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Molecular detection of respiratory pathogens in isolates from two hospitals in Egypt using a microarray chip

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Abstract: Respiratory tract infection is one of the most commonly spread communicable diseases. Among the causative organisms, bacteria are the most common causes of serious disease and deaths. A total of 32 samples were taken from Mansoura Lung Diseases Hospital and Kafr El-Sheikh General Diseases Hospital, in addition to 5 control samples were screened for the presence of different pathogens. In a total of 32 diseased samples, by PCR method, in Mansoura Lung Diseases Hospital, *Hemophilus influenzae* was detected in 2 samples, in addition to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* which were detected in 7 and 7 samples respectively. Mixed microbial infections were detected in 4 samples identified mainly as *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. However, in Kafr El-Sheikh General Diseases Hospital, *Klebsiella Pneumoniae* and *Pseudomonas aeruginosa* were detected in 8 and 8 isolates respectively. Mixed microbial infections were detected in 7 samples identified mainly as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. DNA chip for pathogen detection indicated 100% matching with the test performed by PCR. Control samples were free from any pathogen. These results demonstrate variable results with respect to the geographical distribution and the possibility for fast identification of respiratory pathogens even in mixed culture in very short time.

Keywords: Respiratory infection, polymerase chain reaction, microarrays.

INTRODUCTION

Respiratory tract infection includes any diseases which are caused by pathogenic microbial agents and transmitted directly through air or droplets by the inhalation of these pathogens affecting the activity of respiratory system or even the rest of the body. Many bacterial pathogens such as *Streptococcus pneumoniae, Mycoplasma pneumoniae, Hemophilus influenzae Moraxella catarrhalis, Mycobacterium tuberculosis, Streptococcus pyogenes*, *Neisseria meningitidis, Legoniella pneumophila and Klebsiella pneumonia* can be easily transmitted by air and difficult to be identified or detected. These airborne pathogens target many areas in the human respiratory system. Sinus congestion, coughing and sore throats are examples of induced inflammation of the upper respiratory airway due to these airborne agents. Pulmonary pneumonia is an example of lower respiratory tract infection. Many previous studies have been carried out to develop methods to detect different respiratory pathogens (Khater *et al*., 2016; Wang *et al*., 2008; Xirogianni *et al*., 2013). It was clearly investigated that most of respiratory pathogens are difficult to be identified by using conventional culture-based methods. These pathogens need special media or culture techniques which may not be available in many labs or a long time is needed before isolation of first colonies in each plate (Atkinson *et al*., 2008; Mercante and Winchell 2015). In addition, these methods are extremely biased by selecting one particular microorganism of the population discarding the others. Culture-independent molecular techniques have initiated a new era in microbiology. For detection of mixed microbial samples, a variety of molecular methods have been discovered on the basis of direct isolation and analysis of their nucleic acids. These tools includes: Uniplex PCR, Multiplex PCR, Realtime PCR and other methods. By utilization of these methods, structural and functional information concerning the presence of different pathogens in microbial communities could be revealed. These methodologies have considerably enhanced the sensitivity and the detection speed of microbial pathogens even in mixed microbial samples (Gebert *et al*. 2008; Lehmann *et al*., 2008). However, only a limited number of pathogens can be detected in each reaction even by multiplex PCR (Edin *et al*., 2015; Gadsby *et al*., 2015). In some studies, high-throughput and comprehensive view of microbial communities is required especially when dealing with environmental samples or samples containing mixed culture. DNA microarrays have been used primarily to provide a significant advantage in mixed microbial community analyses with replication. Different detection probes can be designed targeting virulence factors, antibiotic resistance and the ribosomal DNA (Anthony *et al*., 2000; Gadsby *et al*., 2015; Jin *et al*., 2005; Mao *et al*., 2008; Shang *et al*., 2005; Shen *et al*., 2015) which can be used for bacterial identification to the species level. In microarray experiments, DNA probes are immobilized at high-density carrying more than one copy of each target gene in each DNA chip. Each experiment is carried out in different replicates on the same slide. In the literature, only few data concerning the use of Microarray for identification of bacterial respiratory pathogens could be found. In addition, microarrays are rarely used in

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pathogen detection in Egypt. The aim of our study is to test this technology and use it for detection of respiratory bacterial pathogens even in mixed samples and to introduce this technology in microbial detection of pathogens in Egyptian hospitals.

MATERIALS AND METHODS

Specimen Collection and Processing

Sputum samples were collected from patents admitted to the Mansoura Lung Infection and Kafr El-Sheikh General Diseases Hospitals representing two different Governorates. Samples were taken for at least 5 ml from each patient. The isolated samples were immediately placed on ice before transporting to ultra-low temperature freezer in Faculty of Pharmacy in Mansoura University and stored at −80◦C.

Regulatory and ethical considerations

The experimental protocol conducted in this study complies with the ethical guidelines adopted by "The Research Ethics Committee, Faculty of Pharmacy, Mansoura University" which is in accordance with the Code of Ethics of World Medical Association (Declaration of Helsinki involving use and handling of human subjects).

Extraction of genomic DNA and DNA from standard cultures:

Extraction of genomic DNAs from sputum samples was performed according to the manufacturer's instructions using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). The concentration of gDNA was determined using Nanodrop (OPTIZEN NanoQ, Mecasys). Purified DNAs were frozen at -80◦C. In case of pure cultures, genomic material was extracted by colony PCR.

PCR amplification and labelling to get hybridization targets:

Nearly full length 16S rRNA genes were amplified from the DNA isolated directly from sputum samples using Cycler 003 PCR Machine (A & E Lab (UK)). Starting from gDNA as a template, 16S rDNA fragments were amplified using Biotin labelled primers: forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG) and reverse 1492R (5'-GGTTACCTTGTTACGACTT). In a reaction mixture containing 0.5µM of each primer, 1U Taq polymerase (Dream TaqGreen DNA polymerase, Fermentas), 0.2 mM dNTPs, 1.5mM $MgCl_2$, 2µl of template DNA and nuclease free water was added for a total volume of 25µl per reaction. All PCR reactions were performed under the following conditions: 2 min. initial denaturation at 94˚C followed by 35 cycles of denaturation (30 s at 94˚C), annealing (30 s at 52˚C), extension (60 s at 72˚C); and a final extension at 72˚C for 10 min. Finally, PCR products were analyzed by electrophoresis through 1% agarose gel and then stained with ethidium bromide and visualized under UV light.

PCR amplification of strain specific genes:

Amplification of genomic DNA and DNA from standard cultures was performed using primers (Biosearch Technologies) listed in table1. The reaction mixture was prepared as described previously. PCR reactions began with 5 minutes of primary denaturation at 94°C followed by 35 cycles of 94°C for 30 s, annealing temp (as listed in the Table 1) for 30 s, 72°C for 30 s and a final extension at 72˚C for 10 min.

Design and Synthesis of oligonucleotide probes:

Specific probe sequences were selected for each target and have similar lengths, melting and GC content for development of comparable signal intensities. To exclude any theoretical false positive reactions and for identifying the probes that have more than the desired threshold sequence similarity (e.g. 85%), these resulting probes were compared with all available sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Segments of 20-30-mer oligonucleotides with the optimum BLAST hit were selected as potential probes. Oligonucleotides used in this study were listed in table 2.

Oligoarray printing

Each printing oligo solution was prepared using ArrayIt spotting buffer to a final concentration of 50 pmol μl^{-1} in a 384-well printing plate. Oligonucleotides were spotted using Spotbot microarrayer (ArrayIt, USA) spotting machine. The 12 different 20-30-mer oligonucleotides were spotted onto microarry slides (Scienion, Germany). Each probe was spotted in three replicates on the array.

Microarray testing

Labeled samples were resuspended in hybridization buffer (Scienion, Germany) and then applied to the printed microarray chip. Hybridizations were carried out in a hybridization station (ArrayIt, USA) with an initial pre-hybridization step using 20µl of pre-hybridization solution (Scienion, Germany). For hybridization to the array, in a final volume of 16µl, 8µl of hybridization buffer were mixed with 8µl of the PCR products. This mixture was boiled for 2 min, cooled on ice, then applied to the surface of a blocked and immobilized microarray slide that had been pre-warmed to 42°C followed by incubation at 42°C for 12h and washing at room temperature using microarray washing buffers I, II and III (Scienion, Germany). Slides were then detected by colour development using Streptavidin Biotin staining system (Fermentas) under the manufacturer's instructions. ArrayIt Microarray Scanner (ArrayIt, USA) and the Spotware software were used for acquiring images in three technical replicates for each probe. Computational post processing and probe signal intensities were determined by subtracting the local background values from the per-sample median

RESULTS

PCR as a primary test

In PCR screening of *K.pneumoniae*, the resulting amplified PCR products produced bands were detected at approximately 399 bp. Other three respiratory pathogens, *H.influenza P.aeruginosa* and *M. tuberculosis* produced bands at 272 bp, 116 and 72 bp respectively. A total of 20 samples taken from Sputum of patients admitted to the Mansoura Lung Diseases Hospital samples, in addition to 5 control samples were screened for the presence of different pathogens by usual uniplex PCR. Control samples were free from pathogens. However, in a total of 20 diseased samples, *H. influenzae* was detected in 2 samples, in addition to *K.pneumoniae* and *M. tuberculosis* which were detected in 7 and 7 samples respectively. Mixed microbial infections carrying *K. pneumoniae* and *M. tuberculosis* were detected in 4 diseased samples. In Kafr El-Sheikh General Diseases Hospital, *K. pneumoniae* and *P.aeruginosa* were separately detected in 8 diseased sputum samples.

Mixed microbial infections were detected 7 samples identified mainly as *K. pneumoniae* and *P. aeruginosa.*

Microarray testing and detection of bacterial pathogens in clinical samples by DNA microarray

In samples obtained from Mansoura Lung Infection hospital, different bacterial pathogens were detected. In 20 different diseased sputum samples by DNA microarray testing, 12 (60%) were found to be positive by DNA microarray test (fig. 1, 2). The proportions of bacteria identified from the 20 patients were 2 *H. influenzae* (10%), 7 *K. pneumonia* (35%) and 7 *M. tuberculosis*

Table 1: Different primers used in this work to detect different species of bacteria

Primer name		Sequence	Tm	Citation
S.pneumoniae	F	AGCGATAGCTTTCTCCAAGTGG	56	(Greiner *et al.,* 2001)
	R	CTTAGCCAACAAATCGTTTACCG		
M.pneumoniae	F	TTTGGTAGCTGGTTACGGGAAT	54	(Winchell *et al.,* 2008)
	R	GGTCGGCACGAATTTCATATAAG		
H.influenzae	F	ACTTTTGGCGGTTACTCTG	50	(van Ketel *et al.,* 1990)
	R	TGTGCCTAATTTACCAGCAT		
K.pneumoniae	F	TCTGGACCGCTGGGAGCTGG	59	(Cole *et al.,* 2009)
	R	TGCCCGTTGACGCCCAATCC		
M.catarrhalis	F	GTGAGTGCCGCTTTACAACC	54	(Greiner *et al.,* 2003)
	R	TGTATCGCCTGCCAAGACAA		
S.aureus	F	TGCTGGTGGTACATCAAA	49	(Ruimy *et al.,* 2008)
	R	ACGGTCAATGCCATGATTTAA		
P.aeruginosa	F	CGAGTACAACATGGCTCTGG	53	(Feizabadi *et al.,* 2010)
	R	ACCGGACGCTCTTTACCATA		
M.tuberculosis	F	GAACGGCTGATGACCAAACT	53	(Luo *et al.,* 2010)
	R	ATCAGCGATCGTGGTCCTG		
N.meningitides	F	GTGATGGTGCGTTTGGTGCAGAATA	58	(Boving *et al.,* 2009)
	R	CACATTTGCCGTTGAACCACCTACC		

Table 2: Oligonucleotides used in the study were listed in the table.

	Sequence (5' to 3')	Oligo name	Tm	Ref
1	TTGCATGACATTTGCTTAAAAGGTGCAC	*S.pneumoniae*	58	This study
2	TCGGGGCGATCCCCTCGGTA	*M.pneumoniae*	59	This study
3	GCGTATTATCGGAAGATGAAAGTGCG	*H.influenzae*	59	This study
4	CGAAGTTAGGAAGCTTGCTTCTGATA	*M.catarrhalis*	59	This study
5	AACATATGTGTAAGTAACTGTGCACATCTTG	*S. aureus*	59	(Mao *et al.,* 2008)
6	GGGAGGAAGGGCAGTAAGTTA	*P.aeruginosa*	53	(Mao *et al.,* 2008)
7	CGATCCGAACTGAGACCGGCTTTTAAGG	*M.tuberculosis*	64	(Jin *et al.,* 2005)
8	AAAGGCTGTTGCTAATACCAGCGG	*N.meningitidis*	59	This study
9	CGACGGCTAGCTCCAAATGGTTACTG	CON Staphylococcus	62	(Jin *et al.,* 2005)
10	AGGTCGCCCCTTCGCCGCCCTCTGTATC	*L.pneumophila*	68	(Jin *et al.,* 2005)
11	GCAGGTTTTGCCTCTCATATTAAAGTCTT	*S.pyogenes*	59	This study
12	CACTTTCAGCGGGGAGGAAGGC	*K.pneumoniae*	59	This study

(35%). Detection of mixed bacterial infection revealed 4 different samples. In Kafr El-Sheikh General Diseases Hospital, different bacterial pathogens were detected in 12 diseased sputum samples by DNA microarrays. Positive results were detected in 9 (75%) of sputum samples by DNA microarray test (fig. 1, 3) indicating 2 different microorganisms; *K. Pneumoniae* (66%) and *P. aeruginosa* (66%). Detection of mixed bacterial infection revealed 7 different samples detected in 12 diseased sputum samples and identified mainly as *K.pneumoniae* and *P.aeruginosa.* These results obtained by microarray were consistent with the results obtained by positive samples detected by PCR (table 3, 4).

DISCUSSION

Rapid and correct identification of respiratory pathogens is critical for appropriate selection of antibiotic treatment and facilitating early recovery of the patient. In most previous methods, isolation of pure colonies is required before carrying out the detection test.

These methods need facilities that are not available in many labs especially for isolation of slowly growing or microbes requiring specialized media. The use of DNA microarrays for the detection of respiratory bacterial pathogens has been previously described in different studies (Cannon *et al*. 2010; Shen *et al*. 2015). In this

Table 3: Comparison between PCR and microarray results

Comparison of PCR with specific primers and Microarray results			
Organism	No of samples positive		Total number of patients with a positive result
	PCR	Microarrays	
S.pneumoniae	0	0	0
M.pneumoniae	0	0	0
H.influenzae	2	2	2
M.catarrhalis	0	0	0
S. aureus	0	0	0
P.aeruginosa	8	8	8
M.tuberculosis	7	7	7
N.meningitidis	0	0	0
CON Staphylococcus	-	0	0
L.pneumophila	-	0	0
S.pyogenes	-	0	0
K. pneumoniae	15	15	15

Table 4: Comparison between PCR and microarray results in detected pathogens

H. influenzae			*P. aeruginosa*			*M. tuberculosis*			*K. pneumoniae*		
No. of positive samples		Number of positive samples			Number of positive samples	No. of positive samples		Number of positive samples	No. of positive samples		Number of positive samples
PCR	Microarray		PCR	Microarray		PCR	Microarray		PCR	Microarray	
+	+	2	+	+	8	+	+	7	+	+	15
+	-	2	+	-	8	+	-	7	+	-	15
-	+	2	-	+	8	-	+	7	-	+	15
2	2	2	8	8	8	7	7	7	15	15	15

Fig. 1: The layout of the DNA microarray. The species-specific probes were spotted in 3 replicates on the slide.

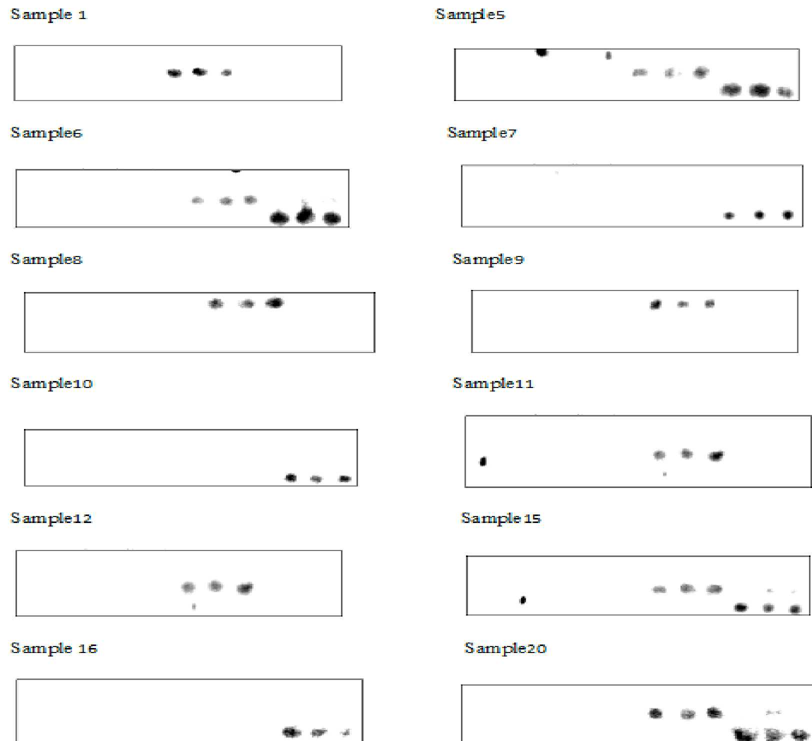

Fig. 2: Hybridization results obtained with the target DNAs from sputum samples obtained from Mansoura Lung Diseases Hospital.

study, a set of 12 oligonucleotide probes were used to detect bacterial 16S PCR products. These probes were designed to distinguish major bacterial species frequently causing pulmonary diseases, including: *H. influenzae, N.meningitidis, S. aureus*, *M. tuberculosis, M. catarrhalis, P. aeruginosa S. pneumoniae, M. pneumoniae*, *S. pyogenes*, Coagulase-negative Staphylococcus, *L. pneumophila* and *K. pneumoniae*.

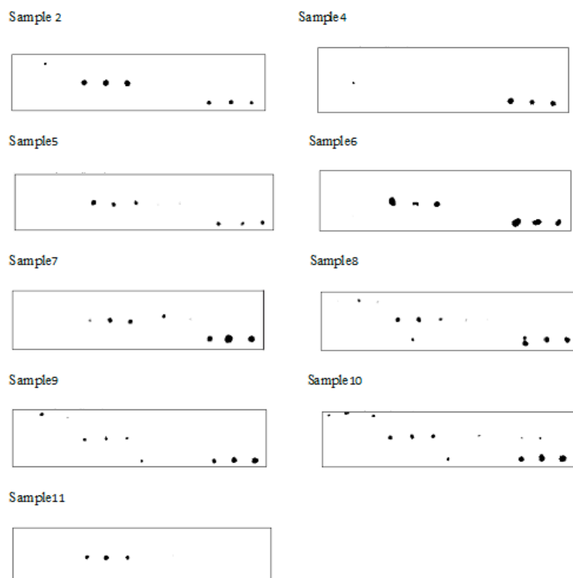

Fig. 3: Hybridization

Shorter oligonucleotide probes were selected in our study to allow independent testing of several species-specific regions of each pathogen which reduces the possibility of misidentification and reducing the chip's estimated cost production. In the current study, oligonucleotide chip-based assay and PCR methods were evaluated for identification of microorganisms directly from sputum samples without culture medium. In Microarrays, each probe is composed of a sequence that is complimentary to a specific gene sequence.

By performing PCR testing, in Mansoura Lung Diseases Hospital, only three species *K. pneumoniae*, *H. influenza* and *M. tuberculosis* could be diagnosed in all 20 diseased sputum samples. Theses bacterial pathogens could be detected in 12 positive PCR specimens then were further hybridized on microarrays. As a result, all 12 PCR positive cases were also positive by DNA microarray chip identifying the same bacterial species. In Mansoura Lung Diseases Hospital, *M. tuberculosis* and *K. pneumoniae* were mainly detected as most common respiratory pathogens. Similar results were obtained in one study in Malaysia, as *K. pneumoniae* were mainly identified in sputum samples (Mustafa *et al*., 2011). In Egypt, high prevalence of *M. tuberculosis* was previously reported by medium inoculation and biochemical testing (Bassiouny *et al*. 2018) and also by PCR detection (Khater *et al*. 2016). In contrast to our study, in one study in Greece (Xirogianni *et al*., 2013), a high percentage of *S.*

pneumoniae could be detected in respiratory diseased patients followed by *H. Influenza*. Similar observations were reported in another study in China (Wang *et al*., 2008) and in Spain (de Roux *et al*., 2006). In Kafr El-Sheikh General Diseases Hospital, *K. pneumoniae* and *P.aeruginosa* were mainly detected in 9 of all 12 diseased sputum samples. Microarray detection was performed using the same samples. DNA chip for pathogen detection indicated 100% matching with the test performed by PCR. Control samples were free from any pathogen. High prevalence of *K. pneumoniae* was also previously reported in Egypt (Agmy *et al*., 2013). Similarly, a high percentage of *P. aeruginosa* was detected in Spain (Cilloniz *et al*., 2011). In addition, both organisms were more prevalently detected in Nigeria (Okesola and Ige 2008) and Malaysia (Mustafa *et al*., 2011). However, *P.aeruginosa* could be rarely detected in another study in Egypt (El Seify *et al*., 2016).

	S.pneumoniae	*M.pneumoniae*	*H.influenzae*	*M.catarrhalis*	*S.aureus*	*P.aeruginosa*	*M.tuberculosis*	*N.meningitides*	*CON Staphylococcus*	*L.pneumophila*	*S.pyogenes*	*K.pneumoniae*
1							■					
2												
3												
4												
5							■					■
6							■					■
7												■
8			■									
9			■									
10												■
11							■					
12							■					
13												
14												
15							■					■
16												■
17												
18												
19												
20							■					■

Fig. 4: Schematic representation of positive microarray results in different samples obtained from Mansoura Lung Diseases Hospital.

	S.pneumoniae	*M.pneumoniae*	*H.influenzae*	*M.catarrhalis*	*S.aureus*	*P.aeruginosa*	*M.tuberculosis*	*N.meningitides*	*CON - Staphylococcus*	*L.pneumophila*	*S.pyogenes*	*K.pneumoniae*
1												
2						■						■
3												
4												■
5						■						■
6						■						■
7						■						■
8						■						■
9						■						■
10						■						■
11						■						
12												

Fig. 5: Schematic representation of positive microarray results in different samples obtained from Kafr El-Sheikh General Diseases Hospital.

Rapid detection of bacterial coinfection is critical for appropriate diagnosis and selection of antimicrobial therapy, thus preventing severe diseases and decreasing the mortality rates (Cilloniz *et al*., 2011; Kumagai *et al*., 2015). Mixed bacterial respiratory infection was detected in different previous studies. The combination *S. pneumoniae* and *H. influenza* was the most frequent identified (de Roux *et al*., 2006). Similar observation was detected in China (Zhang *et al*., 2018) and in Spain (Cilloniz *et al*., 2011). In contrast, in our study, in Mansoura Lung Diseases Hospital, the most common combination was *M.tuberculosis* and *K. Pneumoniae* which could be identified in 4 different samples. However, *K.pneumoniae* and *P.aeruginosa* was the most combination identified in Kafr El-Sheikh General Diseases Hospital isolates. The same observation was previously reported in Nigeria (Okesola and Ige 2008).

For bacterial etiologies, in most studies, in nearly half of respiratory cases, no causative agents can be detected (Mandell *et al*., 2007). Detection rates are reported to be 40% (de Roux *et al*., 2006), 27% (Okesola and Ige 2008) and 44.7% (Jones 2010). In the present study, using the DNA chip and PCR method, the rate of bacterial pathogen detection was 66 %, which can be useful in some of cases with unknown etiologies.

CONCLUSION

DNA chip for pathogen detection indicated 100% matching with the test performed by PCR. *P.aeruginosa,*

K. pneumoniae and *M. tuberculosis* were mainly detected. DNA chip facilitates fast identification of respiratory pathogens even in mixed culture in very short time reducing the time required to choose the proper treatment and avoiding undesired complications.

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