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Microbiological control in the dairy industry

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The dairy industry of Britain and other developed countries has undergone a technological revolution since 1945. The most important changes have been:

1) the merging into large units for both town dairies and manufacturing creameries
2) bulk collection of milk from farms and greatly improved refrigeration throughout the industry
3) perfection of heat-treatment methods for milk and manufacturing methods for dairy products
4) cleaning-in-place (CIP) for equipment
5) mechanisation, automation and computer control of processing.

As examples of modern dairy technology, Figure 1 shows the processing room of a pasteurising dairy, and Figure 2 the computer control room of an ice-cream factory.

These changes have brought about modifications in microbiological control systems, and there has been a change of emphasis from certain groups of bacteria to others. Whereas formerly the problem was to prevent milk from souring, especially in summer, it is the growth of, and enzyme formation by psychrotrophic bacteria which can grow, although slowly, in milk at 5°C which have created new problems for the industry.

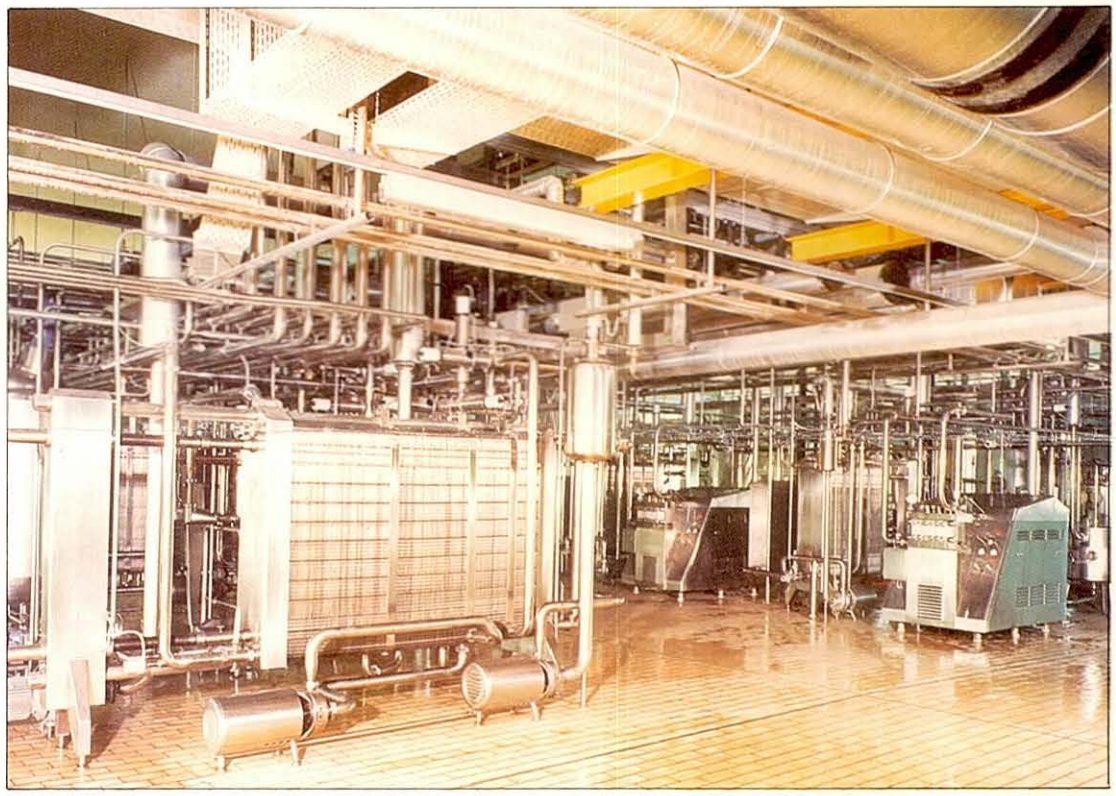

FIGURE 1. The processing room of a pasteurising dairy.

The philosophy of microbiological testing

All tests in the dairy industry should be orientated to the requirements of the product, which are primarily safety and good keeping quality. Some dairy products may be regarded as selective media for the culture of particular types of organisms, for example lactic acid bacteria in Cheddar cheese and yogurt, moulds in Camembert and Stilton cheese, and yeasts in Kefir. In some there is a sequence of flora, one type being succeeded by another. In other products the objective is sterilty or a count as low as possible. The outlook in dairy laboratories thus differs from academic and medical laboratories. Whilst the fundamentals of microbiology are the same for all, there are many differences in the emphasis given to particular aspects of laboratory testing.

Microbiological control of incoming raw materials, processing in the dairy or creamery, and distribution and sale of all products especially of perishable products such as milk, cream, yogurt and Cottage cheese, is essential in an efficient dairy or food organisation. The high quality in dairy products to-day has only become possible because of the evolution of control systems based on the experience of many years, and systematic laboratory testing.

Standard methods in the dairy industry

For many years various organisations all over the world have been publishing standard methods for the laboratory examination of dairy products. Discussion on an international scale is proceeding in an endeavour to reach uniformity.

There is a need for both rapid screening tests for identifying bad samples, and standard reference methods for grading, and official and legal purposes. The former are largely selected by individual countries, and the latter may differ from the recognised medical, public health, veterinary, etc., methods because the nature of the product may require modification of the method.

Keeping quality of retail milk: pasteurisation

Milk is an excellent medium for bacteria and is therefore one of the most perishable of foods. Practically all milk sold in the U.K. is pasteurised, although a few people prefer raw milk and run the risk of contracting undulant fever. Bovine tuberculosis has been eliminated, and to-day our milk is probably the safest of all highly perishable foods.[1] Pasteurisation, which to-day is universally carried out by the high-temperature short-time (HT-ST) method of at least 161°F (72°C) for at least 15 seconds, kills about 99% of the bacteria in raw milk including all the common pathogens and most of the organisms biochemically active in milk. Good pasteurised milk, protected from re-contamination, should keep for a week in the domestic refrigerator (3°C).

The control of pasteurised milk is based on the phosphatase test and a specific form of the methylene blue test.[2]

Organoleptic examination or sensory assessment: the thermoduric count

Examination of milk for odour and taste is the most sensitive test available, far more sensitive than any microbiological test. All foods should be examined first by these subjective tests, as they will sometimes detect taints not detectable by any ordinary chemical or microbiological test.

In spite of the excellence of modern hygiene in milk production and processing, taints sometimes occur and one which affects pasteurised milk in hot weather is not souring, as formerly, but a "dirty" taint. This is usually caused by thermoduric cocci such as *Sarcina lutea* as the result of failure to clean equipment properly, and the build-up of a thin layer of slime.

Apart from such taints, a thermoduric count is a useful test for pasteurised milk. It is made following an ordinary plate count by heating the sample at 63°C for 35 minutes and then plating again. The ratio of the thermoduric count to the total count is a measure of efficiency in cleaning equipment; the higher the ratio the less efficient is the cleaning. If milk comes into the dairy with a high thermoduric count, the dairy cannot deliver a good milk by efficient pasteurisation.

Ultra heat treated (UHT) milk

Any given degree of kill of bacteria in milk can be achieved by selecting a combination of temperature and time of treatment. The higher the temperature the shorter is the time required and the less drastic the chemical effect on the milk. To achieve sterility or a complete kill, a temperature of 121°C for 15 minutes is required, but at 270°F (132°C) sterility is achieved in about 1 second with chemical or nutritional damage only slightly more drastic than with pasteurisation. This method, called "ultra high temperature", is now legal and a simple colony count test is prescribed for control purposes.[3]

The effect of temperature and time of incubation of plates

Bacteriological control methods for milk were derived from water bacteriology in this country, and the plate count and coli tests became standard in the industry. Incubation of plates etc., for 1 – 2 days at 37°C is a general practice in medical and public health laboratories, but this technique is not suitable in dairy microbiology. Counts at 27 – 30°C are often 10 times higher than at 37° and occasionally can be 100 times as high. This aspect has become all the more important recently with the improvement in refrigerated storage and transport of milk. Holding raw milk in silos at less than 5°C for 24 – 48 hours results in an increase in the proportion of psychrophilic and psychroduric bacteria, many of which grow poorly above 27°C and some not at all at 37°C.

The value of dual media

In microbiological control work for special purposes most time and effort are spent in procuring the sample and preparing the dilutions etc., in the laboratory. It requires very little extra effort to plate a product in two media, a procedure which has several advantages. Accuracy of counting is improved, contaminants can be identified, and much more

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information about the identity and biochemical properties of the organisms is automatically obtained. Suitable "pairs" of media are lactose litmus and a caseinate or tributyrin agar for total counts, mannitol salt and egg yolk tellurite agar for staphylococci, Rose Bengal and an osmophilic agar for yeasts, and desoxycholate citrate and bismuth sulphite agar for salmonella.

Another form of the same principle is to use the same (preferably differential) medium at two temperatures, for example at 27 and 43°C or to incubate duplicate plates under aerobic and anaerobic conditions, or to use *both surface culture and double*-pour plates.

The usefulness of differential media

One can safely assert that if an organism is not pathogenic and is biochemically inert in a product, then it can usually be ignored. In practice we are only concerned with organisms causing disease or affecting the product, for example by clotting milk, producing rancidity in butter or yeast taint in cheese.

Although the conventional media such as milk agar and glucose tryptone agar give adequate total counts, they give little information about the nature of the colonies. Microbiologists in the industry use the colony count test for assessing the general quality of products, but little attempt has been made to exploit the value of a simple differential medium as a first step to solving the problems of faults in dairy products. The most useful properties to use for identifying the genera of organisms probably responsible for such faults are colony type, pigment, catalase, oxidase, lactose fermentation, glucose fermentation and growth at 10 and 45°C. Together with cell morphology, Gram stain reaction and heat resistance, these properties not only enable the genus to be determined with a fair degree of accuracy but a knowledge of these properties indicates the probable effect of the organism on any particular dairy product. The use of a differential medium involves a negligible increase in cost and gives very useful additional information to any bacterial count. A suitable differential medium has been described by Donovan and Vincent.[4]

The most important biochemical properties of organisms in dairy products are fermentation of lactose, lipolysis and proteolysis. The last two are well correlated, so that tributyrin agar is a useful medium for identifying organisms attacking fat and protein, although it does not follow that an organism hydrolysing tributyrin can also attack milk fat. Stability of the emulsion in a fat medium can be achieved by the incorporation of an emulsifier such as polyoxyethylene-(20)-hydrated castor oil.[5] Miniaturised test schemes for identifying bacterial genera have been described.[6]

The use of alternative dilutions

The dairy microbiologist is sometimes called on to evaluate products such as butter or cheese of which the microbiological quality and expected counts are unknown. Several dilutions are then necessary in order to make certain of a countable plate, and if various types of organism are to be enumerated, the total number of plates and quantity of media, and incidentally the amount of bench space and incubator capacity required can become considerable. All these can be halved by the simple device of testing only alternate dilutions. For example, if the 1st, 3rd, 5th and 7th dilutions are plated, the four plates would cover a range from less than 10 to 3×10^9 colonies per gram. Bearing in mind the inherent error of the method, a countable plate can always be obtained. Some workers attach a fictitious accuracy to plate counts.

Naturally if one is only working to a standard, for example, not exceeding 10,000 per ml, then only one dilution (the 1/100) need be plated.

Dye reduction tests

The total count and coliform tests for raw and pasteurised milk were replaced by a methylene blue test in 1936, and in the 1939-45 war the reazurin test was developed and used for rejection of unsatisfactory milk (10 minute test) and grading (1 hour – now 2 hours test). Dye tests measure the total biochemical activity in the milk and so are better correlated with souring etc., than are bacterial numbers. They are quicker and cheaper than colony count tests but give no information about types.

The direct microscopic count for milk

For industrial quality control purposes, speed in obtaining the result of a laboratory test is of the greatest importance. Unlike chemical analyses, microbiological tests such as the colony count normally take 2 days although special techniques may reduce this to 24 hours or even less. The only test comparable in speed with rapid chemical tests is the direct microscopic count, which is virtually impossible for solid products, and limited for milk to those of high count. Other drawbacks are the impossibility of distinguishing living from dead cells, and the clumping of cells.

However, Cousins *et al*[7] have used a technique involving the use of a protease and a surfactant followed by membrane filtration to give a count in about 30 minutes using acridine orange staining and epi-fluorescent illumination. With this, actively growing cells fluoresce orange-red and inactive cells green.[8]

Inhibitory substances (penicillin etc.) in milk

The development of antibiotics after 1945 led rapidly to improved methods for treating bovine mastitis, but it had one unexpected consequence. Cheese-making literally came to a standstill in some regions in Britain *ca.* 1950 because the concentration of penicillin in bulk milk was sufficient to prevent normal starter growth. A weak starter nearly always leads to poor cheese, and the loss can amount *to vast sums. The Milk Marketing* Board and the dairy companies corrected the position by organising a comprehensive control system with heavy penalties for farmers who delivered milk containing antibiotics. In addition to preventing the growth of cheese starters and the organisms in yogurt, penicillin can produce an allergic reaction in some persons.

Food poisoning

Food poisoning from dairy products *as such* is rare in the U.K. today. Spray-dried milk powder and cheese were formerly responsible for some outbreaks, but the heat-treatment applied to all raw manufacturing milk today has virtually eliminated the possibility of food poisoning. When it does occur it is usually caused by post-processing contamination, or the incorporation of milk, etc., in compounded foods, which make the food a good medium for staphylococcal growth.

Standard methods for detecting or enumeration of pathogens in dairy products have been issued by the International Dairy Federation[9] and other organisations.

Microbiological tests on dairy equipment

Virtually all sanitising (cleaning and "sterilising") of equipment is carried out today by cleaning-in-place (CIP) methods involving flow in pipes and spray devices for tanks, and using solutions (apart from halogens) at high temperatures. Properly used, such methods can be very effective but unsuspected reservoirs of bacteria can cause persistent trouble. Systematic testing of the hygienic condition of equipment is therefore essential.

The commonly used test for measuring "sterility", or more accurately the bacteriological condition of dairy equipment, is the swab test in which a cotton wool or alginate swab is used over 1 sq ft (about 900 cm²). After dispersal in 20 ml Ringer solution, 1 ml is plated as for milk. The count should be equivalent to less than 1000 per sq ft. Although sides of tanks, etc., are commonly swabbed this is of limited use, because microbiological problems usually arise from contaminated crevices, joints, dead ends, gaskets, etc. Swab tests *should therefore be concentrated* on these.

A very useful and simple device applicable to milk, cream, etc., equipment is to test the 1st, 100th and 200th containers (bottles or cartons) filled. The first contains the residual organisms not removed or killed by the CIP treatment so that the difference between the 1st, 100th, etc., is a measure of the efficiency of sanitation of the equipment. This is a sound and easily performed test because it measures *all* that part of the equipment which extends from the exit of the pasteuriser or steriliser to the filler. If this is sterile then the bacteriological condition of milk, etc., in the first container filled is no worse than that of the subsequent samples. Usually it is worse, and the difference is a measure of the extent of the contamination.

The first batch of bottles, etc., filled should always be taken off and the milk tipped back into the raw milk tank.

Containers

Although glass bottles are the source of most troubles in the liquid milk industry because of misuse by the public, they are still in general use and new dairies are installing bottling equipment. On the Continent there is a marked trend towards non-returnable paper or plastic containers. The glass bottle persists in the U.K. because we have a universal household delivery system for milk, and the housewife prefers the bottle to cartons and sachets. This necessitates a cleaning process and can introduce hygienic problems. A rinse test is used with a standard of not more than 200 colonies per bottle.

Cream

Fundamentally cream is simply fat-enriched milk, but from the point of view of microbiological control there is a significant difference. Whereas milk is pasteurised one day, delivered to the home next day and usually consumed by a day later after storage in a refrigerator, the distribution system for cream is different. As a luxury food the demand is highest at weekends and certain holidays, with the result that it is kept for longer than 1 day. In addition cream is often processed by a large dairy, sent first to a retail distributor and sometimes on to a shop. Thus times and temperatures are not controlled with the result that cream is often much worse than milk bacteriologically when bought. The Public Health Laboratory Service has devised a modified methylene blue method as a screening test for cream involving holding the sample at 20°C for 17 hours and then performing the MB test at 37°C when it should last for 4 hours. This is useful as a screening test for bad cream, but unfortunately some health officials regard the test as being absolutely reliable and having a definite public health significance, which it does not. Some harmless bacteria can grow in cream at 15°C over 2 or 3 days and reduce methylene blue rapidly after 17 hours at 20°C, and some bacteria form spores which survive pasteurisation and similarly *grow at 20°C and reduce the dye.* The MB test should be regarded *only* as a screening test, and failures followed by appropriate tests to identify the types responsible and the cause, e.g. bad raw milk, cream too old, failure to store at 5°C.

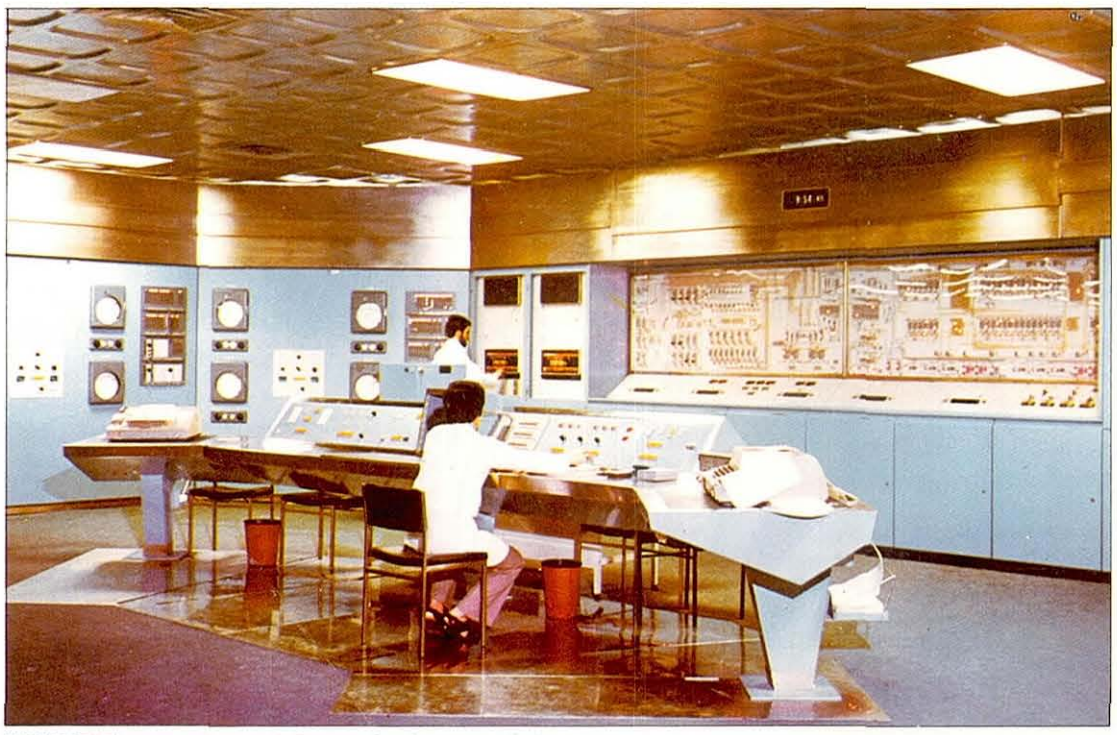

FIGURE 2. The computer control room of an ice-cream factory.

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Legionnaires' disease

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In 1976 the 58th Annual Convention of the American Legion, Department of Pennsylvania was held in Philadelphia from July 21st – 24th. By July 22nd the first case of what was to be known as Legionnaires' disease occurred. Over the next few days many cases occurred so that by August 3rd 149 of those attending the convention had fallen ill. The final count was 182 cases some of which were not associated with the convention, but including some who had been in or near the hotel used as convention headquarters.

This point-source epidemic illness was characterised by an incubation period of 2 – 10 days followed by malaise, muscle pains and headache. A rapidly rising fever developed within the next day accompanied by rigors and cough. Patchy pulmonary consolidation progressed to extensive pneumonia. This severe illness was fatal in 29 cases.

The search for a cause

Such a widespread and severe illness prompted a brisk response in those responsible for the surveillance and control of disease. Extensive investigations[1] revealed no cause attributable to known infective agents. However, when guinea-pigs were inoculated intraperitoneally with lung tissue from fatal cases in a search for rickettsiae[2] they developed fever and prostration.

Following the classical route of rickettsial culture, the spleens of infected guinea-pigs were inoculated into the yolk sac of embryonated hens eggs. After several days the embroyos died and smears of yolk sac were prepared and stained by the method of Giménez.[3] These smears showed the presence of short bacilli with some filamentous forms.

Culturing the organism

After investigating many culture media, the Gram-negative bacilli were found to grow on Mueller Hinton agar supplemented with 1% haemoglobin and 1% Isovitalex, after 4–5 days incubation at 37°C in an atmosphere of 5–10% CO_2. Subsequently organisms were isolated by direct inoculation of this medium with lung from fatal cases. A clear agar medium was developed with haemoglobin being replaced with ferric pyrophosphate and cysteine being substituted for Isovitalax.[4] On this medium the Legionnaires' disease bacterium (LDB) forms a brown diffusible pigment which fluoresces under Wood's light. This is a useful feature in view of the lack of biochemical reactions which may have been used to characterise the organism. LDB is a new species with a unique fatty acid profile[5] and different, in DNA homology experiments, from all bacterial species so far examined.

Seriological studies

Although some of the fatal cases of Legionnaires' disease were shown, by isolation of the organism from post-mortem material, to be due to infection with the LDB the majority of cases were not fatal and were only shown to be due to infection with the LDB by the use of serological methods.

Patient's serum was examined in an indirect fluorescent antibody test using infected yolk sac as the antigen. This test showed[2] that of 111 patients from whom suitably timed specimens were taken, 62 showed the development of antibodies and 39 the presence of antibody at a titre of ≥128. Antibodies were uncommon in sera from healthy persons or patients with pneumonia or psittacosis.

So far, the IFA test is the only serological test showing a good correlation with disease. The more conventional tests such as agglutination or complement fixation have not so far proved successful although a micro-agglutination test has been described.[6]

Further investigations

Once a serological test became available, sera from other outbreaks of pneumonia were examined. LDB was identified as the cause of outbreaks in St. Elizabeth's Psychiatric Hospital, Washington DC in 1965,[7] Pontiac, Michigan in 1968,[8] in a group of Scottish tourists to Spain in 1973,[9] in an Oddfellows Convention in Philadelphia in 1974[10] and in a number of outbreaks since 1976, most of which have occurred in the summer.

The Pontiac outbreak affected employees in an air-conditioned building. However, employees were not infected when the air conditioning plant was not operating. Caged guinea-pigs left in the building developed infection showing that the infection was airborne and investigation revealed a faulty air conditioning plant that could have been contaminated with dirty water. In Bloomington, Indiana the LDB was isolated from the cooling tower water of an air conditioning plant serving a student hostel where an outbreak occurred.[11] Hence one type of point source has been identified.

Many sporadic cases have occurred, both in the U.S.A. and

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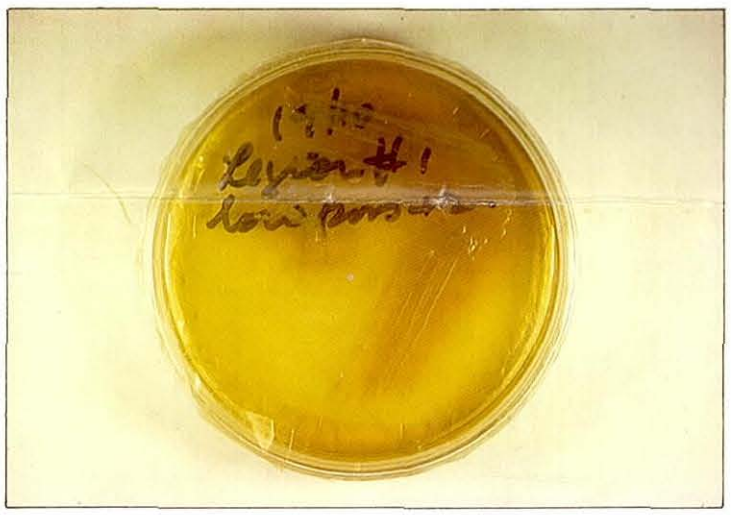

FIGURE 1. Culture of *L. pneumophila* on FG agar, showing brown pigment production.

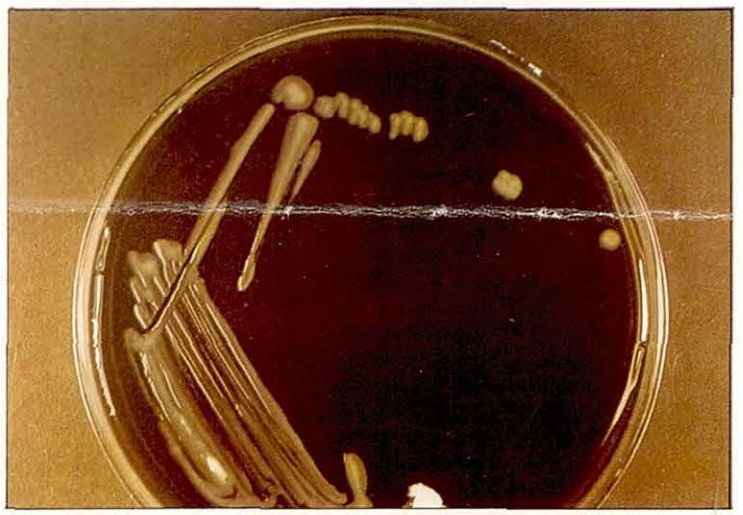

FIGURE 2. Culture of *L. pneumophila* on CYE medium.

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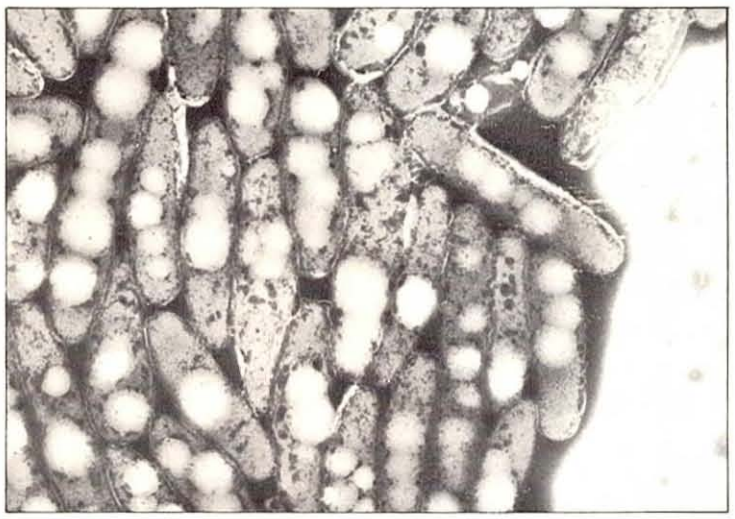

FIGURE 3. *L. pneumophila* negatively stained with 3% potassium phosphotungstate x 15,000. This shows cytoplasmic "granules" (courtesy of Dr C R Madeley).

also in Britain. Their origin is unknown but a considerable proportion of British cases have been associated with overseas travel, especially to Spain. Using the IFA test with agar-grown LDB as antigen we have examined over 800 sera at Ruchill Hospital coming from over 500 patients. The Centre for Disease Control, Atlanta, identified seven patients – six Scots and one from England with antibodies due to disease occurring before 1978. We have identified a further 46 sero-positives (34 confirmed to date by CDC) and one fatal case showed LDB in her lung by direct FA examination. Of the 46 cases, 29 were Scots, 2 Belgians and 15 cases were in hospital in England. Sixteen individuals had fallen ill in Spain, or shortly after returning from that country.

Antibody responses

A variety of antibody responses have been seen – fatal cases with no antibody or high levels, non-fatal cases with an initial high titre of antibody and others who developed antibody only after 3–4 weeks of illness. Antibody has been found in some individuals with no history of illness or with a disease where there was a rising antibody titre to other pathogens such as psittacosis or *Mycoplasma pneumoniae* as well as in some almost certain cases four or more years after a severe respiratory illness. Hence a high titre in a single serum may be difficult to interpret even accepting CDC criteria that a titre ≥ 256 is necessary before it is accepted as likely evidence of infection at some time. As with most diseases a rising titre is the best evidence of infection.

Serotypes

All tests so far have been performed using antigen of a single serotype. However, recent evidence[12] pointed to the likelihood of there being more than one serotype and this was confirmed at the International Conference on Legionnaires' disease held in Atlanta on November 13–15, 1978. There are, currently, four known serotypes[14] of what has tentatively been named *Legionella pneumophila*.[15] Type 1 is the original type which caused the Philadelphia outbreak, type 2 is the Togus[12] strain. Type 3 was isolated at Bloomington, Indiana[11] and type 4 was isolated at Wadsworth Hospital in Los Angeles. This means that for complete coverages of the presently known possible infecting strains, four antigens (in the absence of a "group" antigen) will have to be used in the IFA test. In view of the diversity of serotypes it is important to attempt to isolate strains causing disease in order to define whether other new serotypes are important in the U.K. (or other country as appropriate).

Choice of specimens

What sort of specimens are worth looking at and how should they be dealt with?

There is no evidence, as yet, that specimens other than those from the respiratory tract are of value for isolation studies. The possible specimens are sputum, trans-tracheal aspirates, bronchial aspirates or sputum by direct immunofluorescence. Another exciting possibility for rapid diagnosis is the finding of antigen in the urine of cases[16] by use of the ELISA technique.

Obviously although remarkable progress has been made since the Philadelphia outbreak of 1976, much remains to be discovered about Legionnaires' disease and *Legionella pneumophila*. The important facts to remember are that this severe disease exists and appears to respond well to adequate (i.e. parenteral) erythromycin therapy[1] (although the organism is very sensitive to rifampicin *in vitro*[13]) whereas other antibiotics may not be as effective.

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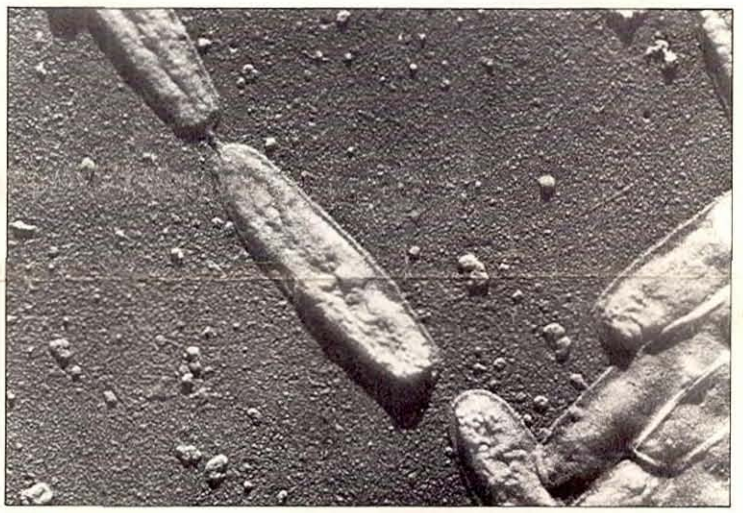

FIGURE 4. *L. pneumophila* shadowed with gold palladium x 15,000, showing dividing bacilli which have not quite separated (courtesy of Dr C R Madeley).

washings, pleural fluid and lung obtained either by biopsy or at autopsy.

As selective media are still under development, the ideal specimen would be sterile on culture on ordinary media and hence suitable for direct inoculation into the yolk sac of embryonated hens' eggs or on to enriched Mueller Hinton or other media. Hence pleural fluid, lung biopsy or transtracheal aspirates could be cultured using these methods, whereas guinea-pig inoculation would be appropriate for potentially contaminated material such as lung obtained at autopsy, sputum or bronchial washings. Spleen, peritoneal exudate, liver and lung from infected guinea-pigs may then be inoculated into eggs or on to culture medium.

At the present time the most sensitive culture 'medium' is the egg. The newly developed charcoal yeast extract (CYE) agar (Feeley and others, in preparation) is, perhaps, more sensitive than the guinea-pig. Gram-negative rods which grow on enriched Mueller Hinton agar, F-G or CYE agar but not on blood agar are examined for pigment production and also by direct immunofluorescence staining. In addition organisms may be visualised in lung tissue and possible transtracheal

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