



(51) International Patent Classification:

C12Q 1/04 (2006.01) *G01N 21/31* (2006.01)
G01N 21/64 (2006.01) *G01N 21/65* (2006.01)

(21) International Application Number:

PCT/IN2016/000028

(22) International Filing Date:

27 January 2016 (27.01.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

6468/CHE/2015 2 December 2015 (02.12.2015) IN

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

(54) Title: A METHOD FOR RAPID DETECTION OF A SINGLE BACTERIUM

(57) Abstract: The invention provides a method for rapid detection of a specific bacterial species. The method includes preparing a sample, irradiating the prepared sample with an electromagnetic radiation of specific wavelength, capturing the electromagnetic radiationscattered by the sample to obtain a Raman spectra and analyzing the Raman spectra to obtain a unique biochemical signature. The unique biochemical signature identifies a single bacterium within the sample. The method also provides for rapid detection of a group of bacterial species within the sample. The method takes about 1 minute to about 1 hour for the detection of specific bacterial species.



WO 2017/094019 A1

A METHOD FOR RAPID DETECTION OF A SINGLE BACTERIUM

5 FIELD OF INVENTION

The invention generally relates to the field of physical chemistry and diagnostic microbiology and particularly to a method for detection of a single bacterium or a group of bacterial species within a sample.

10

BACKGROUND

A variety of diagnostic methods are available in the art for detection and identification of bacterial species in any given biological sample. Examples of sample include but are not
15 limited to those obtained from infectious diseases, food and dairy products. Examples of infectious diseases include but are not limited to pneumonia, tuberculosis, septicaemia, meningitis, urinary tract infection and gastro-intestinal infections. The existing methods are broadly classified as invasive methods and
20 non-invasive methods. Invasive method requires rupturing or lysis of biological material in order to extract a particular biological component for identification. On the other hand, non-invasive methods do not require rupturing or lysis of the biological material.

25 The diagnostic methods available in the art include but are not limited to DNA/protein based methods, culturing based methods, staining based methods and mass spectrometry based methods.

- DNA based methods use specific DNA sequences present in various pathogens as signatures for identification. Examples of DNA based techniques include but are not limited to PCR, RT-PCR and probe hybridization such as molecular beacons. One of the significant disadvantage of the technique is extensive sample processing and preparation steps. Another disadvantage of the technique is the requirement of specific probes for diagnosis.
- 10 Culturing based methods require inoculation of the growth medium with the biological sample containing pathogen. The bacterial colonies grown on the growth medium are then identified. The techniques of identification includes but are not limited to staining, antibiotic selection and metabolic reactions.
- 15 One significant disadvantage of the technique is elaborate procedures involved to perform the test. Another disadvantage is time required for completion of the diagnosis, which normally requires 3-4 days but may extend upto 21 days to detect a certain bacteria.
- 20 Staining and microscopy base methods use specific chemical dyes or fluorescent probes with unique chemistry to stain and visualize various bacterial species. The technique allows species identification by differential staining analysis. Some disadvantages of the technique are requirement of skilled
- 25 persons and has limited potential for low bacterial loads specially for single bacterial identification.
- Mass spectrometry uses a mass spectrometer to analyse extracted DNA or RNA or protein or lipids or other biological

molecules. The mass signatures obtained are analyzed for bacterial species identification. Limitation of the technique is that it has a poor application as a stand-alone technique for single
5 bacterial identification and is often used along with PCR.
Thus there is a need for a diagnostic method that has high specificity and sensitivity and is less time consuming.

BRIEF DESCRIPTION OF DRAWINGS

10 So that the manner in which the recited features of the invention can be understood in detail, some of the embodiments are illustrated in the appended drawings. It is to be noted, however, that the appended drawings illustrate only typical embodiments of this invention and are therefore not to be considered limiting
15 of its scope, for the invention may admit to other equally effective embodiments.

FIG.1 shows an averaged Raman spectra obtained from various kinds of bacteria at lower wave number region, according to an embodiment of the invention.

20 FIG.2 shows an averaged Raman spectra obtained from various kinds of bacteria at higher wave number region, according to an embodiment of the invention.

FIG.3 shows a 2D scatter plot obtained from a PC-Linear Discriminant Analysis of the spectra of various kinds of bacteria,
25 according to one example of the invention.

FIG. 4a shows an confusion matrix obtained for predicting the efficiency and reliability of the detection method, according to one example of the invention.

FIG. 4b shows a confusion matrix for predicting the efficiency of the detection method, according to another example of the invention.

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SUMMARY OF THE INVENTION

One aspect of the invention provides a method for rapid detection of a single bacterium within a sample. The method includes preparing a sample, irradiating the prepared sample with an electromagnetic radiation of specific wavelength, capturing the electromagnetic radiation scattered by the sample to obtain a Raman spectra and analyzing the Raman spectra to obtain a unique biochemical signature. The unique biochemical signature obtained identifies the single bacterium within the sample.

Another aspect of the invention provides a method for rapid detection of a group of a bacterial species within a sample.

Yet another aspect of the invention provides a method for preparation of a substrate for casting the sample. The method includes selecting a silicon wafer and depositing a metal layer on the silicon wafer.

DETAILED DESCRIPTION OF THE INVENTION

Various embodiments of the invention provide a method for detection of a single bacterium within a sample. The method includes preparing a sample, irradiating the prepared sample with an electromagnetic radiation of specific wavelength, capturing the electromagnetic radiation scattered by the sample

to obtain a Raman spectra and analyzing the Raman spectra to
obtain a unique biochemical signature. The unique biochemical
signature obtained identifies the single bacterium within the
5 sample.

The method also includes, preparing a sample, irradiating the
prepared sample with an electromagnetic radiation of specific
wavelength, capturing the electromagnetic radiation scattered by
the sample to obtain a Raman spectra and analyzing the Raman
10 spectra to obtain a unique biochemical signature. The unique
biochemical signature obtained identifies a group of bacterial
species within the sample.

The method of rapid detection is based on Raman
microspectrometry. Raman microspectrometry uses Raman
15 spectrometry to measure the spectra of microscopic samples. A
Raman microspectrometer is used which allows acquisition of
Raman spectra of microscopic samples. The technique captures
molecular bond specific vibrations originating from the
biochemical constituents of the cell. The spatial resolution
20 required for single bacterial biochemical fingerprint is achieved
with the use of microscopy along with the spectroscopy.

The method described in brief hereinabove shall be described in
details with various embodiments. The method involves the
steps of preparing a sample, irradiating the sample with an
25 electromagnetic radiation of specific wavelength, capturing the
electromagnetic radiation scattered by the sample to obtain a
Raman spectra and analysing the Raman spectra to obtain a
unique biochemical signature. The unique biochemical signature

obtained identifies a single or group of bacterial species within the sample.

First step for detection is preparation of a sample. The sample
5 used for detection includes but is not limited to a body fluid sample, a tissue sample, a food sample. Sample preparation is done by first decontaminating the sample. Decontamination is achieved by irradiation or by heat or by chemical treatment. The decontaminated sample is then washed three times with
10 autoclaved Type1 water. Washing is done to remove lysed cellular components. The washed and decontaminated sample is then dried and casted on a substrate. In one embodiment of the invention, prior to casting on the substrate, the decontaminated cells in the sample are fixed by using
15 paraformaldehyde solution.

The substrate is fabricated by depositing a layer of metal on a silicon wafer. The deposition of the metal layer is achieved by first sputter coating and then annealing the metal on the silicon wafer. The thickness of the metal layer is in the range of 100 nm
20 to 1mm. Examples of metal include but are not limited to aluminium, silver and gold. In one embodiment of the invention, aluminium is deposited on the silicon wafer. The prepared sample is then irradiated with an electromagnetic radiation. The wavelength of the electromagnetic radiation is in the range of
25 200 nm to 1400 nm. In one example of the invention, the source of electromagnetic radiation is a laser source. For irradiation, electromagnetic radiation from a radiation source is focused through a plurality of mirrors and lenses or a microscopic

objective on the prepared sample. The electromagnetic radiation scattered by the sample is then dispersed using a grating. The dispersed electromagnetic radiation is then captured by a detector and a Raman spectra is obtained. The Raman spectra obtained is molecular bond specific and represents biochemical composition of the bacterial species. The Raman spectra obtained is then analysed to obtain biochemical signatures specific to the bacterial species. The resolution of the Raman spectra obtained is in the range of 1cm^{-1} to 8cm^{-1} . Further, the time duration for identification of the bacterium is in the range of one minute to 1 hour.

Identification and Validation:

Six bacterial species namely *E.coli*(gram negative, aerobic), *B.subtilis*(gram positive, aerobic), *K.pneumoniae*(gram negative, aerobic), *M.smegmatis*(gram positive, aerobic), *M.vaccae*(gram positive, aerobic) and *M.bovis*(gram positive, aerobic) are cultured. The cultured bacteria are harvested and pelleted by centrifugation at 5000g for 5 minutes. The pelleted bacteria are then washed thrice with autoclaved Type 1 water. After washing the bacteria are dry casted on the substrate.

The substrate is fabricated by first sputter coating and then annealing aluminium as a 200 nm layer on a silicon wafer. The dry-casted bacterial sample are then mounted on a microscope, focused to the appropriate location and then irradiated by a laser source having a wavelength of 633nm. The electromagnetic radiation scattered by the bacterial sample are focused and collected using a 100x, 0.8NA objective. The scattered radiation

after passing the notch filter is focussed on to a monochromator with 1200lines/mm grating and detected using a Peltier cooled CCD camera at 256x1048 pixels resolution.

- 5 During spectroscopic evaluation, for the lower wave number region $500\text{-}1900\text{cm}^{-1}$ the bacteria are exposed for 15 seconds and the spectra is accumulated 5 times to get a good signal to noise ratio. For the higher wave number region $2800\text{-}3000\text{cm}^{-1}$ the bacteria are exposed for 10 seconds and the spectra is
- 10 accumulated three times. After data collection, the spectra is subjected to pre-processing which included, cosmic ray removal, multipoint base line correction, Savitzky- Golay smoothing and vector normalization using Renishaw wire 4.2 and OriginPro 8.5 software. The averaged data is used for univariate analysis and
- 15 the complete dataset after pre-processing is used for multivariate analysis.

FIG.1 and FIG. 2 generally shows averaged Raman spectra obtained from various kinds of bacteria at lower wave number region and at higher wave number region, respectively. The

20 figures show that at both lower wave number region and higher wave number region, various kind of bacteria exhibit distinctly different spectra from one another. The spectra of *M. bovis* clearly reflects the fact that the main discriminatory features are dominated by peaks pertaining to lipids and mycolic acids in

25 their cell wall compared to other five strains. Similarly, *M. vaccae* exhibit distinct spectra because of presence of carotenoids. In *M. smegmatis* comparatively lesser amount of carotenoids is detected but the higher region suggest that these are also rich in

lipids. *E. coli* and *B. subtilis* being gram negative control bacteria and gram positive control bacteria, respectively, yield distinct signatures showing complete demarcation from Mycobacterial species.

FIG.3 shows a 2D scatter plot obtained from a PC-Linear Discriminant Analysis of the spectra of various kinds of bacteria, according to one example of the invention. Each point in the plot denotes the complete spectrum. Each spectrum is colour coded to their respective kind of bacteria, as is evident that the points of same colour cluster together.

FIG. 4a shows an confusion matrix obtained for predicting the efficiency and reliability of the detection method, according to one example of the invention. The confusion matrix reveals that the actual values of all the spectra collected are correctly predicted to their respective sources, i.e. actual values perfectly matches the predicted values. When the number of components used by the LDA is reduced, for example to 6 components the accuracy came down to 96.5% as indicated by FIG. 4b that shows an confusion matrix obtained for predicting the efficiency and reliability of the detection method, according to another example of the invention.

The invention thus provides a method for rapid detection of a single bacterium within a sample. The sample preparation technique provided in the invention do not involve culturing or staining which are time consuming. Thus, the method provides for early and accurate detection of bacterial species in infectious disease and in food contamination even at low bacterial load.

Thus the method can be used as a potential diagnostic tool for rapid detection of bacteria in infectious diseases, in food contamination and in water contamination.

- 5 The foregoing description of the invention has been set merely to illustrate the invention and is not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to person skilled in the art, the invention should be construed to include
10 everything within the scope of the appended claims and equivalents thereof.

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We Claim:

1. A method for rapid detection of a single bacterium within a sample, the method comprising;
 - 5 preparing a sample;
 - irradiating the prepared sample with an electromagnetic radiation of specific wavelength;
 - capturing the electromagnetic radiation scattered by the sample to obtain a Raman spectra; and
 - 10 analyzing the Raman spectra to obtain a unique biochemical signature.wherein the unique biochemical signatures obtained
15 identifies the single bacterium.
2. The method of claim 1, wherein the step of preparation of sample comprises of;
 - decontaminating the sample;
 - washing the decontaminated sample to remove
 - 20 lysed components; and
 - dry casting the decontaminated sample.
3. The method of claim 1, wherein the wavelength of the irradiated electromagnetic radiation is in the range of 200 nm to 1400 nm.
- 25 4. The method of claim 1, wherein the resolution of the Raman spectra for obtaining unique biochemical signatures is in the range of 1cm^{-1} to 8 cm^{-1} .

5. The method of claim 1, wherein the biochemical signatures are molecular bond specific.
6. The method of claim 1, wherein the bacterial species is
5 selected from a group comprising of a gram positive bacteria, a gram negative bacteria, an aerobic bacteria, an anaerobic bacteria and a mycobacteria.
7. The method of claim 1, wherein the sample is selected
10 from a group comprising of a body fluid sample, a tissue sample, a food sample.
8. The method of claim 1, wherein the bacterial species is detected within a time period ranging from about one minute to about one hour.
9. A method for rapid detection of a group of bacterial
15 species within a sample, the method comprising;
 preparing a sample;
 irradiating the prepared sample with an
 electromagnetic radiation of specific
 wavelength;
20 capturing the electromagnetic radiation
 scattered by the sample to obtain a Raman
 spectra; and
 analyzing the Raman spectra to obtain a
 unique biochemical signature.
25 wherein the unique biochemical signatures obtained
 identifies a group of bacterial species.
10. The method of claim 9, wherein the step of
preparation of sample comprises of;

decontaminating the sample;
washing the decontaminated sample to remove
lysed components; and
5 dry casting the decontaminated sample.

11. The method of claim 9, wherein the wavelength of
the irradiated electromagnetic radiation is in the range of
200 nm to 1400 nm.

12. The method of claim 9, wherein the resolution of the
10 Raman spectra for obtaining unique biochemical
signatures is in the range of 1cm^{-1} to 8cm^{-1} .

13. The method of claim 9, wherein the biochemical
signatures are molecular bond specific.

14. The method of claim 9, wherein the bacterial species
15 is selected from a group comprising of a gram positive
bacteria, a gram negative bacteria, an aerobic bacteria, an
anaerobic bacteria and a mycobacteria.

15. The method of claim 9, wherein the sample is
selected from a group comprising of a body fluid sample, a
20 tissue sample, a food sample.

16. The method of claim 9, wherein the bacterial species
is detected within a time period ranging from about one
minute to about one hour.

17. A method for preparation of a substrate for casting
25 the sample, the method comprising;
selecting a silicon wafer;
depositing a metal layer on the silicon wafer;

wherein the deposition of metal layer is achieved by first sputter coating and then annealing the metal on the silicon wafer.

5 18. The method of claim 17, wherein the thickness of the metal layer is in the range of 100 nm to 1mm.

19. The method of claim 17, wherein the metal is selected from a group comprising of aluminium, silver and gold .

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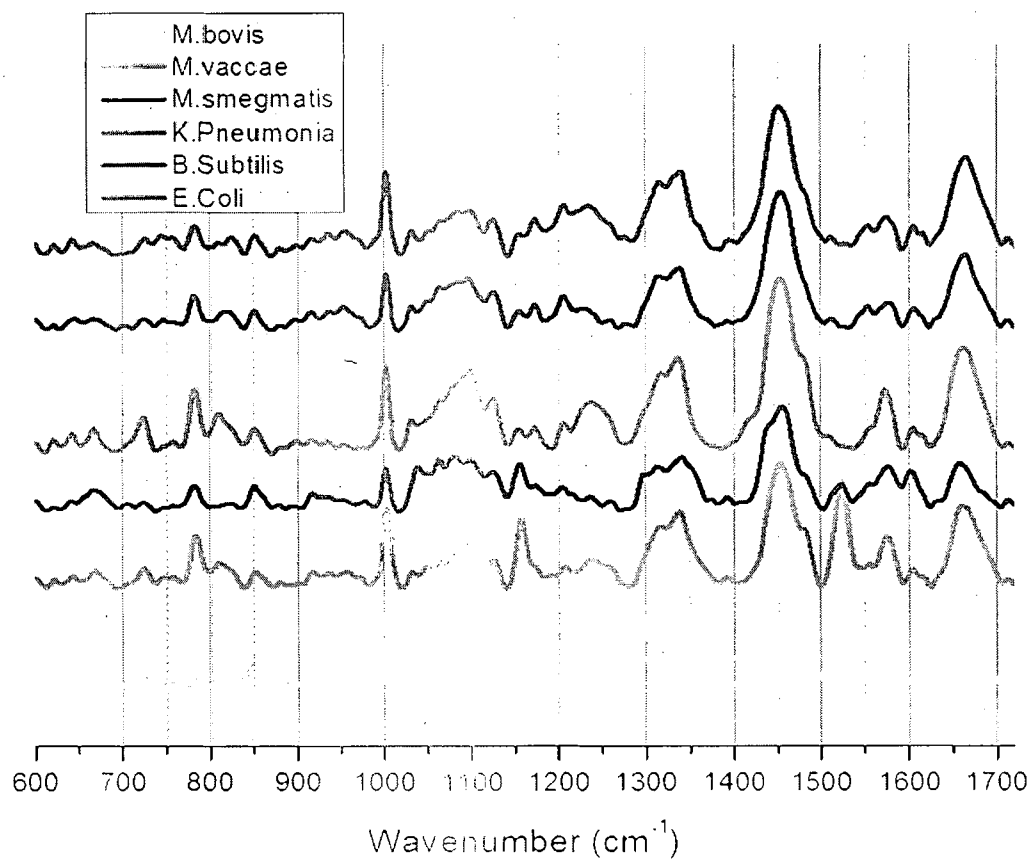


FIG. 1

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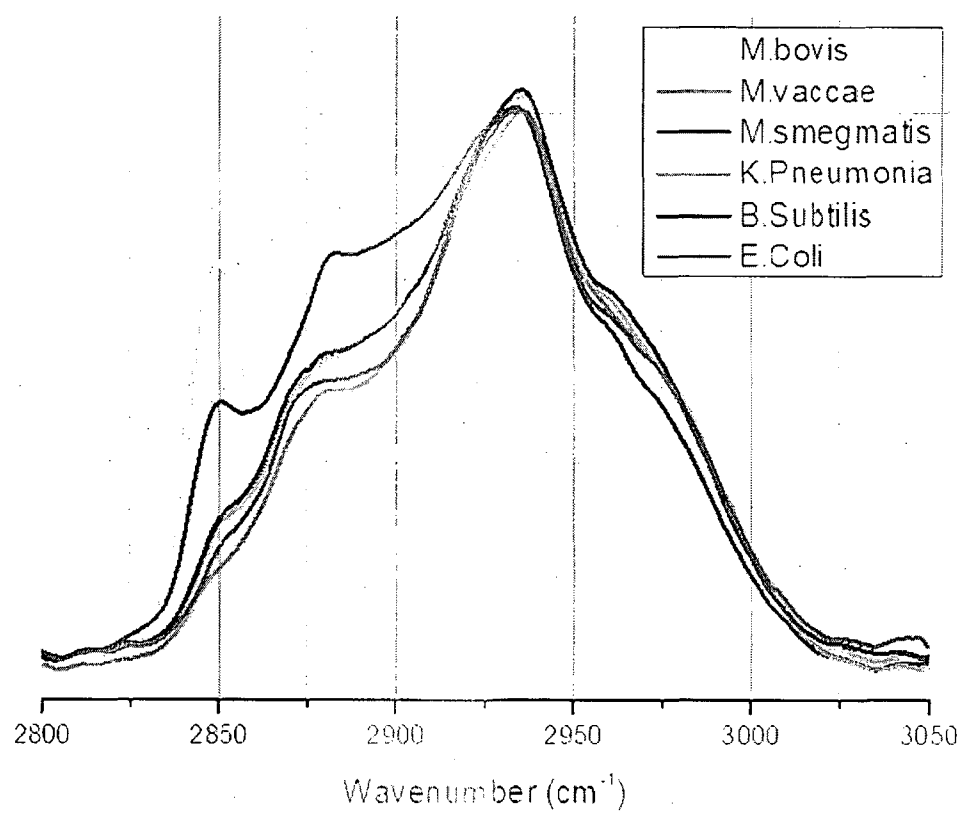


FIG. 2

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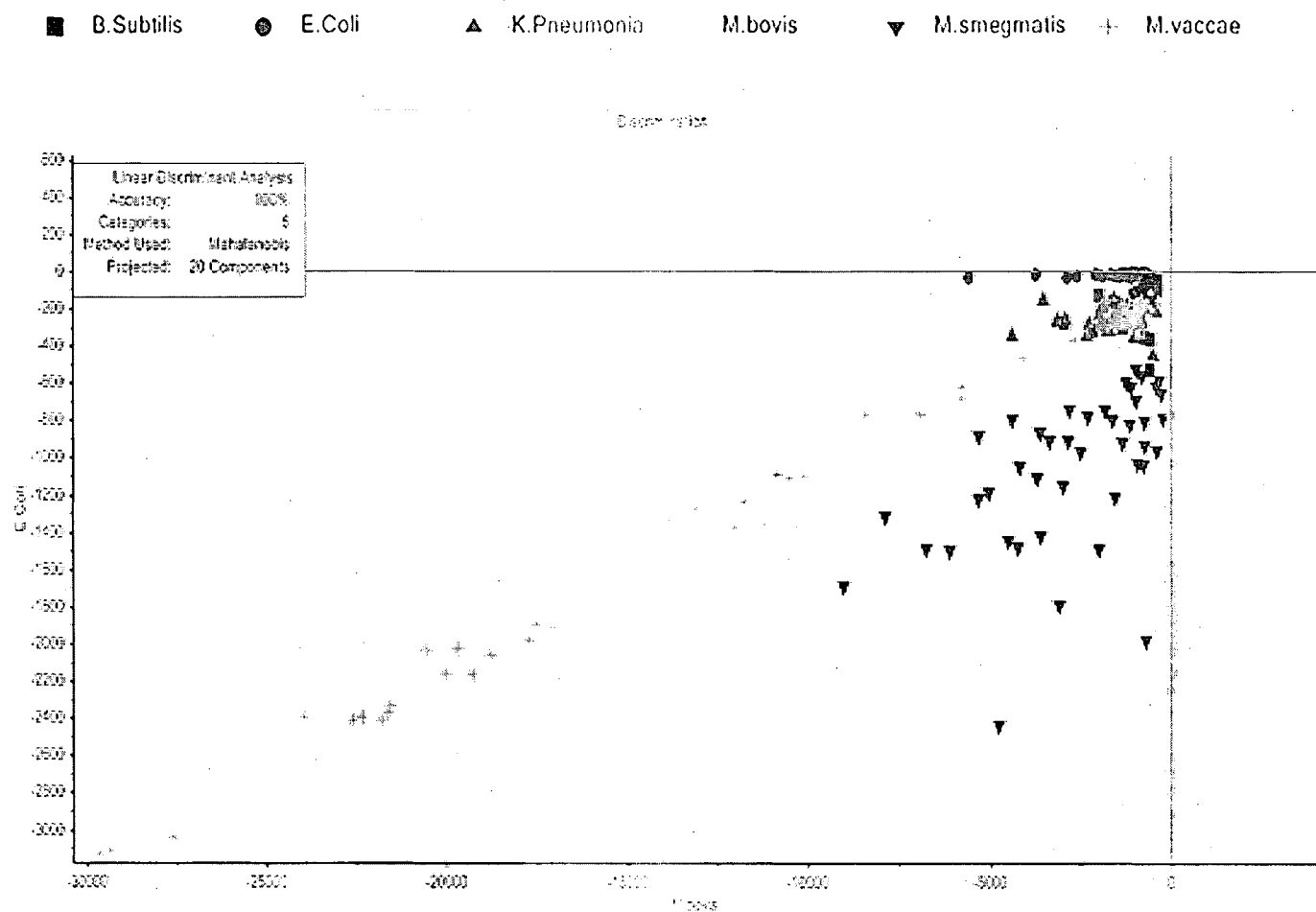


FIG. 3

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Actual ----- Predicted	E.Coli	B.Subtilis	K.Pneumonia	M.smegmatis	M.vaccae	M.bovis
E.Coli	43	0	0	0	0	0
B.Subtilis	0	32	0	0	0	0
K.Pneumonia	0	0	58	0	0	0
M.smegmatis	0	0	0	43	0	0
M.vaccae	0	0	0	0	55	0
M.bovis	0	0	0	0	0	33

FIG. 4a

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Actual ----- Predicted	E.Coli	B.Subtilis	K.Pneumonia	M.Smegmatis	M.Vaccae	M.Bovis
E.Coli	41	0	0	0	0	0
B.Subtilis	2	31	0	1	1	0
K.Pneumonia	0	1	58	0	1	0
M.smegmatis	0	0	0	42	0	3
M.vaccae	0	0	0	0	53	0
M.bovis	0	0	0	0	0	30

FIG. 4b

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN 16/00028

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/04, G01N 21/64, G01N 21/31, G01N 21/65 (2016.01)

CPC - G01N 21/65, G01J 3/44, G01N 21/62, G01N 21/65, G01N 21/31, G01J 2003/4424, G01J 3/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

CPC - G01N21/65, G01J3/44, G01N21/62, G01N21/65, G01N21/31, G01J2003/4424, G01J3/02

IPC(8) - C12Q1/04, G01N21/64, G01N21/31, G01N21/65 (2016.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - G01N21/65, G01J3/44, G01N21/62, G01N21/65, G01N21/31, G01J2003/4424, G01J3/02

IPC(8) - C12Q1/04, G01N21/64, G01N21/31, G01N21/65 (2016.01); USPC - 356/301

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patbase; Google Scholar, FreePatentsonline

Search terms used: Raman scattering bacteria identify detection determine microorganism decontamination lysed wash dry casting bond sample preparation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2008/0151223 A1 (TREADO et al.) 26 June 2008 (26.06.2008), para [0028]-[0044]	1, 3, 4, 6-9, 11, 12, 14-16

Y		5, 13

A		2, 10
Y	US 2007/0134733 A1 (HADDACH et al.) 14 June 2007 (14.06.2007), para [0031], [0035], [0040], [0044], [0071], [0075]	5, 13
A	US 2004/0021860 A1 (GARDNER et al.) 05 February 2004 (05.02.2004), entire document	1-16
A	US 2011/0267614 A1 (REINHARD) 03 November 2011 (03.11.2011), entire document	1-16
A	US 2012/0156716 A1 (WALSH et al.) 21 June 2012 (21.06.2012), entire document	1-16

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 July 2016 (08.07.2016)

Date of mailing of the international search report

05 AUG 2016

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN 16/00028

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

- Group I: Claims 1-16 drawn to a method for rapid detection of a single bacterium within a sample.

- Group II: Claims 17-19, drawn to a method for preparation of a substrate for casting a sample

--See Extra Sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-16

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IN 16/00028

Continuation of Box III: Observations where unity of invention is lacking:

The inventions listed as Groups I through II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

- Group I includes the special technical feature of analyzing the Raman spectra to obtain a unique biochemical signature, not included in the other group.
- Group II includes the special technical feature of deposition of metal layer is achieved by first sputter coating and then annealing the metal on the silicon wafer, not included in the other group.

Therefore, Groups I-II lack unity under PCT Rule 13.

Note: Claim 17, as drafted, lacks antecedent basis for "the sample" For purposes of this determination "the sample" is taken to read "a sample".