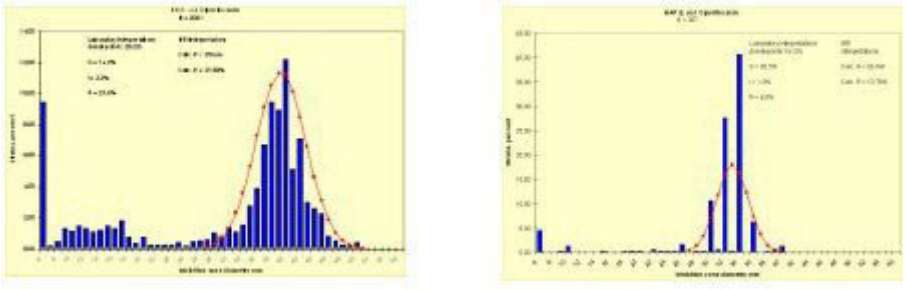
Göran Kronvall projects

Research focus of Göran Kronvall projects.

Antimicrobial susceptibility testing using disc diffusion methods (Göran Kronvall)

Species-related interpretive breakpoints were introduced on a broad scale at the clinical microbiology laboratory in Lund, Sweden, in the late 1970:ies by Göran Kronvall, at that time responsible for clinical bacteriology laboratory services in Lund.



Antimicrobial susceptibility testing using disc diffusion methods, E. Photo: Göran Kronvall

Antimicrobial susceptibility testing using disc diffusion methods, E.

NRI - Normalized Resistance Interpretation

Let us ask ourselves: Whick part of an inhibition zone diameter or MIC distribution (species-wise) for a given antimicrobial is unaffected by the development of resistance?

Answer: The high-zone (or the low MIC) side of the most susceptible population of strains, representing the wild-type population. When resistance occurs in an isolate of that species, the position of that isolate in the distribution changes to lower zone sizes or higher MIC values.

So, if we can use the upper zone size slope for a reconstruction of the whole wild-type distribution, then we have obtained an internal calibrator which will enable us to compare results from anywhere, from any laboratory in the world.

This can be done using the Normalized Resistance Interpretation (see link below) method, NRI.

Normalized Resistance Interpretation method, NRI

A summary of the procedure (see link below) is presented in IJAA.

A detailed analysis of parameter setting (see link below) for the NRI calculations was performed by Joneberg et al.

summary of the procedure

analysis of parameter setting

MIC distributions with regular double dilution steps provided too few points for solving the regression, but Etest results (see link below) with intermediate values included, were precise enough for NRI calculations to work. This was shown in studies of tigecycline susceptibility.

Etest results

A patent application for the NRI method has been submitted by Bioscand AB (International Patent Application WO 02/083935 A1).

Normalized Resistance Interpretation method, NRI.

The solution to standardized resistance surveys.

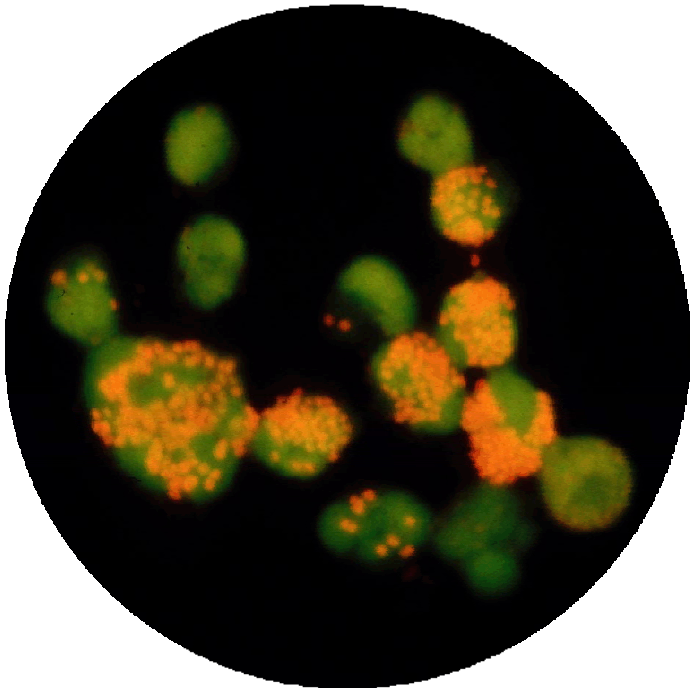
Free downloads

Single strain regression analysis programme for calibration of disk diffusion testing, etc (3.9 Mb installation file).

Publications & Documents

Mini-review on Acridine Orange Staining in Clinical Microbiology

Acridine Orange Staining in Clinical Microbiology
A Mini-Review



Introduction
Before the investigations in 1976-1977 by Kronvall and Myhre on the effect of pH on the staining characteristics of acridine orange and the invention of the acid pH acridine orange staining of clinical specimens (1), the use of acridine orange in clinical microbiology was of no diagnostic value. A regular acridine orange staining at neutral pH stained everything brilliant orange, cell nuclei, bacteria, cell debris, etc., in a fixed specimen on the slide.

On the other hand, acridine orange had been used for several purposes before 1977. Environmental microbiologists used acridine orange to count live versus dead bacteria in soil, water and other materials (2). The immunologists used acridine orange to count per cent blast cells in lymphocyte transformation tests (3,4). In all these applications the staining was performed at a neutral pH.

How it all started
When I was working at A.H.R.I., the Armauer Hansen Research Institute, in Addis Ababa, Ethopia, during 1973 – 1975, I performed lymphocyte transformation tests together with Dr G. Bjune on cells from both patients and apparently healthy individual. The cells were challenged with *M.leprae* antigen and other mitogens or antigens, as had been performed in the laboratory before our arrival. In the absence of isotope methods the transformation tests were read by microscope counting of per cent blasts after acridine orange staining (3,4).

The beauty of the stained preparations was so impressive that I also thought about other applications of staining with acridine orange.

After the return to Lund I engaged a young doctor, E. Myhre, in an investigation of different parameters with effect on the staining of bacteria as well as tissue components.

Publication of the results
With Dr. Myhre I wrote up the results of the acridine orange staining experiments and concluded that we had obtained a **DIFFERENTIAL STAINING EFFECT** of great potential in clinical microbiology.

Such a practical and simple method should be welcomed by clinical microbiologists and we therefore sent the manuscript to the leading journal in the field, Journal of Clinical Microbiology. To our great surprise the manuscript was found "***unacceptable for publication in the Journal***.". The editor wrote: "***The method is much more cumbersome than the gram stain …***". He also wrote: "***Other reviewers have been very concerned about the expense that this technique would generate.***" This contrasts to what later scientists have written about the method, how simple and easy to perform it really is.

The manuscript was then sent to Acta Pathologica et Microbiologica Scandinavica, Section B – Microbiology. It was accepted and then printed (1). This journal is nowadays known as APMIS.

Subsequent reports on the use of the AO method
Already in a few years several clinical microbiologists took up the acid acridine orange staining method and tested it in their laboratories.
Forsum and Hallén, 1979 (5) were first out and wrote: "***Acridine orange staining seems a valuable alternative due to its sharp image contrast***.".

Dr. Jill E.Senne in Chapel Hill, USA, described the test in 1979 in Clinical Microbiology Newsletter (6) and then published a more extensive evaluation of its use in blood culture microscopy in 1980 (7). She wrote in 1979: "***The AO stain's sensitivity, low cost, and potential time-saving make the method attractive for the detection of microorganisms in direct smears*** … ".

Other American microbiologists followed suit with Lauer et al. (8) in 1981, Mirret et al. (9) in 1982, Burdash et al. (10) in 1983, Cashman et al. (11) in 1983, Tierney et al. (12) in 1983, Flejzor and Bokkenheuser (13) in 1984, Kleiman et al. (14) in 1984.

Some conclusions arrived at in these articles were:
"***The acridine orange method represents a rapid and inexpensive alternative*** …" (7)
"***The AO staining procedure is a simple, sensitive, screening technique for the early detection of positive blood cultures***." (9)
"… ***we have substituted AO staining for blind subculturing of BACTEC-negative bottles***." (10)
"***Acridine orange staining … is a rapid, reliable method to detect positive blood cultures***." (12)

Also in laboratories in other countries the use of the AO method was evaluated. For instance Mascart et al. in Belgium wrote: "***Staining with acridine orange yields more positive results than Gram staining and is also simpler***." (15). Meseguer el al. in Spain reported: ***"Acridine orange staining is a sensitive, rapid and reliable method for detecting bacteria in blood***

cultures early during incubation. The method is inexpensive and easy to perform and can be substituted for blind subcultures." (16).

Nor only in blood cultures but also in several other tests on clincal specimens the AO method was found to be of value. For cerebrospinal fluid examinations it was found that "… ***acridine orange staining is a sensitive method for screening clinical specimens*** …" (8). The title of one article was: "***Superiority of acridine orange stain versus Gram stain*** …" (17). Also in urinary tract infections AO staining proved valuable (18,19). One comment read: " … ***the rapid, inexpensive AO procedure*** … " (18). In bacterial keratitis " … ***acridine orange is significantly better than Gram stain in cases with low amounts of oragnisms***." (20), and " … ***Acridine orange was more sensitive that Gram stain***, …" (21), and " … ***this stain*** [AO] ***is reommended as a simple and reliable method for the rapid diagnosis of this disease***." (22).

More rare blood-borne infections have also been successfully analysed using acid acridine orange, for instance infections by *Borrelia* (23), *Bartonella* (24), and *Tropheryma whipplei* (25). *Cryptococcus neoformans* was detected in sets of negative blood cultures, but positive by acridine orange staining (26). In mouse experiments it was noted that "***Acridine orange is a rapid and sensitive method for demonstrating trophozoites of P. carinii in mouse lung tissue***." (27).

Infectious foci in other body compartments have also been studied using acridine orange, for instance in samples from nasopharynx (28), middle ear (29,30), other purulent specimens (31), urethral and cervical smears (5), intra-amniotic infection (32),

In the early history of *Helicobacter pylori* (first designated *Campylobacter pylori*) research after the initial discovery (33) acridine orange staining of specimens at acid pH played an important role. Walters et al. identified *C.pylori* in formalin fixed biopsies (34). They concluded that " … ***this stain was easier to perform than*** … ", and " … ***slides could be examined much more quickly and with less effort than*** …" (34).Haqqani et al., apparently independently found the acid acridine orange staining of gastric biopsies " … ***is inexpensive, quick and easy to perform*** …", and " … ***more sensitive than other stains***, …" (35). After 16 years use Haqqani stated that the AO staining " ... ***has proved to be exremely useful in the identification of H.pylori***." (36). Other authors confirm the valuable use of AO staining in studies of *H.pylori* infections (37-40). For instance Södervik et al wrote " … ***the staining test proved to be a rapid and reliable test particularly in outpatient clinics*** …" (37).

Further developments.

It has been mentioned in several reports that an acridine orange stained smear can subsequently be subject to Gram stain which will enable a report on the Gram stain reaction of the orgnaisms discovered. This possibility is an inportant aspect of the AO staining method.

A very interesting development of the AO method has been described by Dr Ettore Ciancaglini and his group in Italy (41-43). The slide is first stained with acridine orange (10% in acid buffer), then decolorized with ethanol/acetone, 50:50, and finally stained with fluorescein (0.0001%). This procedure produced a differential effect with Gram positive organisms giving yellow fluorescence and Gram negative giving green fluorescence (42,43).

The method was tested on both blood cultures and CSF samples as well as on urine samples, giving higher sensitivity and specificity than regular Gram stain. This procedure has not been evaluated by other clinical microbiologists so far, but that should really be done.

Another alternative to classical Gram stain was described by Mason et al. for staining microorganisms in suspension (44). They used two different fluorescent dyes, hexidium iodide and SYTO 13, with different staining patterns. Also this method should be further evaluated.

AO staining described in manuals

Difco Manual

The 10th edition of "*Difco Manual – Dehydrated Culture Media and Reagents for Microbiology*" included a 3-page description of the acridine orange staining method at low pH of clinical specimens (45). This excellent description together with the availability of acridine orange solution from Difco must have been a perfect guide for clinical microbiologists in the use of this method. Therefore, in many later publications the only reference for the method is to the Difco Manual.

Manual of Clinical Microbiology

The acridine orange staining method at low pH was included 1991 in the 5th edition of the Manual of Clinical Microbiology, but without any references. In the 6th edition in 1995 a more detailed description was included with as many as six references but not to the original one by Kronvall and Myhre. What was more surprising, however, was the lack of any information about the critical pH of the staining procedure. There was a high risk therefore that regular acridine solutions with neutral pH could be used. Therefore, I wrote to the author of the chapter pointing out this fact and I also informed about the original article for the method (1). The response was a kind letter with thanks for the clarification and an assurance that with her as an author the next edition would correct the information.

In the 7th edition in 1999 the information about the low pH was included and also the buffer used, but without any reference to the original work. Five of the earlier six references were included. When the 8th edition in 2003 arrived with no reference to the original description of the method I wrote a letter to the Editor-in-Chief. In my own scientific upbringing it was always important to honour the original work. The reply was that the authors "… ***were one of the first US clinical microbiologists to use the acridine orange stain in the clinical microbiology lab***.". It was apparently more important to honour fellow citizens.

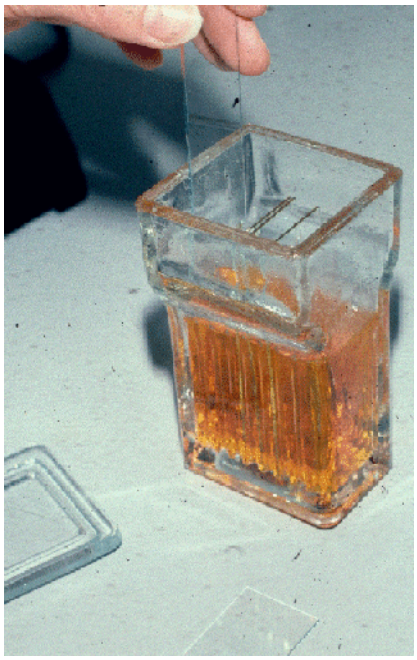
In the 9th edition in 2007 the original reference suddenly appeared, but with no mentioning of its originality, just added to the other five references. Finally, in the 10th edition in 2011 all references were omitted except one of the early American studies (8). It is remarkable that a manual of world-wide importance is so local in its honouring of scientific contributions.

How to stain a clinical specimen with acridine orange at low pH

Acridine Orange (AO) stock solution.
50 mg acridine orange is dissolved in 10 ml of distilled water and stored in the refrigerator, 0.5 per cent.

Staining solution.
1 ml of AO stock solution and 0.5 ml of glacial acetic acid is added to 50 ml of distilled water in a staining trough taking 8 microskope slides. The pH of the solution will be approximately 3 and the AO concentration 0.01 per cent.

Staining procedure.
Dried smears of glass slides are fixed with methanol and after drying put in trough with acridine orange staining solution (0.01 per cent). After 2 minutes of staining, the slides are washed gently with water and dried and then examined in a fluorescent microscope. Bacteria stain orange against a green to yellow background of human cells and debris.



Alternative, commercially available AO solutions.
1. BD BBL Acridine Orange Reagent Droppers, BD Diagnostics No 261182, 50 ampules, each containing 0.5 ml of acridine orange, 0.01 %.
2. Sigma-Aldrich A8097 Acridine Orange stock solution 10 mg/ml in H_2O, 10 ml. For staining solution take 0.5 ml of this 1 per cent solution in the above recipe to make it 0.01 %.

The article on acridine orange staining at low pH in clinical microbiology is one of some 250 publications by Göran Kronvall. For the full bibliography, see web page:
http://www.bioscand.se/kron/gk_bibliography.pdf

2012-02-11
Göran Kronvall

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