A Selective Medium for the Cultivation Of N. gonorrhoeae and N. meningitidis

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A SIGNIFICANT complication in the control of gonorrhea is the large number of women who harbor undiagnosed, asymptomatic infection. Development of better and more rapid laboratory procedures for isolation and identification of the gonococcus would therefore help to control this source of the disease.

For women, diagnosis by culture has always been found greatly superior to gram-stained smears. The culture method, however, becomes much less effective when specimens are contaminated with bacteria that overgrow the more slowly developing gonococci. Complete overgrowth may occur in 10 percent of cervical cultures. It is not known to what extent cultures are lost by contamination when few gonococcal cells are present, as in chronic or asymptomatic gonorrhea or early in the detection of therapeutic failure.

Selective media for the isolation of a single bacterial species from contaminated specimens are seldom absolute; however, growth of the desired organisms may be favored or the undesired contaminants inhibited to the point where pure cultures may be easily isolated.

Since *Neisseria gonorrhoeae* of necessity requires highly enriched media favorable also to bacterial and yeast contaminants, previous attempts to prepare a selective medium have aimed at reducing the contaminating organisms by the addition of inhibitory substances such as thallium acetate (*1*), crystal violet (*2*), Nile Blue A (*3*), tyrothricin (*4*), aerosporin (*5*), boric acid (*6*), and chloral hydrate (*6*). Use of these agents was not successful, primarily because some sensitive gonococcal strains were intolerant to concentrations required to inhibit contaminants.

To accomplish the desired end, the full impact of the inhibiting agent may need to be compromised to allow growth of all or nearly all sensitive strains. Further, when two or more inhibitory agents are used together, antagonistic or synergistic action for the desired and unwanted bacteria must be considered.

In women suspected of gonorrheal infection, cultures are taken routinely from only two sites, the endocervical canal and urethra, although gonococci are known to reside in the posterior fornix of the vagina and in the rectum in many cases.

In an attempt to raise to the highest possible degree the standards of bacteriological investigation employed for diagnosis and test of cure, two studies in England used cultures from the vagina and rectum in addition to those from the cervix and urethra. Of 229 patients, Nicol (*7*) found 19.6 percent positive by vaginal culture, 7.5 percent being positive only from this site. Wilkinson (*8*) examined 224 patients with gonorrhea for rectal infection and found 17.8 percent positive by culture. Gram-stained smears gave a higher proportion, 28 percent, of positive results. Overgrowth of rectal cultures by contaminants resulted in more positive findings by microscopic examination.

Obviously, culture procedures would be improved greatly if a highly selective medium could be devised for the gonococcus. Such a

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medium would probably discover undiagnosed gonorrhea, by cultural examination, particularly of the vaginal and rectal sites.

The selective medium presented here is based primarily on results of laboratory experiments with pure cultures. The work was designed to determine:

1. A single concentration for polymyxin and for ristocetin which would permit undiminished growth of all gonococcal laboratory strains.
2. If all gonococci would tolerate the combined action of the two drugs.
3. Whether a medium incorporating the antibiotics would inhibit bacterial growth in the rectal and vaginal flora.
4. If gonococci when mixed with a menstruum of fecal or vaginal bacteria could be easily selected for purification on the antibiotic medium.
5. If the selective medium was inhibitory for saprophytic species of *Neisseria* and genera of the tribe Mimeae, particularly *Mima polymorpha* var. *oxidans*.
6. If the selective medium permitted undiminished growth of gonococcal strains in specimens taken from infected men and women.

Methods

Media. A conventional chocolate agar (Bacto GC medium base with added hemoglobin and yeast Supplement B) served as control medium for comparison with the selective medium. The latter was of the same composition but had ristocetin and polymyxin B added in concentrations given below. Ristocetin (Spontin lot number 739–3630), potency 1,020 μg./mg., was obtained through the courtesy of Abbott Laboratories, Chicago, Ill. Polymyxin B, potency 7,500 units/mg. (Pfizer P–42M), was obtained from the Antibiotics Section, Food and Drug Administration, Washington, D.C.

The selective medium was prepared by pipetting the required amount of the two antibiotics into sterile, glass-stoppered, graduated cylinders and adding melted chocolate agar cooled to 56° C. After mixing by rotating the cylinders five times from end to end, the medium was poured into plastic culture dishes. The dish tops were set ajar for 2 hours to allow water from syneresis to evaporate from the agar surface. The control plates were prepared in a similar manner.

Trypticase soy broth, Baltimore Biological Laboratory, was used to prepare dilutions of inoculum. The agar and broth media were sterilized at 121° C. for 20 minutes. The reaction was pH 7.2. The prepared selective and conventional chocolate agar plates were stored in plastic food bags at 4° C. Selective properties were retained for at least 6 weeks; however, gonococci grow better on freshly prepared media.

Cultures. Pure cultures of recently isolated *N. gonorrhoeae* and of other *Neisseria* studied were obtained from lyophilized stocks or cultures stored at −70° C. Cultures of Mimeae were provided by Elizabeth King, Diagnostic Laboratory, Communicable Disease Center, Atlanta, Ga.

All cultures were maintained on chocolate agar. Stock suspensions of organisms were prepared by washing off the 24-hour growth on slants with broth and diluted to give a 45 percent transmission of light in a Lumetron photoelectric colorimeter (Photovolt model 402–E, filter 530B). The stock gonococcal suspensions were further diluted to 10^{-6} in the pure culture studies to aid quantitation. The stock suspensions of all other organisms tested were diluted to 10^{-3}. A 3-mm. loopful was used to streak-inoculate the plates.

Vaginal vault flora. Secretions from the vagina were obtained by Dr. Anne R. Yobs, Venereal Disease Research Laboratory, from patients at the Georgia Training School for Girls, Adamsville, Ga. A pledget of sterile cotton was used to absorb secretion in the vaginal posterior fornix. The pledget was dropped into a sterile specimen bottle, containing 10 ml. of broth, for transportation to the laboratory.

At the laboratory, the pledget was squeezed against the side of the specimen bottle in order to release as much vaginal material and broth as possible. This was centrifuged for 20 minutes at 3,000 rpm. The supernatant was discarded and the sediment, primarily vaginal vault bacteria, was suspended in 3 ml. of broth. This inoculum was tested alone for inhibition by selective medium. It also served as a suspending fluid when gonococci were mixed with it for testing.

Rectal flora. Ten grams of fresh, human feces were suspended in 90 ml. of sterile dis-

tilled water and filtered through four layers of sterile cotton gauze. A further tenfold dilution was made in broth. This inoculum was tested alone for inhibition by selective medium and served as fecal suspending fluid when gonococci were mixed with it for testing.

Urethral, vaginal, cervical, and rectal exudate. Material for culture was obtained by Dr. John H. Tiedemann, Fulton County Health Department, Atlanta, Ga. Exudate was collected on sterile cotton swabs which were placed in 0.5 ml. of broth 2 hours before culturing. The swabs were rotated, excess fluid expressed against the side of the tube, and 0.1 ml. of the suspensions was used for plate inoculations after emulsifying by repeated pipetting.

Results

The tolerance for polymyxin B (*9*) of 53 recently isolated strains of *N. gonorrhoeae* was determined. All 53 strains were tolerant to 25 units per ml., 2 were inhibited by 50 units per ml., 7 were partially inhibited by 100 units per ml., and 2 strains did not grow. Numbers and size of colonies were the same as the control cultures grown on conventional medium without polymyxin.

Exudate from 28 patients with clinical gonorrhea was inoculated on medium containing polymyxin. Growth of gonococci occurred without inhibition at 25 units per ml.

Polymyxin, while quite effective in suppressing the gram-negative bacteria of the fecal and vaginal flora had no effect on the gram-positive bacteria. Crystal violet (0.002 percent) and Nile Blue A (0.001 percent) were tried with polymyxin for selective inhibition of gram-positive and gram-negative bacteria in fecal flora. Neither of these dyes, long known for their ability to inhibit contaminants in isolation media for gonococci, was found satisfactory.

Telomycin (*10*), Bristol Laboratories, Inc., with activity only against gram-positive bacteria and streptozotocin (*11*), Upjohn Co., with activity against gram-positive and gram-negative bacteria but with little effect on a *Neisseria* species were tested in combination with polymyxin. Streptozotocin was ineffective as it was unstable in the medium. Telomycin showed some promise as an effective inhibitor of the gram-positive flora in fecal menstruum, with only slight inhibition of gonococci at 32 μg./ml. Further attempts with this antibiotic were abandoned, however, because it was not commercially available.

Ristocetin, with activity for gram-positive bacteria, had been reported to be inactive against gram-negative bacteria including *N. meningitidis* and *N. catarrhalis* (*12*). To determine its activity for *N. gonorrhoeae*, 10 laboratory strains were tested against several concentrations. After 24 hours' incubation, tolerance to 12.5 μg./ml. occurred, a concentra-

Ristocetin activity for 10 strains of *Neisseria gonorrhoeae*

Strain No.	Ristocetin concentrations (μg./ml.)								
	100		50		25		12.5	6.25	0
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	24 hours	24 hours
373	0	0	2	4	2	4	4	4	4
223	4	4	4	4	4	4	4	4	4
338	0	±	2	4	3	4	4	4	4
258	0	0	1	1	2	4	4	4	4
367	0	±	2	4	3	4	4	4	4
1	0	0	0	±	2	4	4	4	4
355	0	0	2	2	3	4	4	4	4
198	0	±	2	2	3	4	4	4	4
369	0	0	0	2	3	4	4	4	4
358	2	4	3	4	4	4	4	4	4

NOTE: 4=maximum growth; 2=50 percent growth; 1=25 percent growth; 0=no growth.

tion inhibitory to enterococci, staphylococci, streptococci, and other gram-positive organisms. The colonies of gonococci were somewhat smaller than the controls at this time but not diminished in numbers. After 48 hours of incubation, the colonies were equivalent in size to the controls and tolerant to 25 μg./ml. (see table).

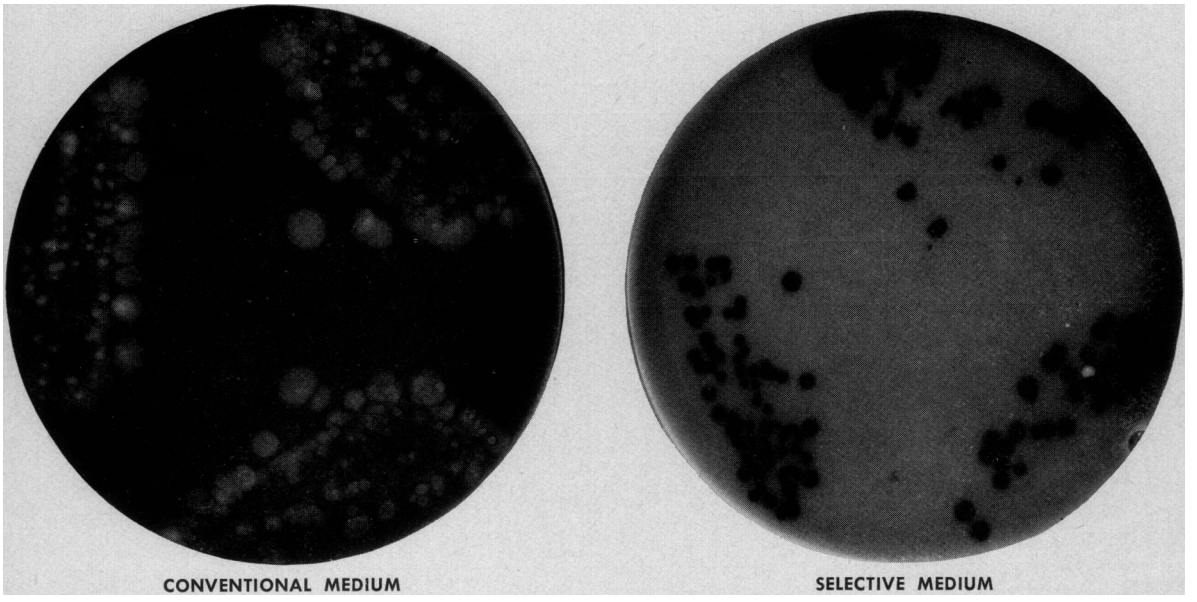
In another experiment, polymyxin and ristocetin were added to the chocolate medium. The same laboratory strains of gonococci and the same concentrations of ristocetin (R) shown in the table were each added to 25 units per ml. of polymyxin (P). Only minor differences in growth were observed from those shown in the table. None of the 10 strains were inhibited by 25 units per ml. of polymyxin alone. All the strains were tolerant to a combination of 25 units P and 12.5 μg. R per ml. at 24 hours' incubation. Six strains were slightly inhibited by 25 units P and 25 μg. R per ml., but by 48 hours growth was the same as that for the controls.

Because of the slight inhibition found with 25 units P and 25 μg. R per ml., 20 freshly isolated strains of gonococci were tested at two concentrations. With 25 units P and 25 μg. R per ml., 3 strains did not grow, 9 were partially inhibited, and 8 were not inhibited. With 25 units P and 12.5 μg. R per ml., 1 strain did not grow, 7 were partially inhibited, and 12 showed the same growth as that of the controls.

Exudate from male patients with clinical gonorrhea was inoculated on a conventional medium and on selective medium containing 25 units per ml. polymyxin and varying concentrations of ristocetin (12.5, 10, and 8 μg./ml.). Of the 28 cultures positive for gonococci, one (No. 19) was negative on the ristocetin medium at all three concentrations and another (No. 22) was greatly inhibited. These two cultures were purified from the control plate and retested in the same manner. Culture No. 19 now grew without inhibition. Culture No. 22 still exhibited great sensitivity, growing only on the 8 μg./ml. ristocetin medium. Less than 0.1 percent of such cultures have been encountered. Contamination of the above specimens was greatly reduced or abolished.

Inhibition of the vaginal vault flora was tested on cultures from four patients. The heavy growth that occurred on each control plate failed to grow in the selective medium. Of 12 vaginal cultures taken from patients suspected of gonorrheal infection, 4 were positive for gonococci on the selective medium. Two of these had failed to grow on the heavily contaminated conventional medium. None of the other eight cultures were positive. On the selective medium, contamination was greatly inhibited

Figure 1. Results obtained with mixture of gonococci and fecal flora suspension



or completely suppressed. Yeast colonies appearing on 3 of the 12 specimens were not inhibited by the selective medium.

The effect of polymyxin and ristocetin, separately and in combination, was tested against eight other species of *Neisseria*. The meningococci were not inhibited by either antibiotic alone or in combination, with the exception of the group D strain which was partially inhibited by both ristocetin and polymyxin alone and in combination.

Of the other *Neisseria* species tested, *N. sicca*, *N. flava*, *N. perflava*, *N. subflava*, *and N. flavescens* failed to grow on polymyxin alone but grew

Figure 2. Results obtained with mixture of gonococci and vaginal flora suspension

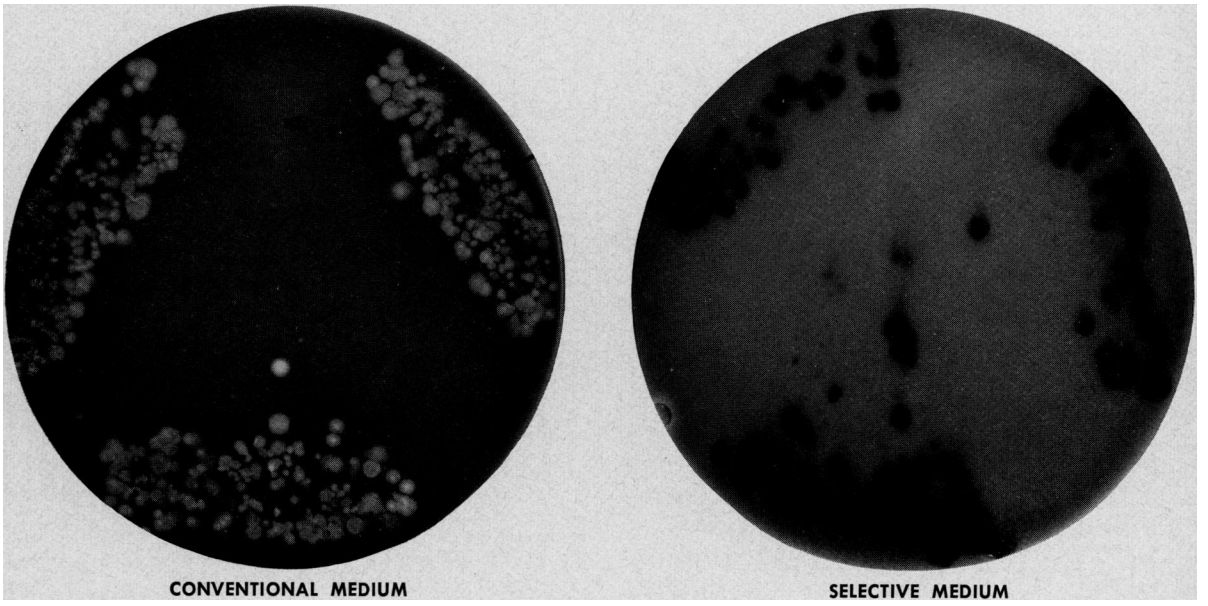
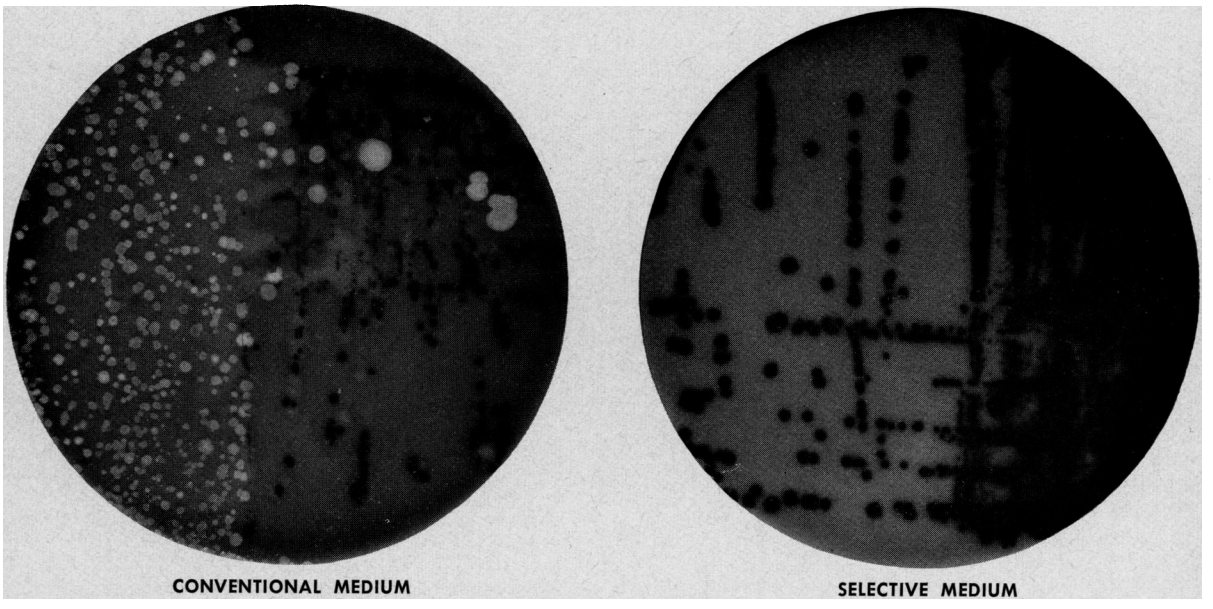


Figure 3. Selective inhibition of contaminants in exudate from a male with gonorrhea



well on ristocetin. However, two strains of *N. catarrhalis* and the *N. hemolysans* grew in the presence of polymyxin. The *N. hemolysans* and *N. catarrhalis* (ATC8176) failed to grow on the ristocetin medium. With the exception of the meningococci and one strain of *N. catarrhalis* (NIH–9), none of the species tested grew on the selective medium at the concentration of 25 units P and 10 μg. R per ml.

Nine isolates of Mimeae were examined for susceptibility to ristocetin and polymyxin. One of three strains of *Mima polymorpha* (835011) showed resistance to both drugs singly and in combination. The other strains of *M. polymorpha*, *M. polymorpha* var. *oxidans* and *Herellea vaginicola*, were all susceptible to 25 units per ml. polymyxin but not to ristocetin (8–15 μg./ml.). All but the *M. polymorpha* (835011) failed to grow in the presence of the combined drugs.

The effectiveness of the combined antibiotics, 25 units polymyxin and 10 μg. ristocetin per ml., of the selective medium for reducing or eliminating contamination is contrasted with conventional medium in figures 1–6. All plates were treated with 1 percent dimethyl paraphenylenediamine hydrochloride, oxidase reagent, before being photographed in order to distinguish gonococcal colonies from those of other bacteria and yeast.

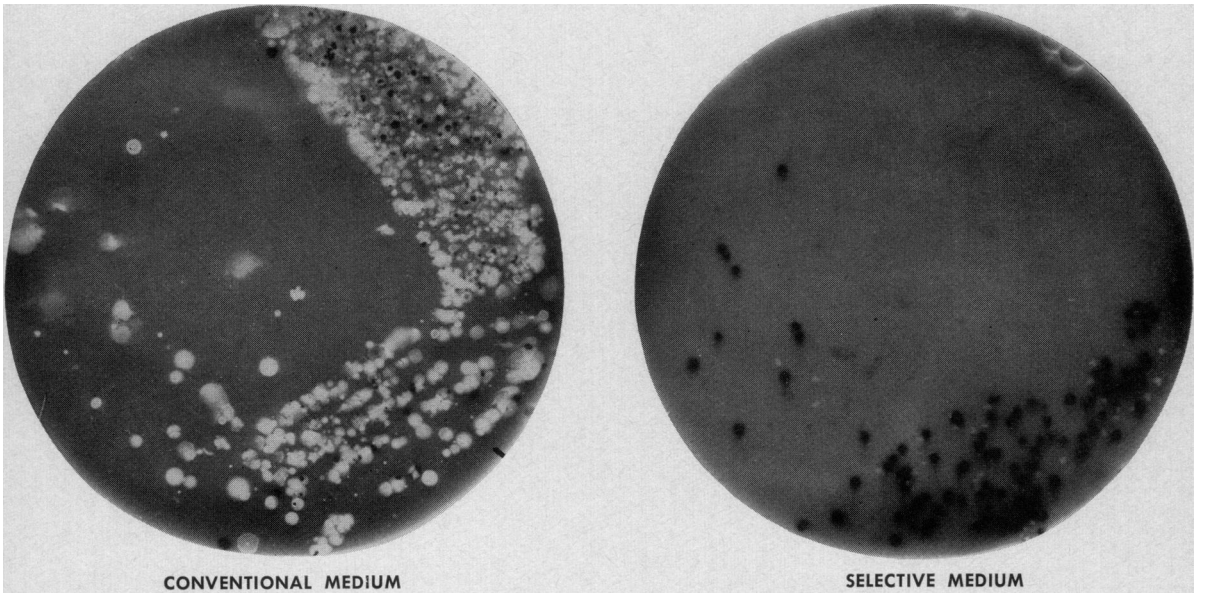
Results obtained when gonococci were mixed with fecal flora suspension are shown in figure 1 and when mixed with vaginal flora suspension in figure 2. Many of the gonococcal colonies on selective medium could be inoculated into sugar fermentation medium without further purification. The selective inhibition of contaminants in exudate from a male with gonorrhea is shown in figure 3.

The selective inhibition of contaminants in cultures from the cervical, vaginal, and rectal sites of infection respectively are shown in figures 4, 5, and 6. With suppression of bacterial micro-organisms of the vaginal specimen, yeast contaminants become evident (fig. 5).

A concentration of 25 units polymyxin and 10 μg. ristocetin per ml. of medium was chosen as the best compromise for selective inhibition of contamination in gonococcal specimens. In a test of this concentration with 59 positive gonorrheal specimens (28 male, 31 female) diagnosed by selective and conventional media, only 1 strain failed to grow; 2 others were greatly inhibited on the selective medium.

Colonies are slightly smaller after 24 hours' growth on the selective medium, but if incubated 48 hours, colony size is usually the same as the control. Not all contaminants are inhibited. Certain yeast and some gram-positive streptobacilli and short, plump, and long, slender

Figure 4. Selective inhibition of contaminants in cervical cultures



gram-negative rods grow. Isolation and purification of gonococci from these uninhibited contaminants has not been difficult. Some strains of *Proteus* are prevented from spreading; however, others are not inhibited and these cultures are lost.

The fluorescent antibody (FA) method (*13*) for diagnosing gonorrhea became practicable when the specimen and swab were cultured on a slant of medium for 16 hours. This resulted in multiplying greatly the numbers of gonococcal cells available for specific FA staining and ultraviolet microscopy. In work underway, selective and conventional media for delayed

Figure 5. Selective inhibition of contaminants in vaginal cultures

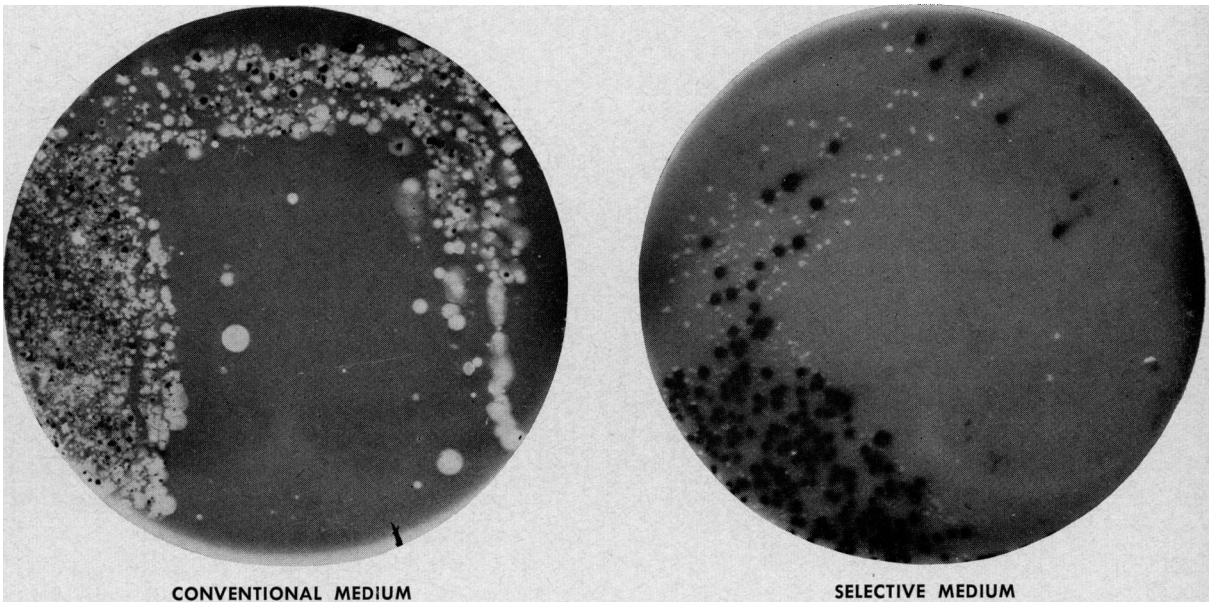
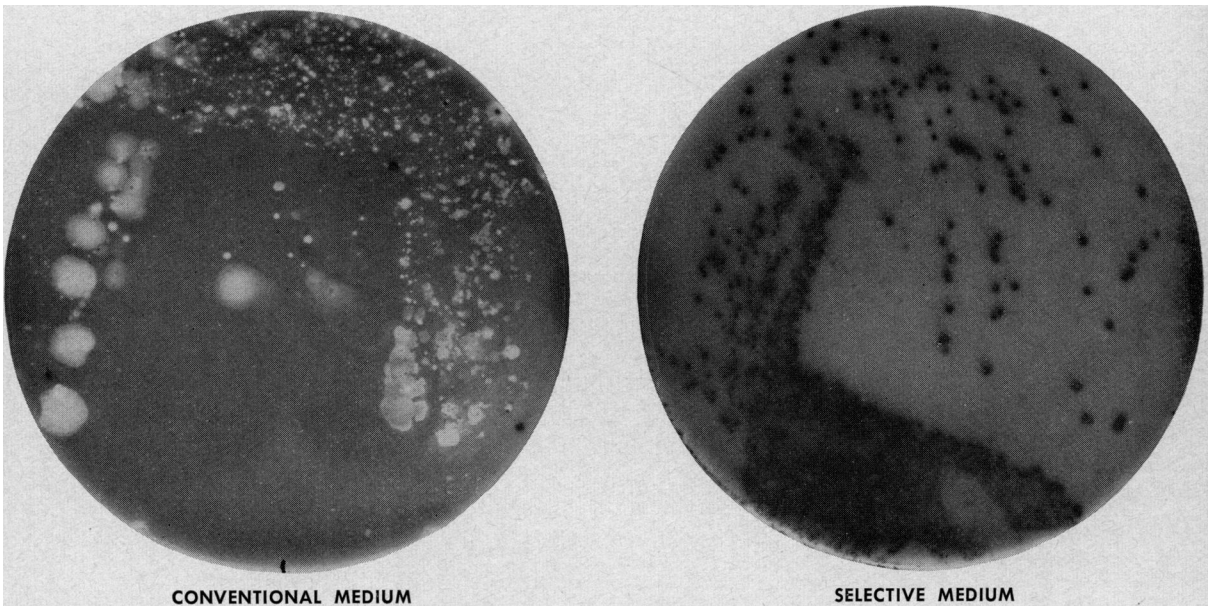


Figure 6. Selective inhibition of contaminants in rectal cultures



FA diagnosis of gonorrhea are being compared. In culturing specimens from the highly contaminated vaginal site of 40 patients, 16 were found positive with selective and 13 with conventional medium. A considerable decrease is noted in fluorescence of non-specific bacteria owing to growth inhibition of contaminants on the selective medium. As a rule, such staining is unimportant when morphology is considered.

Discussion

The inhibition of other *Neisseria* species, except meningococci, is interesting; however, too few isolates have been tested to know the exact value of this finding. In laboratories where only presumptive diagnosis of gonorrhea is made (oxidase-positive colonies of gram-negative diplococci), use of the selective medium should result in fewer false positive results, especially since *N. sicca*, the most prevalent *Neisseria* in secretions, is inhibited.

The finding that *N. meningitidis* grows on the selective medium suggests its use in the epidemiologic search for asymptomatic carriers. The elimination of contaminating bacteria and possibly many saprophytic *Neisseria* of the naso-pharyngeal specimen should make easier the selection of pure colonies for identification and typing (*14*).

The inhibition of Mimeae species by the selective medium is especially gratifying since these micro-organisms are easily confused with *N. gonorrhoeae* by some inexperienced workers (*15*). Only one of the species, *M. polymorpha*, grew without inhibition on the medium, but it gave a negative oxidase reaction. The *oxidans* variants failed to grow and, since the other species are oxidase negative, the possibility of false positive diagnosis with these organisms is restricted.

Bacterial species other than *Neisseria* give positive oxidase reactions on conventional media and thus confuse the cultural diagnosis, especially when reliance is placed on presumptive testing. The latter becomes more valuable because most of these oxidase producers are inhibited by the selective medium.

The selective medium makes possible the use of larger inoculums on culture plates, and it is not uncommon even in primary rectal cultures to find pure colonies suitable for fermentation medium. Many contaminated cultures submitted for testing of susceptibility to penicillin or other antibiotics have been easily purified by subculturing on the selective medium.

An attempt was made to determine the sensitivity of gonococci by inoculating urethral exudate directly on dishes of selective medium containing varying concentrations of penicillin. The procedure failed because of the antagonism of penicillin by ristocetin. It has been found, however, that minimal inhibitory concentrations of penicillin are the same for gonococci isolated on selective medium and on conventional medium.

The selective medium has been used successfully in transporting a few cultures to the laboratory. The specimen is inoculated on a selective medium slant in the usual manner for delayed FA examination. After 16 hours' incubation, the slant is mailed to the laboratory where it is diagnosed by the FA technique. This procedure using the selective medium is being compared with the Stuart transport method (*16*), and good results are anticipated because gonococcal colonies will have grown and contaminants will have been suppressed before the specimen is mailed. Also, the time for a definitive identification will be shortened by FA staining, for it eliminates further purification and sugar fermentation procedures, which take an extra 2 or 3 days at best.

Current tests have shown that use of the selective medium in conventional plate and delayed FA cultures has made laboratory diagnosis of vaginal and rectal specimens feasible and has improved diagnosis of urethral and cervical specimens.

The medium is being used with considerable success in determining the incidence of gonorrheal proctitis among females and homosexual males.

The selective medium should also help in evaluating therapeutic failure of drugs and drug regimens. Gonorrheal proctitis has been suggested as one of the reasons for failure with penicillin treatment (*17*). Penicillinase-producing rectal bacteria are supposed to destroy penicillin and thus leave a focus from which gonococci are able to reinfect the urethra and cervix. This suggestion has persisted despite

work contradicting its validity (*18*, *19*), possibly because of the known difficulty in detecting gonococci in rectal specimens. Further light could be cast on this difficulty by using the selective medium for test of cure of rectal gonorrhea.

Field trials, using the selective medium described here, for gonorrhea and for the asymptomatic meningococcal carrier state will be reported in another paper.

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