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Immunologic detection of bacterial antigens and antibodies

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Early diagnosis and prompt institution of specific antimicrobial therapy are required for the optimal treatment of infectious disease. Traditionally, the laboratory has isolated and identified the specific etiologic agent and when possible, determined its antimicrobial susceptibility profile. If cultural microbiology was not successful, then diagnosis could often be made by observing the appearance of specific antibodies. While the detection of antibodies is still important in infectious disease diagnosis, the process is time consuming in that, for optimal results, an acute and convalescent serum sample should be collected 2-3 weeks apart. A variety of immunologic techniques have been described which enable the rapid detection of specific microbial antigens in body fluids.

Non-traditional approaches to immunologic detection of antibodies and antigens have become popular in clinical microbiology laboratories because the techniques are relatively simple, of moderate cost, and provide diagnostic answers in a time frame which is still of consequence to the treatment of the patient. Tests which are routinely available include counter-immunoelectrophoresis, latex agglutination, latex agglutination inhibition, coagglutination, radio-immunoassay, enzyme-linked immunoassay, and fluorescent immunoassay.

COUNTERIMMUNO-ELECTROPHORESIS (CIE)

Counterimmunoelectrophoresis (CIE) was originally described in 1959 by Bussard [1] and was first used clinically for the detection of "Australia antigen" (HbsAg). Radioimmunoassay soon replaced CIE for HbsAg but CIE has become a valuable immunologic tool for rapid detection of both microbial antibodies and antigens.

CIE is a rapid precipitin reaction in which the reactants are driven by an electric current. In 1901, Vincent and Bellot[2] first described the use of the tube precipitin reaction for the detection of meningococcal antigen in CSF. Dochez and Avery[3] identified the capsular polysaccharide of the pneumococcus in patients' urine as early as 1917. The precipitin reaction is a function of the precipitation of antibody and soluble antigen at the equivalence point. The reaction may take up to 18 hours. CIE, on the other hand, combines the advantages of immunodiffusion and electrophoresis. The antigen (Ag) is placed in a well on the cathodic side of a solid support and the antibody is placed on the anodic side. The antigen, if negatively charged, migrates toward the anode and the antibody which usually has a weak negative charge also migrates toward the anode. Positively charged buffer ions, however, sweep the antibody molecule to the cathode. This is called endoosmotic flow. If conditions of voltage, current, buffer, pH, antigen/antibody concentration, and quality of antisera are optimal, then a precipitin line appears between the two wells after as little as 30 minutes of electrophoresis.

There are a number of variables which must be standardized if CIE is to be a reliable reproducible method in the clinical laboratory:

a) The quality of *antisera* may be the single most important variable in CIE.

b) *Buffer* - The buffer in CIE maintains the pH and the ionic strength. Usually the buffer is alkaline (i.e. barbital buffer, pH 8.2-8.6) and confers a negative charge on the molecules.

Buffer systems may be continuous or discontinuous. A continuous buffer system is one in which the buffer in the electrophoresis chamber is of the same pH and ionic strength as the buffer used to make the agar gel.

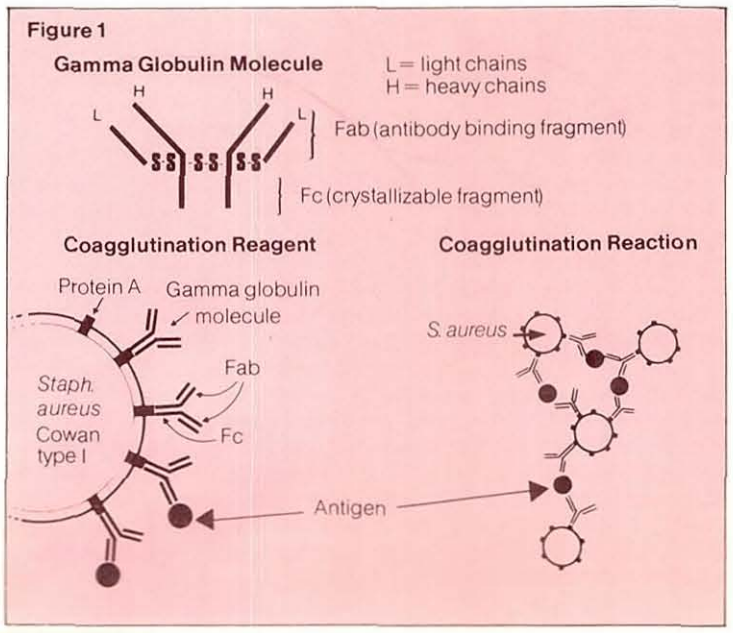
In discontinuous systems, the two buffers are different. Although Wallis and Melnick[4] claimed increased sensitivity for hepatitis antigen detection using a discontinuous system, in the author's experience, discontinuous systems offer no advantage.

c) *Support systems* - Immunologic reactions occur in gels. Several supports have been used for CIE including agarose, cellulose acetate, Noble agar and bacteriological agar. For most applications, agarose is the gel of choice. It is a neutral linear polysaccharide, water soluble, and will form a gel at 0.01%.

The gel may be placed on glass, plastic, or mylar film. The author prefers mylar film as it can be cut to size for the particular application, stained, and entered into the permanent record.

d) *Electrophoresis chamber* - The chamber provides a physical support for the gel, it contains buffer, and it transmits the voltage and current to the gel matrix.

Figure 1



Clinical applications

Anhalt et al[5] list the following five applications of CIE in infectious disease:

1) detection of antigen in body fluids
2) determination of antibody titers (poor sensitivity)
3) prognostic assessment
4) identification and/or typing of clinical isolates
5) elucidation of role of circulating antigens in disease pathogenesis.

CIE is used primarily for the detection of microbial antigens in body fluids and the direct immunologic identification of certain bacteria such as the β-hemolytic streptococci.[6] In some cases, however, it may be necessary to extract the cell associated antigen with either heat, acid, or enzymes. Edwards and Larson[7] noted that on 400 strains of Groups A,B,C,D,E, and F streptococci, CIE was a more sensitive and a faster identification method than the capillary precipitin test.

Virtually any body fluid may be tested by CIE including CSF, urine, serum, pleural fluid, synovial fluid, peritoneal fluid, abscess drainage, and pericardial fluid. In general, those fluids which have less protein (CSF, urine) are easier to process as there are fewer spurious precipitin lines or areas of non-specific precipitation of protein or lipoprotein.

Edwards[8] first demonstrated the polysaccharide capsule of *Neisseria meningitidis* in the serum of patients with meningococcemia. The same polysaccharide was detected by CIE in the CSF of 47/68 patients and the culture in 42/68 patients. Since these early reports, there has been much published on the rapid diagnosis of bacterial meningitis by CIE. The most commonly detected antigenic components are *H. influenzae, S. pneumoniae, N. meningitidis*, Group B streptococci, and *E. coli* K1. The combination of CSF, serum, and urine CIE's can identify virtually 100% of children with *H. influenzae* Type B meningitis, 60% of patients with pneumococcal meningitis, and < 50% of those with meningococcal meningitis[9].

The poor results on meningococcal meningitis, is in contrast to some of the earlier work, probably due to the serogroups involved. It is recognized that Group B meningococcal polysaccharide cannot be detected by a routine agarose CIE system.[10]

The value of CIE for the prospective diagnosis of early onset Group B streptococcal (GBS) infection has been reported. Edwards and Baker[11] found GBS antigen in the CSF of one infant with both meningitis and septicemia, in the serum of 3/7 septicemic infants, and in the concentrated urine of 6/7 patients. In all 7 cases, GBS was eventually cultured from the blood. Jacobs et al[12] report similar utility of CIE for GBS.

Ryan et al[13] have recently described a CIE procedure for detection of *Clostridium difficile* toxin in feces. The early work proved the assay to be sensitive and specific. Later reports[14] on a larger population of specimens revealed that the test was less specific than originally thought. Absorption of the antitoxin with whole cells of *C. difficile* improved specificity with no loss of sensitivity.

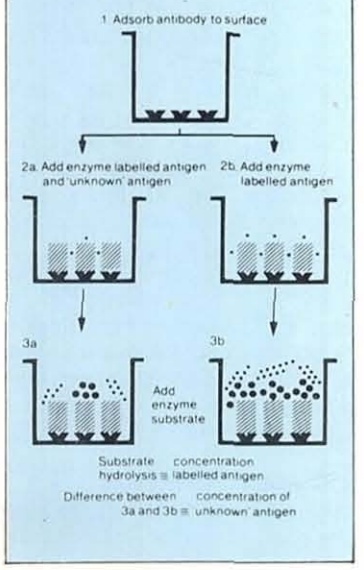
LATEX AGGLUTINATION

Latex polystyrene beads were first used to detect rheumatoid factor in serum. Either antigens or IgG antibody are nonspecifically absorbed to the surface of the latex polystyrene beads of uniform diameter (usually 0.8 μm). Addition of the specific antibody or antigen visibly agglutinates the milky-white latex suspension. Although latex agglutination tests can be done in test tubes, they are usually performed on slides.

Depending on the system, the procedure for the detection of antigen or antibody by latex agglutination is quite simple. A drop or two of the latex reagent is mixed with a suspension of the colony or the body fluid to be tested. The suspension is mixed and incubated at room temperature with occasional rotation of the slide. Agglutination is a positive test. One of the biggest drawbacks to latex agglutination is non-specific reactions with specimens such as urine, sputum, serum, and synovial fluid. False positive agglutination can sometimes be eliminated by heating the specimen to 60°C for 15 minutes. In one study[15], although CIE and latex were similarly sensitive for detection of *H. influenzae* meningitis, false positive latex agglutinations were observed in almost 20% of culture negative CSF's. The following reagents are commercially available for identification of colonies or detection of antigen: streptococci, Group A,B,C,D,F, and G, *Neisseria gonorrheae, Cryptococcus neoformans*, and *Haemophilus influenzae* type B.

Figure 2.

The competitive method of ELISA for assaying antigen.



Clinical applications

The ability of latex agglutination (LA) to detect cryptococcal polysaccharide in serum or CSF has been well documented[16] and is more sensitive than demonstration of the capsule of *Cryptococcus neoformans* by India Ink. [17] Rheumatoid factor may invalidate the test because of non-specific agglutination.

Newman et al[18] detected *H. influenzae* capsular antigen by LA in 27/29 CSF samples positive by culture. Ingram et al[15] showed that both CIE and LA tests were positive in 75% of patients with *H. influenzae* meningitis. Kaldor et al[19] studied 95 patients with purulent meningitis and 63 control patients with other diseases. With the exception of one urine specimen, none of the controls had a positive LA while 62/95 CSF specimens, 10/14 sera, and 11/17 urine specimens were positive by LA. Although these investigators experienced many false positive agglutinations initially, fractionation of antisera and the heating of urine to 100° C increased the specificity.

In a similar study, Leinonen and Herva[20] tested 103 CSF samples for *H. influenzae* (b) and *N. meningitidis* (A,C,D) by LA. They reported that LA was at least as sensitive as CIE. They had little success, however, detecting *N. meningitidis* Group B antigen in CSF by either LA or CIE.

Bromberger et al [12] reported in a preliminary study that Group B streptococci could be detected in body fluids more sensitively by LA than by CIE, especially in urine. For type specific antigens, however, CIE was more sensitive than LA. The authors stated that there was a greater risk of false positive tests with LA than with CIE.

Leinonen and Kayhty[22] compared CIE, LA, and RIA for detection of *H. influenzae* b and *N. meningitidis* A and C polysaccharide. RIA was consistently the most sensitive followed by LA and then CIE.

A latex agglutination inhibition (LAI) card test is commercially available for gentamicin assay in serum. The LAI test is based on the binding of a gentamicin-protein activated latex particle to a rabbit anti gentamicin antibody.

Standiford et al[23] and Doern et al[24] compared LAI results with RIA and a microbiologic method. Standiford et al[23] found that when measuring gentamicin at concentrations >2 μg/ml by LAI, the C.V. was >14%, compared with 15% for the bioassay and 12% for RIA. For those samples containing >2.0 μg/ml, the LAI C.V. ranged from 0-25%. Doern et al[24] found significant variability at all concentrations with LAI. Recent work in our laboratory (unpublished data) indicates that the test in its present form is accurate and reliable. Comparison with RIA revealed C.V.'s of <10%. Because of the dilution scheme, it is not surprising that accuracy and precision suffer when <2 μg of gentamicin is present.

The LAI test should be used primarily in small laboratories or as a "stat" procedure. Performance of more than 3 or 4 tests at one time is laborious. However, a peak and a trough level on a single patient can be done in 15 minutes or less.

CO-AGGLUTINATION

Kronvall[25] was the first to introduce the coagglutination technique for the detection of pneumococcal antigens. Most strains of *Staphylococcus aureus*, in particular, Cowan strain I, contain a cell surface protein, protein A. Antibody proteins (IgG) adhere to protein A by their Fc portion leaving the Fab ends free to complex homologous antigen (Figure 1). The presence of antigen results in the visible agglutination of the staphylococci. The reagent is stable at refrigerator temperatures and is used similar to a latex reagent.

Clinical application

Coagglutination (CoA) reagents can be potentially used to detect any antigen that can be detected by LA. Reagents for *N. gonorrheae*, streptococcus A,B,C,G, and *H. influenzae* are commercially available. Lim and Wall[26] correctly identified 77.8% of 308 clinical isolates of *N. gonorrheae* using the Phadebact (Pharmacia, Inc.) coagglutination reagents. 93.9% of non-*N. gonorrheae* strains were negative by this test.

Other studies have shown that CoA is the equivalent to or better than the capillary precipitin method for grouping of the β-hemolytic streptococci.

In an interesting variation of coagglutination, Essers and Radebold[27] described a test used for the identification of the staphylococci. Latex particles were coated with human plasma. This reagent containing fibrinogen and IgG reacted with both the clumping factor of *S. aureus* and protein A. The absence of these two constituents in *S. epidermidis* resulted in a negative reaction.

RADIOIMMUNOASSAY (RIA)

RIA is a technique which combines the specificity of immunology and the sensitivity of radiochemistry. The principal use of RIA is in endocrinology for the assay of hormones but all areas of laboratory medicine find RIA a useful tool. There are many variations of the competitive protein binding assay, another way to describe RIA. In most applications, however, labelled antigen (usually iodine$_{125}$) competes with unlabelled antigen for available binding sites on a specified amount of homologous antibody. After equilibrium between the bound and the unbound antigen is reached, the bound compound is separated from the unbound by centrifugation or precipitation. In some applications, the antibody is coated on tubes and separation of the phases is as easy as decanting the fluid in the tube.

Although microbiological research employs RIA widely, it is rarely used routinely in clinical microbiology laboratories except for the determination of aminoglycoside antibiotics in body fluids. Kits are currently available for gentamicin, tobramycin, amikacin, kanamycin, and vancomycin. The test results are reproducible, sensitive, and specific.[28] They provide results in 2-3 hours as compared with overnight for a standard microbiologic assay.

Kayhty et al[29] described an RIA procedure for detection of *N. meningitidis* (A,C) and *H. influenzae* antigens in CSF. In patients with *H. influenzae* meningitis, RIA detected antigen in 14/15 patients, with *N. meningitidis* (A), 18/23 patients, and in 2/4 patients with *N. meningitidis* (C). No false positive reactions were observed.

Although the RIA is both sensitive and specific, it has not been widely used in bacterial antigen detection primarily because of the cost of equipment, paucity of standardized reagents, and the general lack of familiarity with RIA on the part of many microbiologists.

ENZYME IMMUNOASSAYS

The search for methodology that has the advantages of RIA and few of the disadvantages culminated in the discovery of the enzyme immunoassay technique. Engvall and Perlman[30] first used the enzyme linked immunosorbent assay (ELISA) to measure rabbit IgG.

ELISA has wide application in clinical microbiology. The concept is similar to RIA. An antibody or an antigen is bound to a solid support, either a plastic tube, tray, or polystyrene beads. The complimentary substance forms an antigen-antibody complex. In the simplest procedure, an anti-species enzyme tagged antibody is added to the developing "sandwich".

After separation of the bound and free enzyme-tagged antibody (or antigen), enzyme substrate is added and the resulting color formation indicates the presence of either antigen or antibody.

Simply stated, there are two major types of enzyme immunoassay: the homogenous assay and the heterogenous assay.

Figure 2 describes the competitive homogenous immunoassay sometimes called EMIT. The substance to be tested, such as gentamicin is usually a low molecular weight compound. There is competition between enzyme labelled gentamicin and free serum gentamicin for binding sites on the specific antibody.

Figure 3 depicts antigen measurement by the ELISA double sandwich technique. The solid phase is coated with specific antibody. The antigen containing body fluid is layered over the sensitized solid phase to which is added an enzyme labelled antibody and substrate. For antibody determinations, the antigen is bound to the solid phase. The sera are incubated and the test antibody binds to the antigen. After washing, an enzyme-labelled antiglobulin and substrate are added.

Although ELISA methodology is not conceptually difficult, there are many variables to be controlled. In the author's experience, the identification and control of the variables is essential for a successful test. These include the enzyme used, the substrate, the reaction termination reagent, the solid phase, incubation times, antigen/antibody concentration, and visual *vs* spectrophotometric reading of results.

The clinical applications of ELISA are many. While ELISA has been used primarily for detection of antibody in the diagnosis of infectious disease (Table 1), antigen detection by ELISA promises rapid diagnosis of etiologic agents directly from body fluids. Table 2 summarizes antigen detection by ELISA.

Table 1. Microbial antigen detection by ELISA.

Aflatoxin B (Asperigillus)
Brucella abortus
Candida albicans
Epstein Barr viral capsid antigen
E. coli toxin (heat labile)
Hepatitis A
Hepatitis B surface antigen
Herpes simplex virus
Rotavirus
Salmonella typhi
Schistosoma mansoni
Staphylococcus aureus (enterotoxin)
Toxoplasma gondii
Vibrio cholera (enterotoxin)
Yersinia enterocolitica
Legionella pneumophila
Clostridium difficile toxin (Toxin A)

In clinical microbiology laboratories in the United States, ELISA is most commonly used for detection of rotavirus antigen in fecal specimens of both children and adults. A recent hospital epidemic of rotavirus infectious diarrhoea at the University of Connecticut Health Center was detected using ELISA. Twenty-five children on an acute care pediatrics floor were screened every other day for both the initial appearance of antigen as well as its disappearance. While there is no specific treatment for rotavirus infection, it was necessary to a) rule out other treatable causes of infectious diarrhoea, and b) monitor the course of the disease particularly in the immunosuppressed children. Alternatives to ELISA for detection of rotavirus are CIE and electron microscopy (EM). Our experience has been that CIE is less sensitive than ELISA and EM is more costly and time consuming.

Although commercial products are not yet available, ELISA for detection of bacterial polysaccharides in body fluids may supplant both CIE and latex agglutination. Pepple et al[31] described ELISA for detection of *Haemophilus influenzae* antigen in serum, urine, and CSF. They noted that the increased sensitivity of ELISA was particularly valuable in diagnosing partially treated meningitis. For example, of 15 CSF specimens obtained from 1-9 days after antibiotic treatment, 11 were positive for *H. influenzae* antigen by ELISA, and only 3 and 4 respectively by CIE and latex agglutination.

The major disadvantage of ELISA for antigen detection is the time required for the test. Work is currently in progress in many laboratories to shorten the processing time.

Some clinical microbiology laboratories are responsible for determining the concentration of antibiotics in body fluids, usually serum. In the broad sense, antibiotics may be considered "antigens" although not always of microbial origin. Competitive protein binding assays such as EMIT have become popular for detecting the aminocyclitol antibiotics as well as drugs of abuse or for the therapeutic monitoring of drugs such as digoxin and theophylline.

Serum containing an unknown concentration of gentamicin is added to anti-gentamicin antibody and glucose-6-phosphate dehydrogenase-labelled gentamicin. Both enzyme-linked gentamicin and free gentamicin compete for binding sites on the antibody. Free, unbound, gentamicin-enzyme complex is measured by the addition of the substrate, glucose-6-phosphate. The reaction is followed by monitoring the reduction of the cofactor, NAD, at 340nm. Syva (Palo Alto, CA) reports no interference with other antibiotics and correlation with

Figure 3.
Double antibody sandwich method of ELISA for assay of antigen.

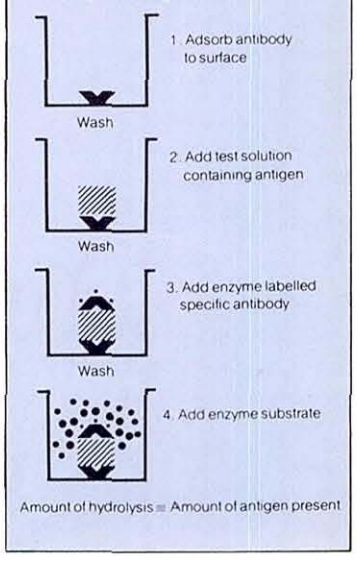


Table 2. Antibodies assayed by ELISA.

Autoimmune Diseases
DNA
Thyroglobulin

Bacterial Infections
Salmonella
Yersinia
Brucella
Vibrio
Ricktsiae
Legionella
Corynebacterium (toxin)
Escherichia (enterotoxin)
Mycoplasma pneumoniae
Streptococcus, Group A, M protein
Clostridium tetani (toxin)
Mycobacterium

Fungi
Aspergillus
Candida

Parasites
Echinococcus
Onchocerca
Plasmodium falciparum
Schistosoma mansoni
Toxocara canis
Toxoplasma gondii
Trichinella spiralis
Trypanosoma

Virus
Adenovirus
Arbovirus
Coxsackie virus
Cytomegalovirus
Hepatitis A
Herpes simplex 1,2
Influenza A,B
Rubella
Measles
Mumps
Rabies
RSV
Rotavirus

RIA from 0.879 to 0.973. Coefficients of variation are reported as ≤15%.

Similarly, fluorescent immunoassay (FIA) systems have not been used yet for detection of microbial antigens but have become increasingly popular for therapeutic drug monitoring. The major difference between FIA and either ELISA or EMIT is that the indicator system is fluorescent. A fluorescent response may be achieved by directly determining the extent to which fluorescent labelled antigens compete with non-fluorescent antigens for binding sites on an antibody or by the use of fluorescent substrates for alkaline phosphatase or β-galactosidase. These high energy substrates do not necessarily increase the sensitivity of an immunoassay procedure. Sensitivity, after all, is a function of the antigen-antibody response. The use of fluorescent substrates, however, markedly reduces the time necessary to achieve the sensitivity limits of the test. Two hour ELISA incubations can be diminished to minutes with fluorescent detection methods. The high energy substrates may allow immunoassays to compete in the same "time to detection" frame as CIE, latex agglutination, and coagglutination.

A fluorescent immunoassay (FIA) kit is commercially available for aminocyclitols. The principle underlying this test is that free antibiotic competes with antibiotic coupled to a fluorogenic substrate for antibody binding sites. An enzyme, β-galactosidase, cleaves the unbound fluorescent substrate and fluorescence is measured at an excitation wave length of 400 nm and an emission wavelength of 450nm.

Ngui-yen et al[32] evaluated RIA, FIA, and EMIT for measurement of gentamicin and tobramycin in serum. They reported that all 3 systems were accurate and precise.

The FIA, however, gave significantly lower values for both aminoglycosides at concentrations <5.0 μg/ml. Phaneuf et al[33] showed similar results when EMIT was compared with RIA. Yet another study by Ratcliff et al[34] compared RIA, microbial assay, adenylation, EMIT, and FIA. Although all systems were acceptable, they indicated that RIA was the preferred test with regard to accuracy, specificity, rapidity, and simplicity.

The basis of the TDX test (Abbott) is a fluorescence polarization immunoassay (FPIA). FPIA like EMIT and FIA is a competitive protein binding process. A gentamicin molecule is labelled with fluorescein. There is competition for antibody binding sites between fluorescent and untagged gentamicin. In FPIA, the binding of fluorescein-gentamicin to antibody is measured directly by determining its fluorescence polarization. The polarization of the fluorescent signal is directly proportioned to the amount of labelled gentamicin bound and inversely proportional to the unlabelled antibiotic. Jolley[35] reported that the correlation between automated FPIA and RIA ranged from 0.958 to 0.979. Coefficients of variation are 1-4%.

Another fluorescent immunoassay (FIAX) system is better known for determination of antibodies to infectious agents such as Toxoplasma, Rubella, Cytomegalovirus, and Herpes. FIAX is a solid phase fluorescent immunoassay and like the others involves competition between labelled and unlabelled gentamicin for binding sites on an antibody bound to a solid phase polymeric surface (StiQ). The solid phase resembles a paddle. In operation, the paddle, or StiQ, is immersed sequentially in fluorescein labelled gentamicin and the serum sample and then placed in a desiccated fluorimeter. The amount of labelled gentamicin that binds to the antibody is inversely proportional to the amount of antibiotic in the serum.

Bruckner et al[36] compared the FIAX assay of tobramycin with RIA. The correlation was 0.93 but the FIAX consistently underestimated the concentration of tobramycin in serum. The advantages of FIAX are that it is faster than RIA and alleviates radioactive waste disposal. Like RIA, a standard curve must be set up for each run. This is not compatible with "stat" testing. On the other hand, with EMIT, FIA, and FPIA, the standard curve is good for 24 hours.

The quality of reagents is still a major obstacle to sensitive and specific immunologic probes for bacterial antigens. However, the discovery of monoclonal antibodies, novel tags for immunoassays, and automated instrumentation pose the real possibility that immunologic techniques for rapid disease detection will be in the forefront of clinical microbiology for the next decade.

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Microbes and Cosmetics

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Almost a quarter of a century ago Baker[1] referred to bacteria as "that unwanted cosmetic ingredient", when he described several examples of cosmetic products which had been ruined by microorganisms. At this time the cosmetic industry was aware of the potential problems attendant upon the ever increasing production of cosmetics. Because products which contain a wide range of ingredients, many of which are susceptible to microbial attack (Table 1), are subject to many possible sources of contamination (Table 2) in a manufacturing environment. It would, however, be true to say that attention was directed only to products which gave trouble and those formulations which appeared to be stable and adequately preserved usually escaped microbiological investigation.

This philosophy, which had largely been shared by pharmacists in respect of non-sterile medicines, was challenged by the findings of Kallings et al[2] of the State Bacteriology Laboratory in Stockholm. They performed bacteriological examinations of a wide range of pharmaceutical preparations not officially required to be sterile, such as tablets, ointments, hand creams, baby creams, liquid medicines, ear and nose drops, baby powders and suppositories. Not only were all of these preparations found to be contaminated but severe eye disorders (including enucleation) were attributed to contaminated cortisone ointment while contaminated thyroid tablets were shown to have caused salmonellosis in over 200 people. The publication of these findings marked the beginning of a concern for, and intense interest in, the microbiological status of pharmaceuticals, cosmetics and toiletries. There followed a series of international surveys, which in America resulted in the recall[3,4] of cosmetics contaminated with organisms as potentially dangerous as pseudomonads and staphylococci.[5] Jarvis et al[6] reported that Gram-negative organisms predominated in high count products.

In contrast to the infections from pharmaceuticals cited by Kallings

Table 1. Examples of cosmetic ingredients which are assimilated by organisms.

Liquid paraffin	Cetostearyl alcohol	Stearic acid
Soft paraffin	Polyethylene glycol	Oleic acid
Vaseline	Propylene glycol	Silicones
Waxes	Glycerol	
Natural gums	Esters	Preservatives

Table 2. Sources of microbial contaminants.

Water	Low demand Gram-negative groups - Pseudomonas, Xanthamonas, Flavobacterium, Achromobacter
Air	Mould spores - Penicillium, Mucor, Aspergillus Bacterial spores - *Bacillus* sp. Yeasts Micrococci
Raw Materials - earths - pigments - starches - gums	Anaerobic spore formers - *Clostridium* sp. Salmonella *Coliforms* Actinomyces
	Also miscellaneous yeasts and moulds
Personnel	Coliforms Staphylococci Streptococci Corynebacteria

et al[2] there is little evidence of contaminated cosmetics causing problems. Where products have been alleged to result in adverse reactions examination has not indicated high microbial counts or the presence of pathogens.[7] This, of course, is not an unexpected state of affairs, because in a situation where cosmetics are applied to the intact skin of healthy individuals, it is, perhaps, only those preparations used to make up the eyes which present a significant hazard. However, it would be unduly complacent to tolerate potentially pathogenic organisms in cosmetics on the grounds that no serious infections had been recorded to date.

The development of microbial standards

A reasonable approach when proposing any standards for cosmetics and toiletries is that they should distinguish between the preparations which have a microbial flora quantitatively similar to that of the environment and those which contain numbers of organisms exceeding the normal environmental contamination.[8] Guidance issued by the Council of the Society of Cosmetic Scientists of Great Britain (1970)[9] on the hygienic manufacture and preservation of toiletries and cosmetics recommended that "relevant known pathogenic microorganisms be absent from cosmetics and that those preparations intended for use on newborn infants, for direct instillation into the conjunctivalsac or for use on broken skin, should be sterile at the time of manufacture". This type of recommendation, which essentially demands the absence of pathogens from cosmetics, can be a source of controversy in respect of the named pathogenic organisms. The genera proposed by the Society of Cosmetic Scientists were Clostridium, Salmonella, Pseudomonas, Escherichia, Klebsiella, Proteus, Streptococcus, Shigella and coagulase-positive Staphylococcus. An alternative type of standard is one which defines the maximum number of organisms permitted in a given volume or weight of a cosmetic product. Thus, guidelines published by the Cosmetic, Toiletry and Fragrance Association (CTFA) of America in 1973 proposed a general numerical limit for all cosmetics of 1,000 organisms per g or per ml and 500 organisms per g or per ml for baby products and those for ophthalmic use.

Figure 1. Typical modern packs of cosmetics.

high manufacturing costs, which in turn adds to the price of cosmetics, for safety never comes cheap.

Figure 2. Mould contamination of cosmetic face cream.

The EEC Cosmetics Directive 1976, has indicated that microbiological standards are to be agreed as part of the programme of work, but as yet no proposals have been published. The gap between the proposing and the enforcing of standards will remain until microbiological techniques are available which will fully satisfy a court of law and be reasonably economic to apply. The development of rapid methods and automation in the detection, enumeration and identification of microorganisms should have a marked influence upon the problem together with the refinement of radiometric screening, flow microcalorimetry and electrical impedance monitoring.[10]

Conclusions

The production of cosmetics is now a worldwide industry, with an enormous financial turnover and a vast consumer consumption. Cosmetic science has reached the high level of sophistication required to meet the many problems created by complex formulations in terms of stability and safety in use. The hygienic manufacture of cosmetics is receiving increased attention and techniques such as gamma-irradiation, applied to raw materials to reduce indigenous microbial flora,[11] together with the integrated use of preservatives [12] and protective packaging are allowing high standards of safety to be achieved. The obvious corollary of this approach is that of

Acknowledgements

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