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DETECTING WILD YEASTS IN BREWERIES

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Yeasts are an important group of micro-organisms that occur as contaminants in breweries. The term 'wild yeast' generally means any yeast not used intentionally and not under full control. It follows, therefore, that brewing yeasts themselves may become 'contaminants' within the brewery. This complicating situation can arise, for example, in breweries operating with more than one brewing strain, such as separate ale and lager yeasts, where undesirable cross contamination can occur. The definition also includes many yeasts that are unable to grow extensively during beer processing and as such are relatively harmless; they merely serve as indicators of general hygiene and raw material problems. Wild yeasts take on greater significance where they are capable of product spoilage which may take the form of haze (cloudiness), deposits and sediments, off-flavours and excessive carbon dioxide formation. This paper is concerned only with conventional culture methods for detecting wild yeasts in brewery quality control and does not include alternative procedures such as immunological or staining methods which are often used alongside cultural methods.

Wild yeasts of many different genera can be isolated from within the brewery. However, it is convenient to allocate them to one of two groups, in addition to brewing strains, namely: **i**) non-brewing Saccharomyces and **ii**) yeast genera other than Saccharomyces (**Table 1**). The significance of this classification is that wild Saccharomyces species give far more problems with spoilage than the non-Saccharomyces. Being able to make this classification, and especially the choice of media, depends a great deal on local circumstances and the type of processing the beer has undergone.

General methods

One of the more straightforward situations is the monitoring of beer pasteurisation, where the heat processing regimen is designed to inactivate all organisms carried through in the beer. Any yeast surviving pasteurisation, whether it be brewers yeast or wild yeast, serves as a warning of process failure and therefore all that is required is a non-selective method for detecting yeasts in general. Many general purpose media will suffice in this case, of which WLN agar (Wallerstein Laboratories Nutrient agar; Oxoid CM309/501) is commonly used, albeit often in a modified form, e.g. pH-adjusted. WLN sometimes has the useful effect of differentiating strains of yeast on the basis of colony staining[1] (see example in **Figure 1**), though this is by no means always the case and is therefore only of supplementary value.

The reliability of modern filters and pasteurisers almost invariably means the brewery microbiologist is seeking to detect low numbers of contaminants in heat-processed or sterile-filtered beer at packaging. A large sample volume of beer (typically 1 to 10 litres) must be membrane-filtered before incubation on nutrient media to achieve a realistic quantitative estimate of contamination. After a suitable period of incubation (2 to 7 days) the contamination level can be assessed by colony counting (**Figure 2**). Incubation times can be shortened significantly by scanning membranes for micro-colony development using a microscope. For this to be effective it is desirable to incorporate suitable stains in the agar before incubation.[2]

Table 1. Yeast types encountered in the brewery

BREWING YEASTS ***Saccharomyces cerevisiae*** ***Saccharomyces carlsbergensis (uvarum)***	
NON-BREWING SACCHAROMYCES eg ***Saccharomyces diastaticus*** ***Saccharomyces bayanus*** ***Saccharomyces cerevisiae***	CULTURE MEDIA Schwarz differential medium Lin's modification of Schwarz Crystal Violet medium
NON-SACCHAROMYCES eg Candida species Torulopsis species Brettanomyces species	CULTURE MEDIA W.L. Actidione medium Lysine medium Lin's Copper Sulphate medium

Non-Saccharomyces yeasts

The majority of samples received by the quality control laboratories contain brewers yeast, often vastly in excess of any contaminants, so that the problem frequently becomes one of detecting a small proportion of wild yeasts against a background of brewers yeast.

The solution of the problem is far easier when the contaminants are non-Saccharomyces than when they are Saccharomyces species. The most widely used medium for the former purpose is lysine agar[3] (Oxoid CM191). This is used on the basis that yeasts have the ability to use lysine as sole nitrogen source whereas Saccharomyces species, and particularly brewing strains, do not normally grow on this medium. The need to ensure a washed inoculum and to regulate the quantity of brewers yeast plated means the procedure is somewhat tiresome in routine use, but even so it is still a very popular method in the industry.

An alternative to using lysine medium is to use Actidione (cycloheximide) as a selective inhibitor.[4] However, this medium also requires careful standardisation to be effective and its use is not as widespread as lysine medium. Recently, another method has been proposed which relies on the selective inhibition of Saccharomyces (in particular brewing strains) by copper ions in a copper-supplemented agar formulation.[5]

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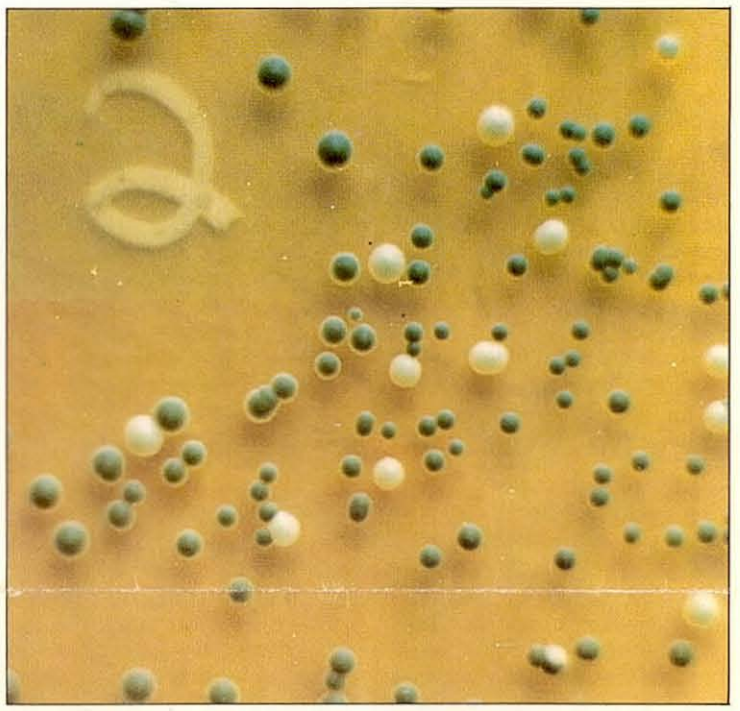


Figure 1: Different colony types on WLN agar showing pale green and dark green staining yeasts.

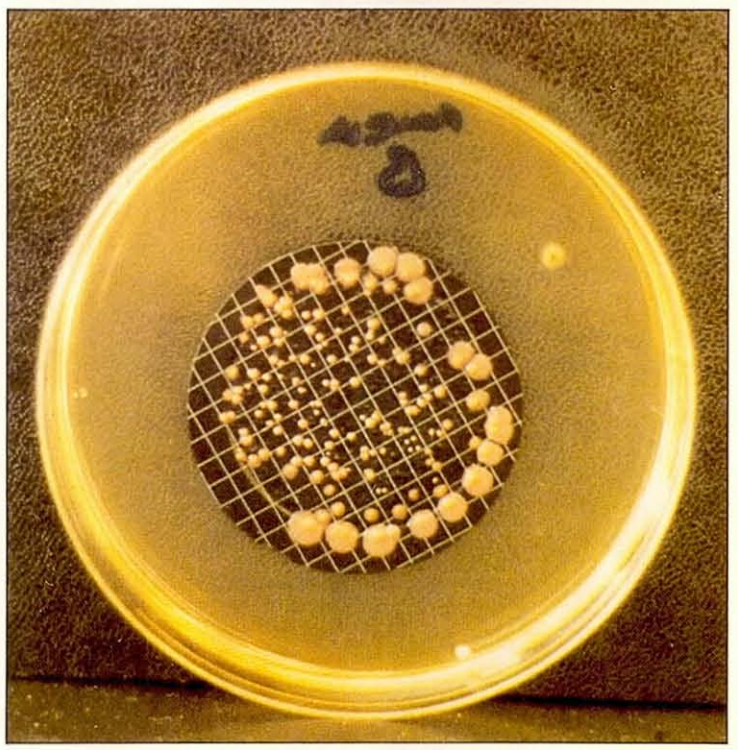
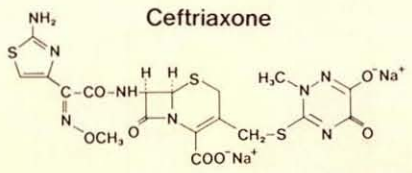


Figure 2: Yeast colonies on a membrane filter.

Addendum: The β-Lactam Antibiotics

In the last issue of *Culture* (Vol. 4 No. 2, September 1983) we provided a review of the nomenclature, structure and β-lactamase susceptibility of the wide range of β-lactam antibiotics available to us for characterisation. Since the article was written, information on a 'third generation' parenteral cephalosporin, ceftriaxone, has recently been made available to us.

Ceftriaxone



Ceftriaxone (Ro 13-9904, see above) shows potent *in vitro* and *in vivo* activity against a wide range of bacteria. Ceftriaxone is stable, as are other cephems of the third generation, in the presence of most β-lactamases, a fact which is reflected in its activity against β-lactamase-producing organisms. The antibacterial spectrum and the activity *in vitro* of this antibiotic against both gram-positive and gram-negative organisms is similar to that of cefotaxime.[1]

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Allan Brown and Christopher Reading

Campylobacter jejuni in food products

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The role of *Campylobacter jejuni* as a causative agent of acute enteritis is well established. As more and more reports come in it is clear that the infection is found in all areas of the world.[1,2] It is most common in tropical under-developed countries where the majority of infected children under 9 months of age are symptomatic, whilst older infected children usually are asymptomatic.[3,4] In developed countries the great majority of cases are symptomatic. This is probably due to a better control of the infection with fewer subclinical cases and hence less acquired immunity. The infection is usually mild, but large outbreaks of campylobacteriosis[5,6] may temporarily reduce the working capacity of the affected community. This is particularly true when outbreaks hit military units under field conditions.[7]

Source of infection

The infection is exceedingly common in nature. Birds, domestic or wild, appear to be the most important source of infection. Reports from many countries indicate that about 80% of chickens and turkeys are infected and that they carry about 10^6 *C. jejuni* organisms per gram of faeces.[8–12] The organisms seem to have a preference for the caeca (**Figure 1**) but whether or not caeca are important for the persistence of the infection is unknown. Most wild birds, including pigeons, migratory waterfowl, swans and flamingoes, are also infected.[13,14] The organism has been isolated from most domestic animals[15] and from numerous species of wild animals, ranging from cheetahs to crocodiles.[14]

Infected humans may also be a source of infection. In the acute phase there are about 10^6 *C. jejuni*/g faeces (N.J. Richardson, personal communication), falling off over the next 3-6 weeks until spontaneous bacteriological cure is attained.[16] In tropical and subtropical countries the carrier state may last from a few weeks to more than a year.[17]

Young pets may also harbour the infection. From time to time they suffer from acute Campylobacter enteritis; the stools are liquid, often bloody and teeming with *C. jejuni*.[18,19]

Transmission of *C. jejuni* to man

Although man-to-man and pet-to-man infections have been reported, they are rare and of limited epidemiological importance. Outbreaks involving large populations are water-borne[5,20] or milk-borne.[6,21] The evidence is essentially epidemiological and circumstantial. The organisms have rarely been recovered from the incriminated vehicles which supposedly have been contaminated with faecal material. Many smaller outbreaks have also been attributed to ingestion of raw milk.[22,23]

The great majority of clinical cases occur sporadically or in small clusters and appear to be due to consumption of infected, undercooked food. Meat from domestic birds is probably the single most important source. Infection rates as high as 70-90% have been reported.[9,12,24,25] About one third of the carcasses are positive for *C. jejuni* after overnight soaking in chilled, chlorinated water.[9] The organisms survive up to 7 days at 4°C on the skin and abdominal wall of the carcasses and up to 21 days in meals frozen at -25°C.[26] There is no evidence that *C. jejuni* multiply on the infected products — indeed their number dwindles with time.

How does meat or poultry become infected? The answer has a bearing on the preventive measures that should be adopted to provide a clean consumer product. The seeding may take place *in vivo* secondary to bacteraemia[27–29] or after slaughtering due to unhygienic handling of the offals. To distinguish between the two possibilities we examined 117 chicken livers purchased in retail outlets in New York City. Fifty-six livers (48%) were positive for *C. jejuni* (**Table 1**). More than half of these yielded growth from the surface only. When the organism was recovered from both surface and tissue; the tissue infection was invariably lighter than the surface infection. In the two cases where the organisms were isolated from the tissues only, no more than two colonies were observed. Moreover, no liver showed signs of abscess or scar formation. It was concluded that unhygienic handling of poultry parts is the cause of the infection. Other types of meat are also infected. For example, Bolton *et al*[30] investigating *C. jejuni* infection of carcasses in English abattoirs found 32% infection of beef, 70% of sheep and 56% of pork. Other authors concur that the organism may be recovered from all three types of meat but have generally found a lower infection rate.[31–33] To the best of our knowledge *C. jejuni* has not been isolated from cured meat.

Detection of *C. jejuni* in meat products

The basic questions about detection are: should direct seeding of selective media be supplemented with enrichment procedures; and if so, how much do we gain?

Some investigators employing enrichment media report improved recovery of the organisms[34–36] but systematic inquiries are missing. This led us to examine the problem. First, we compared the sensitivity of selective and non-selective *solid* media for the isolation of wild strains of *C. jejuni*. This was accomplished by counting *C. jejuni* in selective filtrates[3] of caecal contents on the media under investigation. Blaser's medium (Oxoid: 10mg vancomycin; 2,500 IU polymyxin B; 5mg trimethoprim; 2mg amphotericin B; and 15mg cephalothin per litre) was as sensitive to wild strains of *C. jejuni* as chocolate agar. Butzler's medium (Oxoid: 25,000 U bacitracin; 50mg cycloheximide; 10,000 U colistin sulphate; 15mg cephazolin sodium; and 5mg novobiocin - per litre) was less sensitive but more selective. In experiments designed to identify the important components in a *liquid* medium we found that best results were obtained with thioglycollate broth supplemented with 5% lysed sheep blood. If this medium (TBS) was further supplemented with Blaser's antibiotic mixture it supported good growth of *C. jejuni* but it did not adequately suppress growth of contaminants. TBS supplemented with Butzler's antibiotic mixture and 0.1% lauryl sulphate supported growth of as little as one colony-forming unit of wild strains of *C. jejuni* occurring in caecal contents, and at the same time suppressed growth of contaminants. In experiments where this enrichment medium was inoculated with 5ml diluted caecal suspension, and Blaser's solid medium with 0.1ml of the same suspension, the sensitivity of the enrichment was approximately 50 times higher than the sensitivity of the solid medium.

Table 1. Site of *C. jejuni* in 117 chicken livers

Site	Number positive for *C. jejuni*
Surface and tissue	18 (15%)
Surface only	36 (31%)
Tissue only	2 (2%)
Uninfected	61 (52%)

Conclusion

From a practical point of view, all meat should be considered to be infected with *C. jejuni* due to unhygienic handling after slaughtering. The organisms tend to diminish in number with time. Heat treatment, smoking and brine will eliminate the risk of infection. Systematic examination of meat for *C. jejuni* is costly and of little epidemiological significance. In the rare cases where it may be justified to conduct a search for *C. jejuni*, the sensitivity of the test may be increased significantly by the use of enrichment media.

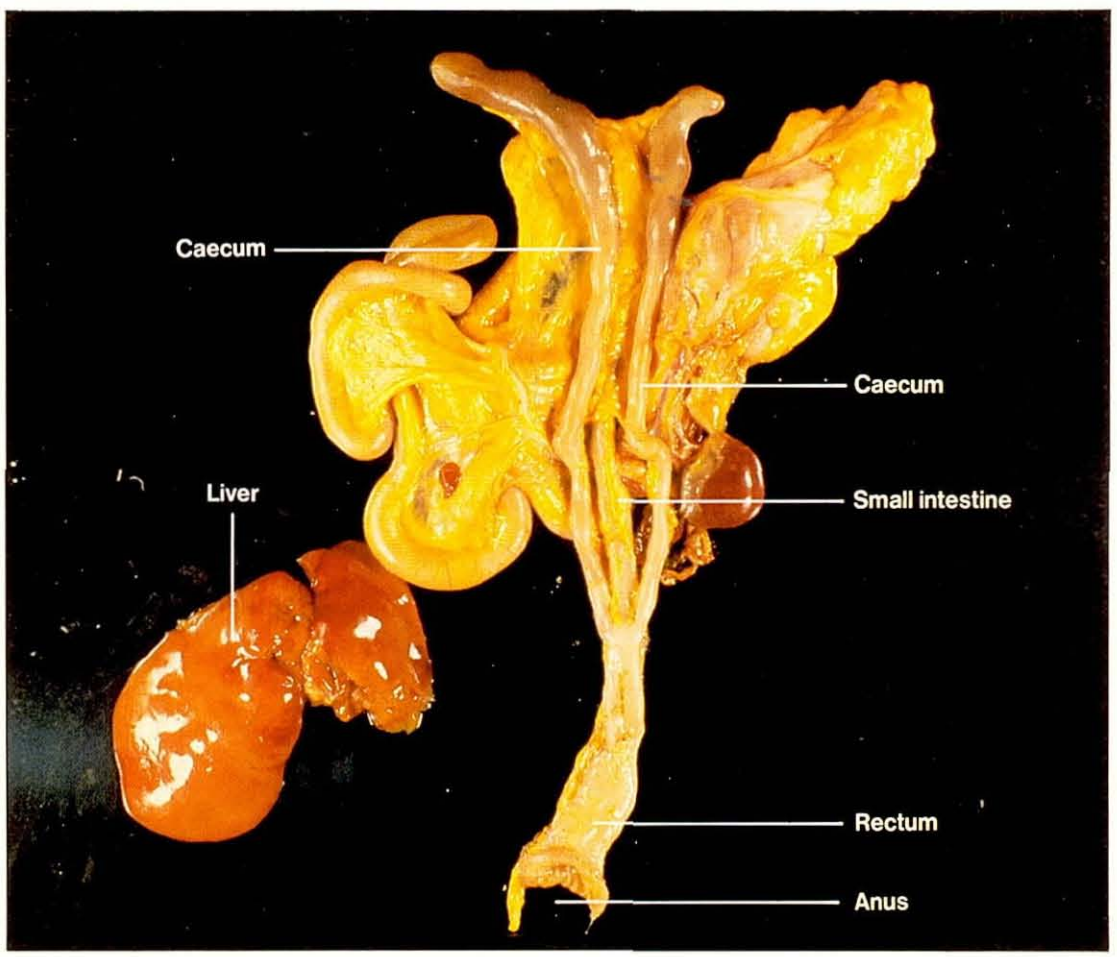

Figure 1: Caeca of chicken.

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Serological Tests for Syphilis

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The accurate diagnosis of syphilis depends to a great extent on the results of laboratory investigation. In primary and secondary syphilis, material from the lesions can be examined for *Treponema pallidum* by dark-field microscopy, but in all stages of the disease the detection of circulating antibodies is a most valuable procedure.

Early Test Procedures

During the complex interaction between *T. pallidum* and its host, a series of antibodies is produced, some specific and others non-specific (**Table 1**). The first serological test for syphilis was introduced by Wassermann in 1906; this was a complement fixation test. The original antigen was an extract of syphilitic tissue from stillborn foetuses with congenital syphilis. It was thought at the time that the active component in the extract was *T. pallidum*, but it is now known to be a diphospholipid called cardiolipin which is present not only in treponemes but in many mammalian tissues as well. The Wassermann reaction (**WR**) is thus a non-specific serological test. In its most refined form it was performed with a chemically pure cardiolipin antigen supplemented by lecithin and cholesterol. Antibodies to cardiolipin have been called 'reagin', a term which has been condemned as misleading but which is still in general use.

The WR held the field in syphilis serology for many years, but flocculation tests are easier to perform and more sensitive for the detection of reagin. The Venereal Disease Research Laboratory test (**VDRL**), introduced in 1946, is the most popular of these. The patient's serum is reacted with a mixture of cardiolipin, lecithin and cholesterol on a slide, and after a few minutes flocculation is detected with a microscope.[1] The rapid plasma reagin test (**RPR**) is essentially the same test, but carbon particles are added to the antigen mixture and the results can be observed with the naked eye.[2] The RPR can be used with autoanalyser equipment; this automated reagin test (**ART**) is very useful for the examination of large numbers of sera. All these flocculation tests can readily be quantitated to provide a titre of reagin.

These tests are easy to perform, sensitive and familiar. Unfortunately, they are not specific and positive reactions are given by some people who do not have syphilis. These biological false-positive (**BFP**) reactions can be acute or chronic. Acute reactions are usually due to febrile conditions; chronic reactions, lasting 6 months or more, may indicate the development of immune disorders such as lupus erythematosus.[3] Some completely healthy people (less than 1% of the population) also have circulating antibodies to cardiolipin. Because of the problems created by BFP reactions, a search was made for alternative serological tests which would be specific for treponemal disease.

Table 1. Serological Tests for Syphilis

Tests for Non-specific Antitreponemal Antibodies (Reagin Tests)

- Wassermann Reaction (**WR**)
- Venereal Disease Research Laboratory (**VDRL**) Test
- Rapid Plasma Reagin (**RPR**) Test

Tests for Specific Antitreponemal Antibodies

- *T. Pallidum* Immobilisation (**TPI**) Test
- Fluorescent Treponemal Antibody-Absorbed (**FTA-ABS**) Test
- *T. Pallidum* Haemagglutination Assay (**TPHA**) Test

More Specific Tests

The first of these was the *Treponema pallidum* immobilisation test (**TPI**), which was introduced in 1949. Live treponemes are incubated with the heat-inactivated test serum in the presence of complement and the preparation examined by dark-field microscopy; a positive reaction is reported if more than 50% of treponemes are immobilised.[1] This test is specific for treponemal disease but it is expensive, hazardous and technically demanding; it can be performed only in specialist centres, and is now obsolescent.

The fluorescent treponemal antibody – absorbed (**FTA-ABS**) test was developed in 1968. It is an indirect immunofluorescence procedure and uses *T. pallidum* as antigen. It is important that the test serum is first treated with a sonicate of Reiter treponemes to remove group-reactive antibodies, and the specificity of the test depends on the use of a really effective absorbent.[1] The FTA-ABS test is sensitive and specific; false-positive reactions are rare but are seen in some patients with collagen diseases. The test can be modified by the use of non-specific fluorescein-labelled anti-human globulins (e.g. anti-IgM) to identify the immunoglobulin class of the antibody. The FTA-ABS test is tedious to perform and is not suitable for screening large numbers of sera, but it is a useful confirmatory test and is of value in the diagnosis of primary syphilis (see below).

The *T. pallidum* haemagglutination assay (**TPHA**) was introduced in 1966. Sheep or fowl erythrocytes coated with particles of *T. pallidum* are mixed with the test serum in wells where agglutination caused by specific immunoglobulins is observed.[4] Non-specific reactions are eliminated by pre-treatment of the serum with an extract of Reiter treponemes, rabbit testis and erythrocyte membranes. The TPHA test is highly specific, easy to perform and can be quantitated. It is increasingly used for the routine screening of sera — because of the cost of the reagents, a micromethod is usually employed.

Table 2. Results of serological tests for syphilis in various conditions

Condition	Results of Serological Tests		
	VDRL or RPR	TPHA	FTA-ABS
Primary syphilis	±	–	+
Secondary syphilis	++	+	+
Latent syphilis	+ or ± or –	+	+
Late syphilis	+ or ± or –	+	+
Non-venereal treponematoses	+ or ± or –	+	+
Biological false-positive reaction	+	–	–

++ strongly reactive + reactive ± weakly reactive – non-reactive

Natural History of Syphilis

It may be of interest to outline the natural history of syphilis before the use of these serological tests in clinical practice is discussed. The incubation period of acquired syphilis is between 9 and 90 days. **Primary syphilis** is manifested by the appearance of a primary sore, or chancre, at the site of inoculation of *T. pallidum*: this may be on the penis, vulva, cervix or on extragenital sites such as the anus and mouth. There is usually an associated regional lymph node enlargement. Although these signs are local, in primary syphilis dissemination of treponemes throughout the body has already occurred. In **secondary syphilis**, which develops 6-8 weeks after the primary stage, this dissemination combined with immunological reactions results in a variable but widespread disease: malaise, fever, weight loss, skin rashes, enlargement of lymph nodes and disease of the abdominal viscera and central nervous system may all occur. If untreated, secondary syphilis runs a fluctuating course for up to two years. The disease then becomes latent and there are no symptoms or signs for many years. In about one-third of the patients **late syphilis** then appears, characterised by disease of the cardiovascular system, the central nervous system or by gummatous destruction of various organs and tissues. During primary, secondary and latent syphilis it is possible for a mother to transmit the infection to the foetus in the uterus, resulting in **congenital syphilis**.

When a person contracts syphilis, the first serological test to become reactive is the FTA-ABS test, followed by the reagin tests (VDRL or RPR tests). The TPHA becomes positive next, and the TPI test last.

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Thus, the FTA-ABS and VDRL or RPR tests are useful for the serological diagnosis of primary syphilis, particularly if dark-field microscopy for *T. pallidum* is not practicable (**Table 2**). In secondary syphilis, all serological tests are strongly positive and the titre of antibodies in the VDRL and RPR tests is maximal. As the disease becomes latent this titre slowly falls, although the specific tests remain positive. In most patients with untreated late syphilis, reagin tests are reactive in low titre or they may even be negative; the TPHA and FTA-ABS tests however, are always positive. In a small proportion of patients with late syphilis the TPI test may give negative results.

What effect does antitreponemal treatment have on this serological pattern? The VDRL and RPR tests usually become negative one year after the effective treatment of primary syphilis and two years after treatment of secondary syphilis, but the specific tests may remain positive for many years. The FTA-ABS (IgM) test, which is always positive in untreated primary and secondary syphilis, usually becomes negative within 9 months of treatment. When patients are treated for latent or late syphilis, tests for cardiolipin antibody often show a decline in titre, but this may be slow, and in a few patients the titre becomes stationary and unaffected by further treatment. The specific tests remain positive indefinitely.

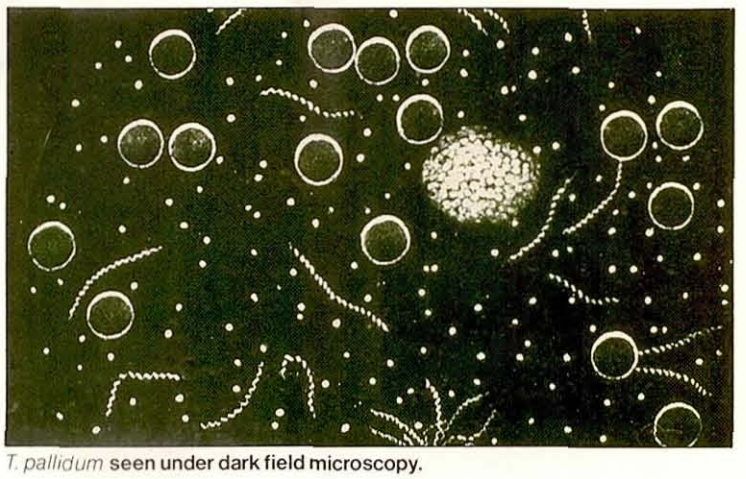
Serological Tests in Clinical Practice

It is not possible to use a single serological test for the diagnosis of syphilis and for the follow-up of a patient after treatment. If a reagin test alone is used, many patients with latent and late syphilis may escape diagnosis; conversely, patients with BFP reactions may be wrongly suspected of having syphilis. The best combination is a quantitated reagin test (VDRL, RPR, or ART) and a TPHA test. An FTA-ABS test should be available for the diagnosis of early syphilis and for the resolution of discordant results between reagin and TPHA tests. For the assessment of the results of treatment, quantitated reagin tests are most useful; specific tests are of little value.

The serological reactions to syphilis and non-venereal treponematoses, such as yaws, are identical. Patients who have had yaws in childhood show positive specific tests and reagin tests which are weakly reactive or negative, a picture identical to that seen in patients with latent or late syphilis, or a previously treated infection. This means that unless there are positive features in the history or physical examination it may be impossible to decide whether a patient from an area in which yaws is endemic has yaws or syphilis.

It is customary to perform serological tests on all pregnant women and this, together with treatment of infected mothers, explains the very low incidence of congenital syphilis in developed countries. The interpretation of syphilis serology during pregnancy is no more difficult than at any other time, but it must be remembered that BFP reactions can occur during pregnancy, so serological screening must always include a specific test.

If a mother has been adequately treated for syphilis, and has not been reinfected since treatment, there is no risk of congenital syphilis in the baby. Occasions arise when there is doubt about the adequacy of treatment or the possibility of reinfection when it is important to ensure that the baby is not infected. Serological tests on the neonate will reflect those of the mother because of the transplacental passage of antibodies. These maternal antibodies normally disappear within 3 months of birth. Persistently positive serology, or a rising titre in the reagin tests, or a positive FTA-ABS (IgM) test, all suggest congenital infection. In some babies the onset of congenital syphilis may be delayed; therefore, it is customary to continue serological examination for the first 6 months of life if there is a possibility that the mother had untreated or inadequately treated syphilis before the baby's birth. The virtual disappearance of congenital syphilis from developed countries is one of the major successes of modern medicine and no relaxation of the present screening procedure should be contemplated.



T. pallidum seen under dark field microscopy.

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WILD YEASTS IN BREWERIES

CONTINUED FROM FRONT PAGE

Once again, inhibition of Saccharomyces includes many wild yeasts as well as culture yeast, though by comparison with lysine agar the incubation times are shorter and sample manipulation is minimal, making the procedure at least worthy of a closer examination. Often, though, non-Saccharomyces yeasts do not cause problems within the brewery and under these circumstances their selective detection serves as a measure of general brewery hygiene.

Saccharomyces yeasts

Over the years a number of media have been proposed for estimating wild Saccharomyces yeasts in the presence of brewing yeasts. Kato's [6] medium employing crystal violet as selective inhibitor was probably one of the forerunners, though it has been reported subsequently to be unreliable on a number of occasions. Schwarz Differential Medium (SDM) [7] uses fuchsin sulphite as inhibitor, but again its total reliability has been called into question. In a further variation, Lin combined crystal violet and fuchsin sulphite into one medium and reported superior results compared with the individual inhibitors on their own.[8] The efficiencies of these various differential media have been reviewed.[9]

Longley and co-workers have taken a different approach to the problem of detecting Saccharomyces species by exploiting the well known obligate requirement of *Saccharomyces cerevisiae* under anaerobic growth conditions for both ergosterol and an unsaturated fatty acid. In fact, almost all species of Saccharomyces have this requirement and grow adequately in the absence of oxygen on media supplemented with ergosterol and Tween 80 (ET 80), whereas yeasts from other genera only produce microcolonies.[10] Thus, media can be made selective for Saccharomyces species in general using ET80 and strictly anaerobic conditions. More importantly, media can be made selective for wild Saccharomyces using ET80-supplemented crystal violet agar or ET80-supplemented Lin's medium.[11]

Summary

The diversity of wild yeasts encountered in the brewery and their not infrequent similarity to brewing strains means there is no 'universal' wild yeast medium that suffices in all situations. It is now recognised that in most situations at least two media are required, one to detect the Saccharomyces range and one to detect the non-Saccharomyces range. Lysine medium is widely used in the latter instance with Lin's medium gaining favour for detecting wild Saccharomyces species. However, this combination of media is not ideal and the brewery microbiologist is faced with the need to adjust techniques to suit local conditions and specific problems. There is certainly further scope for developing multi-purpose wild yeast media for brewery use.

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