C10G-E043

SHIMADZU
Excellence in Science

# Clinical Research Reviews



Printed in Japan 0000-0000-XXXXX

# Endocrine disorders

## Fast accurate serum analysis of testosterone, androstenedione and 17-OH progesterone

In the past, immunoassays were the primary methodology for analysis of steroids in biological samples because they are rapid and easy to perform. However, these methods were shown to suffer from the lack of specificity for measuring many of the diagnostically important steroids. LC-MS/MS overcomes many of the limitations of immunoassays, enhances diagnostic utility of the testing, and expands diagnostic capabilities in endocrinology. In addition to the superior quality of the measurements, LC-MS/MS allows high throughput testing using small sample volume with minimal sample preparation, and frees the laboratory from dependence on suppliers of assay specific reagents. LC-MS/MS is being widely employed for routine measurement of steroids, and the methodology plays an important role in the standardization and harmonization of measurements among clinical laboratories.

### Scope

The LC/MS/MS assay was focused on measuring serum testosterone (T), androstenedione (A) and 17-OH progesterone (OHP) with a rapid sample preparation to help the clinical management of hyper- and hypoandrogenic diseases.

### Key highlights of the methodology

- **A simple and rapid extraction from serum**
  100uL serum; add deuterated internal standards and protein precipitate
  Vortex, centrifuge, dilute with water and inject onto the LC/MS/MS systems
- **High sensitivity LC/MS/MS analysis (LCMS-8050) ; sample turnover was 15 minutes per sample.**
  On-line integrated sample purification (2 minutes)
  LC/MS/MS analysis (5.5 minutes)
  Clean up and re-equilibration (7.5 minutes)

### Key highlights of the results

A simple and rapid extraction from serum samples. High sensitivity analysis by tandem mass spectrometry (Shimadzu LCMS-8050).

| | Testosterone | Androstenedione | 17-OH progesterone |
|---|---|---|---|
| Sensitivity I Lower limit of quantitation | | | |
| Concentration (pg/mL) | 19.5 | 39.1 | 78.1 |
| Amount on column (pg) | 0.13 | 0.26 | 0.52 |
| Accuracy % | 102.5 | 106.0 | 111.0 |
| CV % | 10.0 | 15.8 | 3.5 |
| Precision I Serum matrix (n=3) | | | |
| Mean concentration (pg/mL I CV %) | 54.5 (CV; 9.3%) | 173.4 (CV; 3.7%) | 254.7 (CV; 9.3%) |
| | 427.7 (CV; 3.7%) | 1308.7 (CV; 5.6%) | 1207.5 (CV; 4.5%) |
| | 7104.8 (CV; 1.8%) | 4972.3 (CV; 5.5%) | 2396.7 (CV; 3.7%) |
| Functional Sensitivity I BSA matrix | | | |
| Mean concentration (pg/mL Accuracy %) | 21.1 (CV; 2.5%) Accuracy 108.2% | 38.7 (CV; 2.5%) Accuracy 99.1% | 80.0 (CV; 3.8%) Accuracy 102.4% |

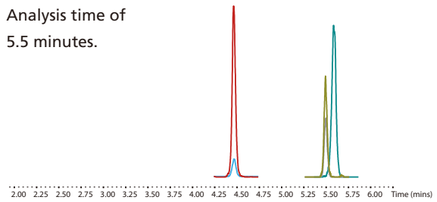


### Validation

The 2D LC/MS/MS method was validated against established protocols (immunoassay and 1D LC/MS/MS1). Testosterone was validated in both male and female patient groups (the female group also included pediatric patients).

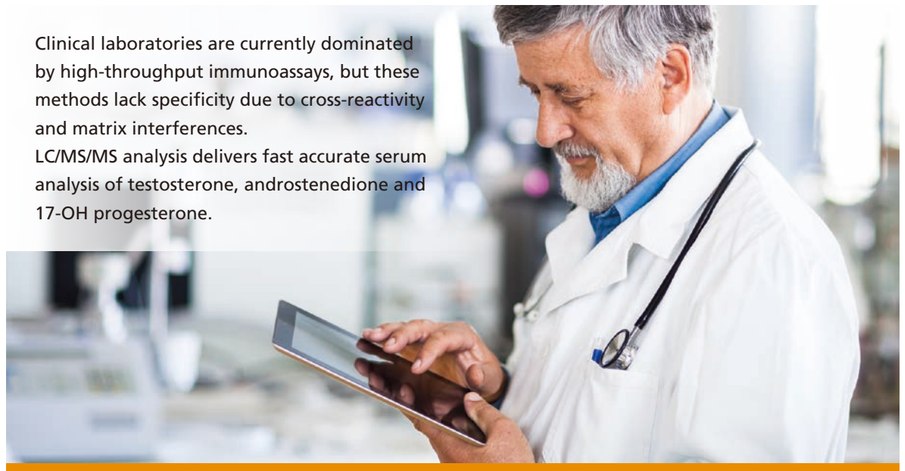
### Rapid automated LC/MS/MS analysis

Analysis time of 5.5 minutes.




Highly specific and sensitive detection of testosterone, androstenedione and 17-OH progesterone.
A key advantage of LC/MS/MS is the ability to add other steroids and quantitate a steroid panel in a single test.

Clinical laboratories are currently dominated by high-throughput immunoassays, but these methods lack specificity due to cross-reactivity and matrix interferences.
LC/MS/MS analysis delivers fast accurate serum analysis of testosterone, androstenedione and 17-OH progesterone.



## Biography

**Markus Herrmann, MD, FRCPA**

Dr. Markus Herrmann, MD, FRACP, Bolzano Hospital (Italy). Dr. Herrmann is a Clinical and Chemical Pathologist. He directs the Central Laboratory of Clinical Pathology at the District Hospital of Bolzano and is an Associate Professor at the University of Sydney. Dr. Herrmann is involved in numerous clinical and experimental research projects in the area of vitamin D, bone and energy metabolism.

References
1 Serum steroid profiling by isotopic dilution-liquid chromatography–mass spectrometry: Comparison with current immunoassays and reference intervals in healthy adults. Fanelli et al; Steroids Volume 76, Issue 3, February 2011, Pages 244–253

# New born screening

## Detecting 40 inborn errors of metabolism in a single test

The objective of a newborn screening program is to detect inborn errors of metabolism quickly and accurately. Its also important to achieve a fast turn around time in delivering results to the clinician as, in some disease states, treatment must be started rapidly to prevent irreversible damage to the infant.

Tandem mass spectrometry is an established technique which can identify and quantify a panel of metabolite markers from a single dried blood sample in an analysis cycle of less than 2 minutes. The metabolite panel includes acylcarnitines, aminoacids, succinylacetone, and more recently, some purines.

### Scope

A protocol has been developed for the analysis of acylcarnitines, aminoacids, succinylacetone and purines from a single dried blood spot sample by LC/MS/MS.

### Key highlights of the methodology

- Dried blood spot sample extraction
  25uL blood sample; extract with methanol and aqueous solution of hydrate hydrazine and add deuterated internal standards
- Flow injection LC/MS/MS analysis (LCMS-8040) ; sample analysis cycle was less than 2 minutes per sample.
  The standard concentrations were in the range of 500-2500 µmol/L for amino acids, and in the range 7.6-152 µmol/L for acylcarnitines.

### New born screening panel of target compounds

| | | | | | |
|---|---|---|---|---|---|
| CO | C12 | C5:1 | C10:2 | Met | Met MRM |
| C5 | C12:1 | C5DC | C4DC | tyr | SuAc |
| C6 | C14 | C5OH | C6DC | Asp | ADO |
| C8 | C14:1 | C12OH | C10OH | Glu | Deoxi ADO |
| C2 | C16 | C14OH | C8DC | Phe | |
| C3 | C18 | C16OH | C18:2 OH | Gly | |
| C4 | C18:1 | C18OH | C8:1 | Cit MRM | |
| C10 | C18:2 | C18:1OH | Ala | Arg MRM | |
| C10 | C2DC | C14:2 | Val | Arg Succ MRM | |
| C10:1 | C4OH | C16:1OH | Xlue | Orn | |

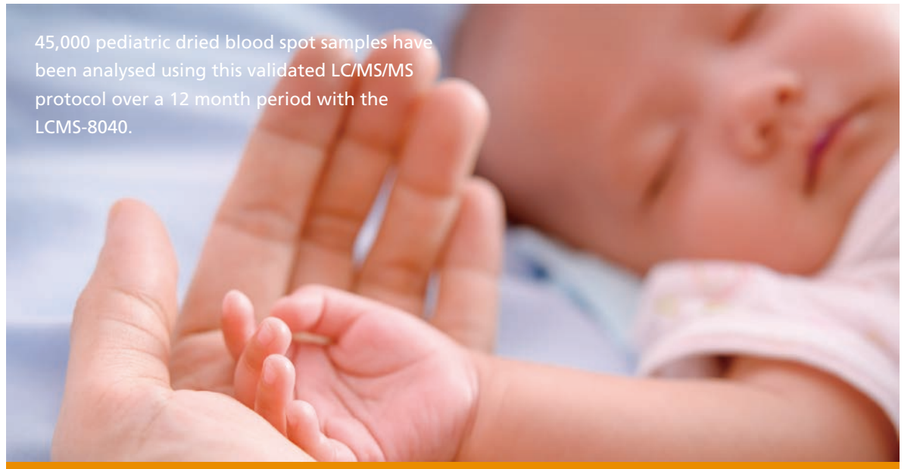
### Key highlights of the results

The development of electrospray tandem mass spectrometry (LC/MS/MS) in recent years has helped the introduction of expanded newborn screening programmes in many countries. This techonology has increased the capacity to test newborns for rare metabolic disorders during the neonatal period. LCMSMS can identify and quantify several acylcarnitines, amino acids, adenosine, deoxyadenosine, succynilacetone1, 2 and is able to detect more than 40 inborns errors of metabolism in a single test, allowing a change from the concept “one spot, one test, one disease” to “one spot, one test, many disease”.
In collaboration with Dr. G. la Marca (Meyer Children’s Hospital, Metabolomic Unit, Florence, Italy), we are developing a method for identify and quantify rare metabolic disorders using Shimadzu LCMS-8040.



### The role of MS/MS in new born screening

MS/MS can quickly and accurately identify 40 or more metabolites in less than 2 minutes (screening for acylcarnitines, aminoacids, succinylacetone, and more recently, some purines).
Several expanded newborn screening programs are now not only screening for PKU, CH, and, more recently, for cystic fibrosis (CF), and congenital adrenal hyperplasia (CAH), but also for other like aminoacidopathies, β-oxidation fatty acid defects, organic acidurias, urea cycle defects, and, since 2011, for some severe combined immunodeficiencies (SCID).



45,000 pediatric dried blood spot samples have been analysed using this validated LC/MS/MS protocol over a 12 month period with the LCMS-8040.

## Biography

**Giancarlo la Marca, Pharma Sc**

Mass Spectrometry Lab in the Newborn Screening Centre of Meyer Children’s Hospital, Florence, where he coordinated the pilot project on expanded newborn screening. Since 2004 he has coordinated the Expanded Newborn Screening in the Tuscany Region, since 2006 the Expanded Newborn Screening for the Umbria Region, and in 2012 the Expanded Newborn Screening for the Sardinia Region. He has authored more than 100 peer-reviewed publications dealing with areas of pharmacology, clinical chemistry, mass spectrometry, and pediatrics.

Further details
http://www.shimadzu.eu/sites/default/files/PO-CON1355E_BIOM-31_HPLC%202013.pdf

# Pain Management I Drugs of Abuse

## Patient monitoring for drug compliance (and abuse)

As clinicians seek evidence for both drug compliance and abuse, urine drug testing (UDT) by immunoassay and by LC/MS/MS has seen a great change in the number of samples sent to diagnostic testing laboratories. In the US in particular, the growing use of urine tests has mirrored the rise in prescriptions for narcotic painkillers, or opioids. Whilst the qualitative test detect provides a fast and simple approach to detect the class of drug, such as opioids, amphetamines, barbiturates and cocaine, the sensitized strips have a high rate of both false positive and false negatives reporting. The use of such tests has increased sharply in recent years as a growing number of states have passed laws requiring recipients of welfare and other types of public assistance to undergo drug screening.
In the case of pain patients, suspect samples are routinely subjected to quantitative analysis test by LC/MS/MS rather than by immunoassay. The scale of the problem is described by the CDC as a 'growing, deadly epidemic of prescription painkiller abuse' . Nearly three out of four prescription drug overdoses are caused by prescription painkillers—also called opioid pain relievers. The unprecedented rise in overdose deaths in the US parallels a 300% increase since 1999 in the sale of these strong painkillers. These drugs were involved in 14,800 overdose deaths in 2008, more than cocaine and heroin combined. The misuse and abuse of prescription painkillers was responsible for more than 475,000 emergency department visits in 2009, a number that nearly doubled in just five years. More than 12 million people reported using prescription painkillers non-medically in 2010 (painkillers are used without a prescription or for the feeling they cause).

### Scope

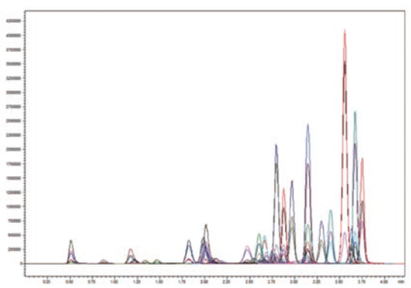
Pain management and drugs of abuse sample analysis can be achieved using a number of different approaches. This protocol describes a method for opiates, amphetamines and cocaine in whole blood, plasma and urine by UHPLC-MS/MS using a QuEChERS sample preparation.

### Key highlights of the methodology

- **Biological sample extraction (whole blood, plasma or urine).**
  100uL of sample; add deuterated internal standards and 50 mg of QuEChERS salts (MgSO4, NaCl, Sodium citrate dehydrate, Sodium citrate sesquihydrate)
- **LC/MS/MS analysis (LCMS-8040) ; LC/MS/MS sample analysis cycle was less than 6 minutes per sample.**
  Lower limit of quantification was 5 ug/L for all compounds while the upper limit of quantification was set at 500 µg/L.

### Key highlights of the results

The speed and simplicity of this approach resulted in an effective method for a panel of drugs in differing biological samples (whole blood, serum and urine). 38 filled the validation conditions in term of intra- and inter-assay precision and accuracy were less than 20% at the lower limit of quantification and less than 15% at the other concentrations.



Target list

**Cocaine and metabolites**
Anhydroecgonine methylester, Benzoylecgonine, Cocaethylene, Cocaine, Ecgonine methylester.

**Amphetamines or related compounds.**
2-CB, 2-CI, 4-MTA, Ritalinic acid, Amphetamine, BDB, Ephedrine, MBDB, m-CPP, MDA, MDEA, MDMA, MDPV, Mephedrone, Metamphetamine, Methcathinone, Methiopropamine, Methylphenidate, Norephedrine, Norfenuramine, Porpseudoephedrine, Pseudoephedrine.

**Opiates**
6-monoacetylmorphine, Dextromethorphan, Dihydrocodeine, Ethylmorphine, Hydrocodone, Hydromorphone, Methylmorphine, Morphine, Naloxone, Naltrexone, Noroxycodone, Oxycodone, Pholcodine.



### Conclusions

There are a number of methods that can be used to monitor pain management compliance and also test for drugs of abuse screening. In this paper, QuEChERS have been applied to the LC/MS/MS analysis of a panel of drugs of abuse which has several key advantages as it is a quick method to prepare samples and delivers near zero carry over (conventional on line sample preparation method are often characterized by sample carry over).
(It is also important to note that this approach is not only used in pain management and drugs of abuse screening but has been validated in a forensic toxicology context3).

CDC Policy Impact Paper
Prescription Painkillers Overdoses.
In 2012, of the 41,502 drug overdose deaths in the United States, 22,114 (53%) were related to pharmaceuticals.
Of the 22,114 deaths relating to pharmaceutical overdose in 2012, 16,007 (72%) involved opioid analgesics (also called opioid pain relievers or prescription painkillers).

## Biography

**Pierre Marquet, MD, FRCPA**

Pierre MARQUET has been a Doctor of Medicine since 1986 and obtained a master in statistics and epidemiology followed by a PhD in physiology in 1992. After several years as a hospital pharmacologist at Limoges University Hospital, he has been an associate professor of pharmacology since 1998, and in 2001, became a full professor at the Faculty of Medicine of Limoges, France. Pierre Marquet is currently the Head of the "Pharmacology, Toxicology and Pharmacovigilance" Department at the University Hospital of Limoges and Director of the INSERM U850 research unit "Pharmacology of immunosuppressive drugs in transplantation".

Further details
1https://solutions.shimadzu.co.jp/an/s/en/lcms/fro114022.pdf?return=http%3A%2F%2Fwww.shimadzu.com%2Fan%2Fliterature%2Flcms%2Ffro114022.html
2http://www.cdc.gov/HomeandRecreationalSafety/pdf/PolicyImpact-PrescriptionPainkillerOD.pdf
3https://solutions.shimadzu.co.jp/an/s/en/lcms/jpo114021.pdf?return=http%3A%2F%2Fwww.shimadzu.com%2Fan%2Fliterature%2Flcms%2Fjpo114021.html

# Screening methods for Colorectal Cancer

## A Novel Serum Metabolomics-Based Diagnostic Approach for CRC

Colorectal cancer is one of the most common causes of cancer death in developed countries. Treatment methods based on colonoscopy and surgery have advanced rapidly, and a large number of patients with colorectal cancer achieve improvements after therapy. However, advanced stage colorectal cancer reduces the quality of life of patients receiving operative treatment or chemotherapy. Therefore, methods that allow the early detection and diagnosis of colorectal cancer are currently being sought. The fecal occult blood test (FOBT) is the most commonly used screening method for diagnosing colorectal cancer and is a noninvasive and inexpensive method. However, the FOBT has low sensitivity, especially for early stage colorectal cancer. Colonoscopy is a more accurate and reliable approach for diagnosing colorectal cancer, but it is difficult for elderly or severely ill patients to undergo colonoscopy, and its high cost is also a problem. Thus, examinations involving a combination of conventional screening methods have been used for the diagnosis of colorectal cancer; however, such examinations only detect about 40% of colorectal cancers. Therefore, it is necessary to establish new screening methods for the early diagnosis of colorectal cancer that are highly sensitive, specific, easy, and noninvasive.

### Scope

We performed serum metabolome analysis using gas-chromatography/mass-spectrometry (GC/MS). First, the accuracy of our GC/MS-based serum metabolomic analytical method was evaluated by calculating the RSD% values of serum levels of various metabolites. Second, the intra-day (morning, daytime, and night) and inter-day (among 3 days) variances of serum metabolite levels were examined. Then, serum metabolite levels were compared between colorectal cancer patients (N = 60; N = 12 for each stage from 0 to 4) and age- and sex-matched healthy volunteers (N = 60) as a training set. The metabolites whose levels displayed significant changes were subjected to multiple logistic regression analysis using the stepwise variable selection method, and a colorectal cancer prediction model was established.

The prediction model was composed of 2-hydroxybutyrate, aspartic acid, kynurenine, and cystamine, and its AUC, sensitivity, specificity, and accuracy were 0.9097, 85.0%, 85.0%, and 85.0%, respectively, according to the training set data. In contrast, the sensitivity, specificity, and accuracy of CEA were 35.0%, 96.7%, and 65.8%, respectively, and those of CA19-9 were 16.7%, 100%, and 58.3%, respectively. The validity of the prediction model was confirmed using colorectal cancer patients (N = 59) and healthy volunteers (N = 63) as a validation set. At the validation set, the sensitivity, specificity, and accuracy of the prediction model were 83.1%, 81.0%, and 82.0%, respectively, and these values were almost the same as those obtained with the training set. In addition, the model displayed high sensitivity for detecting stage 0–2 colorectal cancer (82.8%).

### Key highlights of the methodology

Serum metabolomics analysis by GC/MS.
A 50 µl of serum was mixed with 250 µl of a solvent mixture (MeOH:H2O:CHCl3 = 2.5:1:1) containing 10 µl of 0.5 mg/ml 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) dissolved in distilled water as an internal standard, and then the solution was shaken at 1,200 rpm for 30 min at 37°C, before being centrifuged at 16,000 x g for 3 min at 4°C.
A 225 µl of the resultant supernatant was transferred to a clean tube, and 200 µl of distilled water were added to the tube ,and the solution was shaken at 1,200 rpm for 30min at 37°C, before being centriguged at 16,000 x g for 3min at 4°C. A 250uL of the resultant suppernatant was transferred to a clean tube, and freeze-dried for 6 hours at 4°C, after being concentrated under vacuum pressure at 16,000 x g. For oximation, 40 µl of 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich, Tokyo, Japan) dissolved in pyridine were mixed with a lyophilized sample, before being shaken at 1,200 rpm for 90 min at 30°C. Next, 20 µl of N-methyl-N-trimethylsilyl-trifluoroacetamide(MSTFA) (GL Science, Tokyo, Japan) were added for derivatization, and the mixture was incubated at 1,200 rpm for 30 min at 37°C. The mixture was then centrifuged at 16,000 x g for 5 min at 4°C, and the resultant supernatant was subjected to GC/MS measurement.

| Biomarkers | Coefficient | Standard error | P value | Lower 95% CI | Upper 95% CI |
|---|---|---|---|---|---|
| (intercept) | –8.32 | 1.539 | <0.0001 | –11.71 | –5.621 |
| 2-hydroxy-butyate | 286.59 | 71.90 | <0.0001 | 155.0 | 440.1 |
| Aspartic acid | 33.87 | 14.29 | 0.0178 | 7.390 | 63.85 |
| Kynurenine | 1634.96 | 569.3 | 0.0041 | 559.1 | 2.830E+03 |
| Cystamine | 78.78 | 26.82 | 0.0033 | 31.53 | 137.3 |

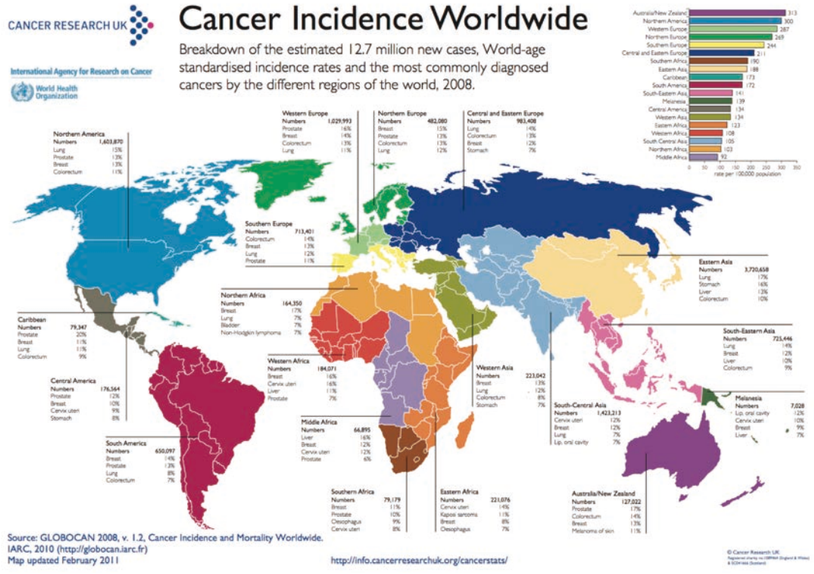
The 4 metabolites selected by multiple logistic regression analysis using the stepwise variable selection method. The results of the analysis are shown in Table 4. The 95% confidence interval (95% CI) for the AUC (0.9097) obtained from ROC analysis ranged from 0.8438 to 0.9495.
doi:10.1371/journal.pone.0040459.t004



### Conclusions

We developed a metabolomics-based prediction model for colorectal cancer involving multiple biomarkers, and the sensitivity of the model for detecting early stage colorectal cancer patients was the same or better than those of previously described methods. In this study, serum metabolome analysis was able to describe the status of colorectal cancer patients rather than simply detect the presence of colorectal cancer, which might be explained by our use of multiple biomarkers.

Determining the concentrations of the 4 metabolites selected in this study is crucial for the future clinical application of our model, and moreover the development of easier methods, for example enzyme-linked immunosorbent assay (ELISA) systems and procedures based on enzyme chemistry, is also important. Taken together, our findings will hopefully lead to an improved quality of life via the early detection of colorectal cancer.




## Biography

**Masaru Yoshida, MD, PhD**

Masaru Yoshida is the chief of Metabolomics Research at Kobe University Graduate School of Medicine. Dr. Yoshida received his Ph.D. training at Kyoto University and carried out postdoctoral training in Brigham and Women's Hospital & Harvard Medical School. His laboratory study focus on metabolomics research by mass spectrometer and these technologies have been used for Clinical Medicine. He has published more than 100 articles in scientific journals/books.

Further details
1http://asms.inmerge.com/ASMS13PDF/ASMS201311399.6500VER.1.pdf