RESPIRATORY INVESTIGATION 57 (2019) 337–344

Contents lists available at ScienceDirect

Respiratory Investigation

journal homepage: www.elsevier.com/locate/resinv

Original article

HIRA-TAN detects pathogens of pneumonia with a progressive course despite antibiotic treatment

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ARTICLE INFO

Article history:
Received 21 October 2018
Received in revised form
9 January 2019
Accepted 5 March 2019
Available online 26 April 2019

Keywords:
Empiric antibiotics
Microbiological techniques
Molecular typing
Multiplex polymerase chain reaction
Pneumonia

ABSTRACT

Background: Empiric antibiotics are administered for pneumonia when the causative pathogens are unidentified. Pathogen-directed therapy is impeded by negative culture results and/or culture time lag. This circumstance necessitates a salvage method for pathogen identification, especially when antibiotic therapy has failed. Here, we aimed to preliminarily investigate the HIRA-TAN method in pneumonia with a progressive course despite prior empiric antibiotic therapy.

Methods: This prospective study was conducted for patients who were referred to Dr. Zainoel Abidin Hospital, Aceh, Indonesia, from December 2016 to January 2017, owing to pneumonia with a progressive course. Sputum or pleural effusion was subjected to culture and the HIRA-TAN assay. The HIRA-TAN identified the candidate causative pathogens based on the difference in the cycle threshold (Ct) between the targeted pathogen and the single-copy human gene.

Results: Patients (n=27) were predominantly males (22 patients, 81.5%), with a median age of 62 years. All patients had comorbid disease and were classified as hospital-acquired pneumonia (25 patients, 92.6%) with multilobar infiltrates (22 patients, 81.5%). Bacterial culture identified causative pathogen(s) in some (14 patients, 51.8%), whereas the HIRA-TAN identified pathogen(s) in most (23 patients, 85.2%). The rapid pathogen identification by the HIRA-TAN will provide valuable information in guiding pathogen-directed therapy.

Conclusions: The result warrants a larger clinical trial to confirm the clinical efficacy of the HIRA-TAN in patients with progressive pneumonia despite previous antibiotic treatment.

Abbreviations: PCR, polymerase chain reaction; CAP, community-acquired pneumonia; TNF, tumor necrosis factor

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https://doi.org/10.1016/j.resinv.2019.03.001
2212-5345/

1. Introduction

Pneumonia is a significant cause of morbidity and mortality worldwide [1,2]. Empiric broad-spectrum antibiotics have been used to reduce mortality, anticipating all pathogens that may be the cause of pneumonia [3]. This strategy often leads to an overuse of broad-spectrum antibiotics and risks the emergence of drug-resistant pathogens [4]. Therefore, adequate antibiotic management based on the prompt identification of the pathogen(s) will help to de-escalate the antibiotic use; however, this practice is hindered by the culture time lag and/or negative culture results. Rapid and sensitive bacterial diagnostics and antibiotic susceptibility tests are required [5].

Polymerase chain reaction (PCR) has been widely applied in diagnostic microbiology during recent decades [6]. Ironically, the highly sensitive nature of PCR obscures the clear discrimination between pathogens that actually cause pneumonia and those simply colonizing the site. The HIRA-TAN was established to overcome this problem, and has successfully detected pathogens in untreated, community-acquired pneumonia (CAP) [7,8].

An important clinical question regarding the HIRA-TAN is whether it shows clinical benefits in pneumonia cases for which first-line antibiotic treatment has failed. Progressive disease during antibiotic treatment is a serious situation, in which the empirical choice of antibiotics may no longer be effective. Rapid and accurate identification of pathogens is required as salvage microbiology [9]. The HIRA-TAN may be effective even in such circumstances. The aim of this study was to preliminarily investigate the utility of the HIRA-TAN in patients who have progressive pneumonia, even under treatment with empirical antibiotics. The result will give evidence that the HIRA-TAN provides information on pathogens causing progressive pneumonia, even in the patients for whom culture provides negative results.

2. Methods

2.1. Study subjects

This prospective study was carried out between December 2016 and January 2017 in Dr. Zainoel Abidin Hospital, Banda Aceh, Indonesia. The inclusion criteria for patients were to have cough, chest discomfort, productive sputum, fever, and dyspnea on physical examination; leukocytosis on laboratory examination; and progressive pulmonary infiltrates on chest radiology. Written informed consent was obtained from all patients.

2.2. Sample preparation

The clinical specimens were sputum or pleural effusion that was collected by thoracentesis. All the clinical specimens were collected at Dr. Zainoel Abidin Hospital with sterile screw-top containers, then transported to the laboratory. The specimen was divided into two portions: one was used for conventional culture and the other was used for the HIRA-TAN. The specimen for the HIRA-TAN was diluted with an equal volume of phosphate-buffered saline and homogenized by vortexing. DNA was isolated using the Blood/Cell DNA Mini Kit GB100 (Geneaid, Taipei, Taiwan). A 200-μl portion of homogenate or pleural effusion was added to 200 μl of GB buffer in a 1.5-ml centrifuge tube and thoroughly mixed. The reaction mixture was incubated at 60 °C for more than 30 min. DNA was eluted in 100 μl deionized distilled water. Genomic DNA was stored in a 4 °C refrigerator until quantitative PCR was performed.

2.3. The HIRA-TAN

The first step of the HIRA-TAN is real time PCR. The PCR is a multiplex TaqMan assay performed in 5 separate reactions [7,8]. Each reaction detects 2 to 4 target genes, with a total of 18 target genes being detected (Tables 1 and 2). The reactions contain a 25-μl reaction mixture consisting of 12.5 μl Takara Premix Ex Taq (Takara Bio Inc., Shiga, Japan), primers for one or more pathogens (each 250 nM), primers for the human tumor necrosis factor (*TNF*) gene, fluorescence-labelled detection probes for the pathogens (250 nM), fluorescence-labelled detection probes for the human *TNF* gene (250 nM), and a 1-μl sample, where the average DNA concentration was 7.13 ng/μl in the current study. The PCR was performed in the SmartCycler II (Cepheid Inc., Sunnyvale, USA) with a cycling profile of 95 °C for 30 s, followed by 35 cycles of 95 °C for 8 s, 61 °C for 25 s, and 72 °C for 20 s.

The second step of the HIRA-TAN is the evaluation of the quality of the samples by the cycle threshold for the human TNF gene: Ct_{Human}. The *TNF* gene comprises a single copy in the human genome, and thus its copy number in the reaction directly reflects the number of human cells. Sputum samples with a $Ct_{Human} < 27$ contain many human cells and have been classified in M2-P3, according to the Miller and Jones' classification, and are expected to provide highly reliable results [7,10].

The last step is to normalize the number of pathogen cells to the number of human cells. Human cells in purulent sputum or in para-pneumonic pleural effusion are mostly inflammatory cells (neutrophils and lymphocytes). Therefore, the ratio of the number of pathogen cells to the number of human cells is likely to reflect the balance of power between them [7,8]. The log of the ratio of pathogen cells to human cells is expressed as $\Delta Ct_{Pathogen}$, that is,

$$\Delta Ct_{Pathogen} = -\left(Ct_{Pathogen} - Ct_{Human}\right) = \log\left(\frac{Number\ of\ pathogen\ cells}{Number\ of\ human\ cells}\right)$$

Pathogens with a $\Delta Ct_{Pathogen}$ above the cut-off value overwhelm inflammatory cells in numbers and are thus considered to be the pathogens causing pneumonia. Otherwise, the pathogens are considered to be the colonizing ones.

2.4. Microbial and human genomic DNA

Escherichia coli (12713G), *Pseudomonas aeruginosa* (106052G), and *Klebsiella pneumoniae* subsp. *pneumoniae* (14940G) genomic DNA was purchased from Biological Resource Centre, NITE (Chiba, Japan). *Staphylococcus aureus* subsp. *aureus* Rosenbach

Table 1 – Primers and probes of the target genes.

No	Target	Gene (Accession number)		Oligonucleotide	Amplicon (bp)
1	*Homo sapiens*	TNF (NC_000006.12)	F[a]	5′-GTGGAGCTGAGAGATAACCAGC-3′	153
			B	5′-GACCTTGGTCTGGTAGGAGACG-3′	
			P	FAM-CTGTACCTCATCTACTCCCAGGTC-BHQ1	
2	*Pseudomonas* spp.	16S rRNA (AY486350.1)	F	5′-GTGAGTAATGCCTAGGAATCTGC-3′	135
			B	5′-CTAATCCGACCTAGGCTCATCTG-3′	
			P	ALEXA532-AGTGGGGGATCTTCGGACCTC-BHQ1	
3	*Enterobacter* spp.	tusB (AH002539.2)	F	5′-CGTCAGACTTACGGTTAAGCAC-3′	193
			B	5′-GTACCAGCTGGTTAACTGTTGC-3′	
			P	ALEXA594-AGCCAGATGGCCTGGTGATG–BHQ2	
4	*Staphylococcus* spp.	Tuf (NC_007168.1)	F	5′-CTCAATCACTGGTCGTGGTACTG-3′	163
			B	5′-GTCACCAGCTTCAGCGTAGTC-3′	
			P	ALEXA647-CGTGTTGAACGTGGTCAAATCA-BHQ2	
5	*Pseudomonas aeruginosa*	23S rRNA (AJ549386.1)	F	5′-GTTGTCCAAGTTTAAGGTGGTAGG-3′	97
			B	5′-CCACTTCGTCATCTAAAAGACGAC-3′	
			P	FAM-TTCAAGGCCGAGAGCTGATGAC-BHQ1	
6	*Klebsiella pneumoniae*	gapA (M66869.1)	F	5′-CATCGAGATCGTTGCAATCAACG-3′	81
			B	5′-CGACCGTGAGTGGAGTCATAC-3′	
			P	ALEXA532-AGACGCAGAGTACATGGCTTACATG-BHQ1	
7	*Streptococcus pneumoniae*	Pneumolysin (NC_003098.1)	F	5′-CAAGGTAAGGAAGTCTTGACTCC-3′	193
			B	5′-GCTTACGCACTAGTGGCAAATCGG-3′	
			P	ALEXA594-AGGGAATGTTCGTAATCTCTCTGTC-BHQ2	
8	*Staphylococcus aureus*	Thermonuclease (NC_007795)	F	5′-GTCCTGAAGCAAGTGCATTTACG-3′	282
			B	5′-GACCTGAATCAGCGTTGTCTTC-3′	
			P	ALEXA647-CGAAGCTTTAGTTCGTCAAGGCTTG-BHQ2	
9	*Acinetobacter baumannii*	ompA (AY485227.1)	F	5′-GAACTATACAGCTCTTGCTGGC-3′	87
			B	5′-CTCTTGTGGTTGTGGAGCAAC-3′	
			P	FAM-GTTCTTGGTGGTCACTTGAAGC-BHQ1	
10	*E. coli*	phoA (M29670.1)	F	5′-CGAAGAGGATTCACAAGAACATACC-3′	88
			B	5′-CATTAAGTCTGGTTGCTAACAGC-3′	
			P	ALEXA532-TCAGTTGCGTATTGCGGCGTATG-BHQ1	
11	*Streptococcus* spp.	Tuf (AY267003.1)	F	5′-CACTGGACGTGGTACAGTTGCTTC-3′	188
			B	5′-GAACACCACGAAGAAGGACACCTAC-3′	
			P	ALEXA594-CAACTTGACGAAGGTCTTGCTGG-BHQ2	

Table 1 (*continued*)

No	Target	Gene (Accession number)		Oligonucleotide	Amplicon (bp)
12	*Haemophilus influenza*	16S rRNA (Z22806.1)	F	5′-GACATCCTAAGAAGAGCTCAGAG-3′	266
			B	5′-CTTCCCTCTGTATACGCCATTG-3′	
			P	ALEXA647-CCTTCGGGAACTTAGAGACAG-BHQ2	
13	*Moraxella catarrhalis*	CopB (U69982.1)	F	5′-GTGCGTGTTGACCGTTTTGAC-3′	134
			B	5′-GTGGCATAGATTAGGTTACCGCTG-3′	
			P	FAM-CCGACATCAACCCAAGCTTTG-BHQ1	
14	*Bacteroides fragilis*	16S rRNA (AP006841.1)	F	5′-GACTGCAACTGACACTGATGCTC-3′	316
			B	5′-CAACCATGCAGCACCTTCACAG-3′	
			P	ALEXA532-AGATACCCTGGTAGTCCACACAG-BHQ1	
15	*Proteus* spp.	16S rRNA (NC_010554.1)	F	5′-CTCTTCGGACCTTGCACTATC-3′	127
			B	5′-CGTGTCTCAGTCCCAGTGTG-3′	
			P	ALEXA594-CGACGATCTCTAGCTGGTCTG-BHQ2	
16	*Staphylococcus aureus (MRSA)*	mecA (AY786579.1)	F	5′-CGGTAACATTGATCGCAACGTTC-3′	108
			B	5′-CTTTGGTCTTTCTGCATTCCTGG-3′	
			P	ALEXA647-TGGAAGTTAGATTGGGATCATAGCG-BHQ2	
17	*Mycobacterium* spp.	16S rRNA gene (NC_000962)	F	5′-GTTCACCGAAGTACTGCTGGAC-3′	445
			B	5′-CAACCACAGGAAGTACTCGACATTG-3′	
			P	ALEXA532- CTGTCGTTCATCTCGTTGGCTAC-BHQ1	
18	*Mycobacterium tuberculosis*	cyp141 (NC_000962)	F	5′-GACAAGCACCTCGATTCCGAC-3′	169
			B	5′-GGACAGCACTCCCTTTACATCG-3′	
			P	ALEXA647- CACACAGCATGGCTCGTCACTC-BHQ2	

[a] F: Forward primer; B: Backward primer; P: Taqman probe; BHQ: Black Hole quencher™.

Table 2 – Multiplex PCRs.

Reaction	FAM	Cy3	Texas Red	Cy5
I	*Homo sapiens* (*TNF*)	*Pseudomonas* sp. (*16S rRNA*)	*Enterobacter* sp. (*tusB*)	*Staphylococcus* sp. (*Tuf*)
II	*P. aeruginosa* (*23S rRNA*)	*K. pneumoniae* (*gapA*)	*S. pneumoniae* (*Pneumolysin*)	*S. aureus* (*Thermonuclease*)
III	*A. baumannii* (*ompA*)	*E. coli* (*phoA*)	*Streptococcus* sp. (*Tuf*)	*H. influenzae* (*16S rRNA*)
IV	*M. catarrhalis* (*CopB*)	*B. fragilis* (*16S rRNA*)	*Proteus* sp. (*16S rRNA*)	Methicillin-resistant *S. aureus*; MRSA (*mecA*)
V	n/a	*Mycobacterium* sp. (*16S rRNA*)	n/a	*M. tuberculosis* (*cyp141*)

The PCR was performed in 5 separate multiplex reactions. The Smart Cycler is able to simultaneously detect 4 fluorescent channels. We used 4 fluorophores: FAM for the FAM channel, ALEXA532 for the Cy3 channel, ALEXA594 for the Texas Red channel, and ALEXA647 for the Cy5 channel. n/a: not applicable.

ATCC 700699D-5, *Streptococcus pneumoniae* (Klein) Chester ATCC BAA-255D-5, *Moraxella catarrhalis* (Frosch and Kolle) Bovre ATCC 25240D-5, *Haemophilus influenzae* (Lehmann and Neumann) Winslow et al. ATCC 51907D, *Proteus mirabilis* Hauser ATCC 12453D, *Acinetobacter baumannii* ATCC BAA-1605D-5, *Bacteroides fragilis* (Veillon and Zuber) Castellani and Chalmers ATCC 25285D-5, and *Mycobacterium tuberculosis* (Zopf) Lehmann and Neumann ATCC 27294D-2 genomic DNA were purchased from the American Type Culture Collection (Rockville, MD, USA). Human placental genomic DNA was purchased from Promega Corp. (Madison, WI, USA).

2.5. Real-time PCR design

We targeted pathogens that are the most frequent causes of pneumonia in Indonesia (Table 1) [11,12]. A primer pair plus a fluorescent-labelled probe forms one detection unit. A total of 18 detection units implemented in 5 multiplex PCRs (Table 2) were able to detect 6 genera (*Pseudomonas* spp., *Enterobacter* spp., *Staphylococcus* spp., *Streptococcus* spp., *Mycobacterium* spp., and *Proteus* spp.); 10 species (*P. aeruginosa, E. coli, S. aureus, S. pneumoniae, M. tuberculosis, K. pneumoniae, A. baumannii, H. influenza, M. catarrhalis,* and *B. fragilis*); 1 drug-resistant bacterial gene (*mecA*); and 1 human gene (*TNF*). Some pathogens were detected by multiple detection units. For example, methicillin-resistant *S. aureus* (MRSA) was detected by the detection units for *Staphylococcus* spp., *S. aureus*, and the *mecA* gene; *M. tuberculosis* was detected by those for *Mycobacterium* spp. and *M. tuberculosis*.

Each detection unit was designed so that it specifically amplified and detected the target sequence. Firstly, the specificity was confirmed *in silico* by the primer BLAST program searching the entire Genbank database. Next, the nucleotide sequence of the amplified fragments from the microbial genomic DNA or human placental genomic DNA (for the *TNF* gene) was confirmed by Sanger sequencing. Finally, detection of the specific fluorescence signals was confirmed by running the real-time PCRs for a variety of mixtures of multiple microbial genomic DNA and human placental genomic DNA.

2.6. Ethics

The research protocol for the current study was approved by the institutional review boards of Dr. Zainoel Abidin Hospital, Syiah Kuala University (07/KE/FK/2016 dated on 24 June 2016), and Jichi Medical University (Rindai16–057 dated on 11 Jan 2017).

3. Results

3.1. Patients

A total of 27 patients were enrolled in this study. Sputum was collected from 25 patients, and pleural effusion from the remaining 2. All patients were referred from the primary (19 patients) and the secondary (8 patients) health care centers. Accordingly, the predominant type of disease was hospital-acquired pneumonia (HAP: 25 patients, 93%). The patients had median age of 62 years (range: 21–90 years) and were predominantly male (22 patients, 88%). All patients had comorbid disease, including lung cancer and other neoplastic diseases (11 patients, 41%), chronic respiratory diseases such as COPD (9 patients, 33%), cardiovascular disease (3 patients, 11%), cerebrovascular disease (1 patient, 4%), thyroid disorder (2 patients, 8%), and mandibular fracture (1 patient, 4%). As expected from the high rates of HAP and comorbid disease, many patients (82%) had pneumonia with multilobar infiltrates.

3.2. The HIRA-TAN

The cut-off values that discriminate the pathogens causing pneumonia were determined for *P. aeruginosa*, *K. pneumoniae*, *M. catarrhalis*, and *S. pneumoniae* in a previous HIRA-TAN study that investigated untreated pneumonia [8]. We adopted the same cut-off values in this study because it was considered to be a good starting point to preliminarily investigate the utility of the HIRA-TAN in progressive pneumonia without any preceding studies targeting the similar patient group. The 3 steps for the HIRA-TAN are usually completed in 6 h. The graph drawn for these 4 pathogens indicates the samples in which these pathogens are likely the cause of pneumonia (Fig. 1A). The HIRA-TAN clearly discriminated *S. pneumoniae* (Fig. 1A, rightmost plots) from *Streptococcus* spp. (Fig. 1B). In many samples, the HIRA-TAN provided 2 pathogens suggestive of being the causative agent, which is also clear from the HIRA-TAN results plotted for each sample (Fig. 2). The results of the culture-positive samples and those obtained by the HIRA-TAN were in close agreement (Table 3, 13/14 samples agreed). Moreover, the HIRA-TAN provided pathogens that may be the cause of pneumonia in 12 samples for which the

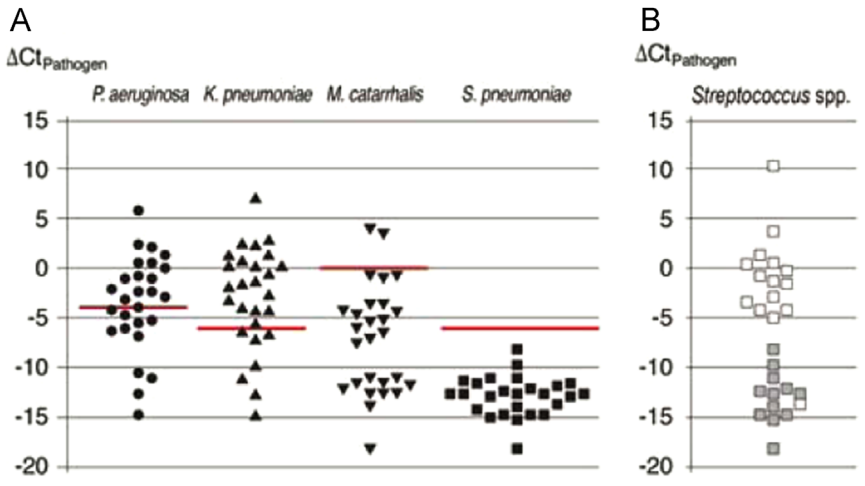

Fig. 1 – The results of the HIRA-TAN. (A) $\Delta Ct_{Pathogen}$ values for the 4 pathogens for which the cut-off value has been reported in a previous study [7,8]. The cut-off values (shown as red lines) were −4 for *P. aeruginosa*, −6 for *K. pneumoniae*, 0 for *M. catarrhalis*, and −6 for *S. pneumoniae*. (B) The $\Delta Ct_{Streptococcussp.}$ for *S. pneumoniae* was obtained by the detection unit for both *S. pneumoniae* and *Streptococcus* spp. (gray boxes), whereas that for other bacteria belonging to *Streptococcus* spp. was detected only by the detection unit for *Streptococcus* spp. (white boxes) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

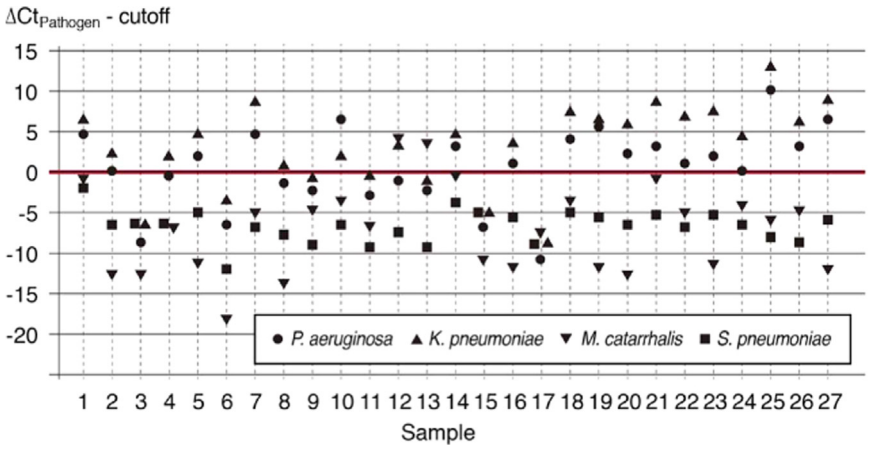

Fig. 2 – $\Delta Ct_{Pathogen} - cutoff$ for each sample. The $\Delta Ct_{Pathogen} - cutoff$ value was graphed for each sample. The pathogens that show values above 0 (a red line) are likely to be those causing pneumonia. Samples are in the same order as in Table 3: samples 1–25 are sputum, whereas samples 26 and 27 are pleural effusion (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

culture failed to provide any information (Table 3). There was no detection from other detection units, and we did not consider their involvement.

4. Discussion

In the current study, we aimed to preliminarily evaluate the clinical utility of the HIRA-TAN for pneumonia with a progressive course after a failure of antibiotic treatment. In many cases, the HIRA-TAN identified pathogen(s) with numbers overwhelming those of inflammatory cells, thus judging them as likely causative agents of the pneumonia.

More sensitive and quicker results within 6 h were demonstrated by the HIRA-TAN method than the culture. Bacterial culture that was performed in parallel often failed to detect any pathogens. This disparity may be explained by the low performance of culture. We suspect that, in some cases, a trace amount of the previous antibiotics remaining may have prohibited the growth of colonies [13,14].

Detection of a bacterial genome by PCR does not necessarily indicate that the bacterium is viable in the sample, because PCR detects DNA in both living and dead cells. However, a report of the HIRA-TAN for CAP demonstrated that the copy number of bacterial DNA in a sample quickly decreased after effective antibiotic treatment [7]. Therefore, practically, when a pathogen(s) is detected as the causative

Table 3 – Results of the culture and the HIRA-TAN.

Sample name	Previous antibiotics	Culture	HIRA-TAN Pathogen 1	Pathogen 2
Sputum 1	CTRX	*Staphylococcus* coagulase negatives	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 2	LVFX	No growth	*Klebsiella pneumoniae*	
Sputum 3	LVFX	No growth	Negative	
Sputum 4	CTRX	No growth	*Klebsiella pneumoniae*	
Sputum 5	CTRX	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 6	CTRX	No growth	*Klebsiella pneumoniae*	
Sputum 7	CTRX+AZM	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 8	CTRX	*Staphylococcus* coagulase negatives	*Klebsiella pneumoniae*	
Sputum 9	LVFX	*Staphylococcus* coagulase negatives	Negative	
Sputum 10	LVFX	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
Sputum 11	CTRX	*Pseudomonas aeruginosa*	Negative	
Sputum 12	CTRX	*Moraxella catarrhalis*	*Moraxella catarrhalis*	*Klebsiella pneumoniae*
Sputum 13	LVFX	*Moraxella catarrhalis*	*Moraxella catarrhalis*	
Sputum 14	LVFX	No growth	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 15	CTRX	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	
Sputum 16	CTRX+AZM	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 17	LVFX	No growth	Negative	
Sputum 18	CTRX	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 19	LVFX	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 20	LVFX	No growth	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 21	CTRX	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 22	CTRX+AZM	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 23	CTRX	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Sputum 24	CTRX+AZM	No growth	*Klebsiella pneumoniae*	
Sputum 25	CTRX+AZM	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Effusion 1	CTRX	No growth	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Effusion 2	CAZ	No growth	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*

Abbreviations: CTRX, ceftriaxone; LVFX, levofloxacin; AZM, azithromycin; CAZ, ceftazidime. The pathogen 1 was that which gave the highest $\Delta Ct_{Pathogen}$, while pathogen 2 was that which gave the second highest value.

pathogen by the HIRA-TAN, it should be considered viable and set as the treatment focus.

K. pneumoniae, *P. aeruginosa*, or both were detected in most patients. On the other hand, *S. pneumonia*, which is the most common pneumonia pathogen worldwide, was not detected. This suggests that the antibiotics used in the area investigated in the current study may be effective for *S. pneumonia*, pending confirmation. Our data suggests that *K. pneumoniae* and *P. aeruginosa* survived the treatment and became the causative pathogen(s) for the subsequent pneumonia. This finding is slightly different from that of a previous report, in which most of the causative pathogens in HAP and VAP were *S. aureus*, *K. pneumoniae*, or *P. aeruginosa* [15]. This difference may reflect hospital ward representation differences or geographical variance. However, such information is important for installing region-specific therapeutic strategies.

The overgrowth of *K. pneumoniae* and *P. aeruginosa* is important from the perspective of the development of drug resistance. The list of antibiotics previously administered (Table 2) shows the predominance of a few kinds of antibiotics in the hospitals studied. Some of these antibiotics are expected to be effective against *K. pneumoniae* and *P. aeruginosa*; however, these pathogens are detected. outgrew. The HIRA-TAN may also be effective in identifying local tendencies of bacterial drug resistance and the biased use of specific antibiotics in the region. The detection of multiple pathogens by the HIRA-TAN is in clear contrast to the results of the HIRA-TAN in untreated CAP, where only 1 pathogen was suggested in most of the samples [8]. Our result was consistent with that of a previous report, in which multiple pathogens were found in nearly half the HAP cases that occurred in patients with risk factors, including prior antibiotic use, older age, and existing comorbid disease [16]. The management of nosocomial infection is complex, and the treatment should be directed by the accurate knowledge of the pathogens involved [17]. The detection of multiple pathogens by the HIRA-TAN will help to control nosocomial infection, not in an empirical manner, but in a pathogen-oriented way.

5. Conclusions

We consider that the HIRA-TAN is useful as an alternative diagnostic test and can provide important information while waiting for confirmation from cultures. Early initiation of a pathogen-directed, second-line therapy will become possible by employing the HIRA-TAN as salvage microbiology. However, the study was limited by a small sample size within only one ward, pending confirmation by a larger clinical trial. We conclude that the HIRA-TAN provided valuable information for determining the second-line treatment for pneumonia that fails the initial round of antibiotic therapy.

Acknowledgements

Ferry Dwi Kurniawan would like to thank the Indonesia Endowment Fund for Education (LPDP) for the study support.

Conflict of interest

The authors have no potential conflict of interest.

Summary at a glance

Patients with progressive pneumonia that persisted with antibiotic treatment were investigated for causative pathogens by the HIRA-TAN, a multiplex PCR-based test using human cells as the control. The HIRA-TAN was able to list candidate pathogens, which would be helpful for narrowing the spectrum of second-line antibiotics.

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