (5'–3') |
|---|---|---|---|---|---|---|---|
| 7 | *Staphylococcus aureus* | | | 18 | *Mycobacterium tuberculosis* | | |
| | femB | Fw | TGGCCACTATGAGTTAAAGCTTGC | | MPB64 | Fw | ATCCGCTGCCAGTCGTCTTCC |
| | (DQ352467) | Rv | TCATAATCAATCACTGGACCGCGA | | (NC_000962) | Rv | CTCGCGAGTCTAGGCCAGCAT |
| | 162 bp | Dp | CGAGGTCATTGCAGCTTGCTTACTTA | | 238 bp | Dp | CCGGACAACAGGTATCGATAGCGCC |
| 8 | *Escherichia coli* | | | 19 | *Mycobacterium intracellulare* | | |
| | phoA | Fw | CGAAGAGGATTCACAAGAACATACC | | ITS 16-23S rRNA | Fw | AGCACCACGAAAAGCACTCCAATT |
| | (M29670) | Rv | GGTCTGGTCGGTCAGTCCAA | | (AM709724) | Rv | CGAACGCATCAGCCCTAAGGACTA |
| | 94 bp | Dp | CGGGCCATACGCCGCAATACGCA | | 243 bp | Dp | CCTGAGACAACACTCGGTCGATCC |
| | | | | 20 | *Mycobacterium avium* | | |
| | | | | | 16S rRNA | Fw | CAAGTCGAACGGAAAGGCCTCT |
| | | | | | (M29572) | Rv | GCCGTATCTCAGTCCCAGTGTG |
| **Drug resistance-related genes** | | | | | 257 bp | Dp | TACCGGATAGGACCTCAAGACGC |
| 9 | Metallo-beta-lactamase | | | 21 | *Mycobacterium kansasii* | | |
| | IMP | Fw | GGCAGYATTTCCTCTCATTTTCATAGC | | dnaJ | Fw | ACCCGTGTGATGAGTGCAAAGGC |
| | (AY625689) | Rv | AATTTGTRGCTTGAACCTTACCGTCTT | | (AB292544.1) | Rv | GTAAAGCTGACCGGAACTGTGACG |
| | 134 bp | Dp | ATTCTCGATCTATCCCCACGTATGCA | | 231 bp | Dp | AGGACGGACAGCGGATCAGACT |
| 10 | Methicillin-resistant *S. aureus* | | | 22 | *Pneumocystis jiroveci* | | |
| | mecA | Fw | AACTACGGTAACATTGATCGCAAC | | 5S rRNA | Fw | GTGTACGTTGCAAAGTACTCAGAAGA |
| | (AY786579) | Rv | CTTTGGTCTTTCTGCATTCCTGGA | | (AF461782) | Rv | GATGGCTGTTTCCAAGCCCA |
| | 112 bp | Dp | AGATGGTATGTGGAAGTTAGATTGGGA | | 346 bp | Dp | CTAGGATATAGCTGGTTTTCTGCGAA |
| | | | | 23 | *Nocardia* spp. | | |
| | | | | | 16S rRNA | Fw | CCTTCGGGTTGTAAACCTCTTTCGAC |
| | | | | | (DQ659898) | Rv | TTGGGGTTGAGCCCCAAGTTTTCA |
| | | | | | 191 bp | Dp | AAGAAGCACCGGCCAACTACGTGC |

A database search was used to select potential DNA sequences to identify microorganisms of interest. The primers and probes were validated using genomic DNA from the microorganism of interest as well as screened against 74 other microorganisms [12]. Fw, forward primer; Rv, reverse primer; Dp, detection probe (TaqMan).

**Table 2.** Primers and probes for the real-time PCR.

### 4.4. Determination of the $\Delta Ct_{pathogen}$ cutoff

We then confirmed that the primers and probes were specific to seven representative commensal organisms (*S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* (*M. catarrhalis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* (*E. coli*)) and the human genome and were able to quantify their cell numbers by counting the copy numbers (Ct value) for each in sputum. The pathogen-to-human cell number ratio was measured using 533 sputum samples collected in the past clinical study between May 2007 and January 2009 (**Figure 4**). Based on the microbiological examination and its criteria (see Section 3.4), the $\Delta Ct_{pathogen}$ cutoff to discriminate the causative pathogens was determined by the smallest $\Delta Ct_{pathogen}$ value in seven organisms (**Figure 4**).

### 4.5. HIRA-TAN system

Accordingly, we devised a PCR-based test for sputum samples that can distinguish causative pathogens from detected commensal organisms. Moreover, combining the described PCR system for "commensal organisms" with a PCR detection system for "foreign organisms" constitutes the HIRA-TAN (human cell-controlled identification of the respiratory agent from "TAN," which means sputum in Japanese), which involved 23 PCR with organism-specific target genes for quantifying 7 commensal organisms, 13 foreign organisms, 2 drug resistance-related genes (DRRG), and the human specific gene (as the internal control) (**Table 2**). HIRA-TAN was capable of screening 23 target genes simultaneously and diagnosing the therapeutic targets among commensal and foreign organisms in a single assay, which was able to be completed within 4 h. The technical details in the real-time PCR and the HIRA-TAN system were discussed in more detail in Refs. [12, 14].

### 4.6. Criteria for detected organisms to be the therapeutic target by HIRA-TAN system

In the HIRA-TAN system, the cutoff value was determined for each commensal organism, which enabled us to discriminate the *bona fide* therapeutic target from other commensal organisms detected by real-time PCR,. with the assumption being that the high relative levels of any of the given candidate pathogens would increase its likelihood of being the causative pathogen. Thus, commensal organisms were diagnosed as the "therapeutic target" when $Ct_{pathogen}$ exceeded the given cutoff value. Foreign organisms were diagnosed as the "therapeutic target" when detected by real-time PCR.

Technically, since opportunistic organisms, such as *Pneumocystis jiroveci* and *Nocardia* spp., were not normally identified from healthy individuals, they were classified as foreign organisms. Methicillin-resistant *S. aureus* (MRSA) was judged as therapeutic target in HIRA-TAN both when *S. aureus* (femB gene) was detected above −7 in the $\Delta Ct_{S.\ aureus}$ cutoff and when DRRG (mecA gene) was also detected above −8 in the $\Delta Ct_{MRSA}$ cutoff altogether. IMP-producing *P. aeruginosa* (multidrug resistance *P. aeruginosa*, MDRP) was judged as therapeutic target in HIRA-TAN both when *P. aeruginosa* was detected above −4 in the $\Delta Ct_{P.\ aeruginosa}$ cutoff and IMP gene was detected altogether.

## 5. Materials and methods

### 5.1. Respiratory specimen

In Section 5, samples and their treatment will be described. Sputum, induced sputum, or sputum obtained by intratracheal aspiration (sputum hereafter) was collected from patients with pneumonia. The sample was homogenized by pipetting and dispensed into two tubes; one was submitted for a standard microbiological test (microscopic examination and culture) and the other for nucleic acid extraction and real-time PCR analysis. To assess the pathogenic role with an appropriate sample, sputum with M2–P3 macroscopic appearance and a $Ct_{human}$ <27 (the human-specific gene with Ct (threshold cycle) value by the real-time PCR) were studied [15]. Classification of the gross appearance of the sputum (M1, M2, P1, P2, and P3) was according to Miller and Jones [16].

### 5.2. DNA preparation from sputum

There are several kit options available for DNA extraction depending on the sample type; however, only column-based extraction has been used for most respiratory specimens due to their viscosity [14]. The sample was diluted with an equal volume of phosphate-buffered saline (PBS) and homogenized by vortexing. 200 μL of the homogenate was mixed with 200 μL AL buffer (Qiagen, Tokyo, Japan) containing 20 μL proteinase K (Takara Bio Inc., Shiga, Japan), and the resultant mixture was incubated at 56°C for 1 h. The DNA was extracted with 100 μL TE buffer using QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). The DNA concentration, based on the absorbance, was determined in a spectrophotometer GeneQuant Pro (GE Healthcare, Tokyo, Japan). The ratio of nucleic acid to protein absorbance (260 nm/280 nm) was calculated as an index of the purity of DNA samples [14].

### 5.3. Real-time PCR

Although a variety of PCR methodologies and devices are available, an illustration of the details of our approach is given. The final solution of the PCR contained 12.5 μL of the Takara Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 300 nM of each primer, 100–300 nM of the fluorescence-labeled TaqMan probe, 1.0 μL of purified DNA, and deionized distilled water up to 25.0 μL. The PCR for 23 target genes was multiplexed in 16 reactions and amplified using in a single assay. The PCR was performed by starting at 95°C for 30 s followed by 40 cycles at 95°C for 8 s, 61°C for 25 s, and 72°C for 20 s using the SmartCycler II (Cepheid, Sunnyvale, CA). The sequences of primer and probe were described in **Table 2**.

## 6. Practical application of HIRA-TAN system

### 6.1. Prospective study

We designed a prospective study to investigate the validity of the cutoff values we set up for the commensal organisms in the HIRA-TAN system. The aim of the study was the proportion

of samples in which $\Delta Ct_{pathogen}$ was greater than the cutoff value (diagnosed as the therapeutic target by HIRA-TAN), compared to the proportion of the samples in which each commensal organism was shown to be the causative pathogen (diagnosed as the causative pathogen by microbiological methods (**Table 1**)). The study was performed between February 06, 2009, and October 14, 2010, at the Saitama Medical University Hospital and other six participating institutes. Five-hundred and sixty eight patients with pneumonia were enrolled, and the results of the microbiological examinations were summarized in Ref. [12] and **Figure 2**. The identification rates for *S. pneumonia*, *H. influenzae*, *M. catarrhalis*, *P. aeruginosa*, *K. pneumoniae*, MRSA, and *E. coli* by the HIRA-TAN were 91.6% (87/95; 95% CI 84.1–95.6%), 96.7% (29/30; 95% CI 82.8–99.2%), 90.9% (10/11; 95% CI 58.7–97.7%), 93.2% (41/44; 95% CI 81.3–97.5%), 80.6% (29/36; 95% CI 64.0–89.9%), 78.1% (25/32; 95% CI 60.0–88.5%) and 87.5% (14/16; 95% CI 61.6–96.0%), respectively (**Figure 5A–G**). The response of the antibiotics and the relief of pneumonia for the cases in which such commensal organisms were the causative pathogens showed a consistent clinical course as predicted from the results of the HIRA-TAN.

### 6.2. Overall identification capacity of therapeutic targets by real-time PCR-based test

Overall performance of the HIRA-TAN system to identify both the therapeutic targets (commensal organisms judged by the ΔCt cutoff and foreign organisms detected by the real-time PCR) was altogether 60–70% en masse, which was comparable to what was attained by an extensive search using multiple detection methods [10, 18, 19]. However, it is supposed to reach its limit to identify the causative pathogens using primers and probe of only bacteria, and for a thorough investigation, incorporating PCR systems for viruses, anaerobes, and fungus will be required.

### 6.3. Beneficial aspects of PCR-based system for identifying pathogen

The most prominent feature of the HIRA-TAN is its ability to identify the causative pathogen for the pneumonias from among the commensal organisms detected in the sputum. Clinically, without this ability, this system would have been only partially useful, since more than half of pneumonias are caused by commensal organisms (**Figure 2**). And this system does not require the use of pathogen-by-pathogen identification methods (unique agar or a specific antibody for an organism). The easily performed comprehensive test covers a wide variety of pathogens in a single assay, which will reduce the time and labor spent on cumbersome procedures. The HIRA-TAN procedure now provides a comprehensive detection system for causative pathogens of pneumonia.

The HIRA-TAN system can also be expanded to include more pathogens, thereby increasing its abilities. The addition of any respiratory viruses or particular fungi to the screening protocol is straightforward [6, 9–11, 20]. Likewise, the inclusion of other commensal organisms, such as *Acinetobacter baumannii*, *Burkholderia cepacia*, or *Stenotrophomonas maltophilia*, will also be beneficial in nosocomial pneumonia. In addition, the most challenging category is anaerobes. Since investigating the anaerobe's contribution to respiratory tract infection by molecular techniques has not been attempted, indicating that setting a cutoff value for anaerobes with HIRA-TAN system will be beneficial. Thus, the commensal organisms that have conformed to

the scheme shown in **Figure 3A–D** and all non-commensal organisms become candidates, which will make the greater detection rate by the HIRA-TAN than the current 60–70% performance.

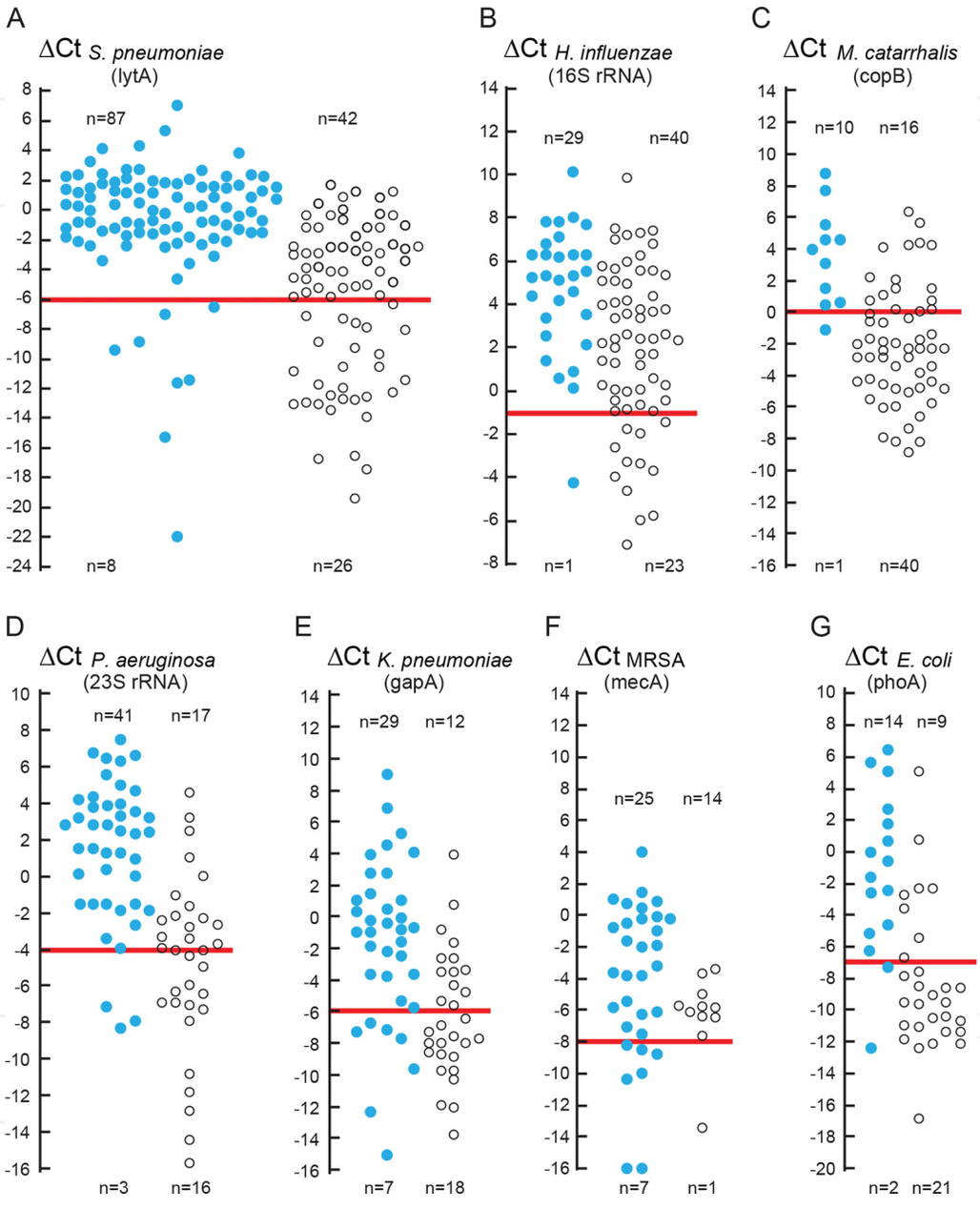


**Figure 5.** Evaluating $\Delta Ct_{pathogen}$ for each commensal organism in the prospective study (n = 568). Samples from patients with pneumonia in which a causative pathogen was identified using criteria (1)–(3) (see Section 3.4) are shown as blue circles, and samples from patients with pneumonia in which none of criteria (1)–(3) was met were shown as white circles. The HIRA-TAN identified the causative pathogens: (A) 91.6% (87/95; 95% CI 84.1–95.6%) for *S. pneumoniae*, (B) 96.7% (29/30; 95% CI 82.8–99.2%) for *H. influenzae*, (C) 90.9% (10/11; 95% CI 58.7–97.7%) for *M. catarrhalis*, (D) 93.2% (41/44; 95% CI 81.3–97.5%) for *P. aeruginosa*, (E) 80.6% (29/36; 95% CI 64.0–89.9%) for *K. pneumoniae*, (F) 78.1% (25/32; 95% CI 60.0–88.5%) for MRSA, and (G) 87.5% (14/16; 95% CI 61.6–96.0%) for *E. coli*. Brackets indicate the target gene specific for each pathogen. The 95% confidence interval (CI) was calculated using the formula for the binomial probabilities by the software Mathematica Ver.8TM (Wolfram Research, IL, USA) [17].

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### 6.4. Drawback of newly emerged PCR system

Currently the ability of the HIRA-TAN system to determine if MRSA is the causative pathogen is lacking. This is largely due to the fact that the determination and establishment of the cutoff value for a given microorganism in the HIRA-TAN system still require the conventional sputum examination. To date we have not been able to determine the $\Delta Ct_{MRSA}$ cutoff, and this will take more time and the availability of properly analyzed clinical samples to be established. Likewise, the diagnosis of *Pneumocystis jiroveci* pneumonia is hardly confirmed since the definitive diagnosis for this organism by conventional method has not been established.

## 7. Conclusion

In this chapter, the principle and utility of a real-time PCR-based diagnostic test for the causative pathogen in respiratory samples was described. Although rapid and accurate identification of pathogens and corresponding treatment based on the microbiological results are required in the healthcare setting, the current clinical tests lacking high sensitivity and a comprehensive approach have not been able to work these issues out. Development of molecular techniques and their usefulness enables the detection of organisms from the clinical specimens speedily as well as precisely and aids the settlement of such issues. With our novel approach that employs the relative quantification, we successfully set up the cutoff value to differentiate the causative pathogen from colonizing commensal organisms by PCR, with which a real-time PCR-based diagnostic system was devised and validated through clinical sample testing. Although this may be only one instance among many comprehensive systems, innovating such systems will help patients struggling with these disorders in the future.

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