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Trichomonas vaginalis
II. Laboratory investigations in trichomoniasis

RUTH NIELSEN
Neisseria Department, Statens Seruminstitut, Copenhagen, Denmark

Trichomonas vaginalis was first described by Donné (1836), who observed the organism in purulent secretions in both women and men, and stated that it was not present in normal vaginal secretions. *T. vaginalis* was successfully cultured *in vitro* by Lynch (1915), and there have since been a number of publications containing descriptions of the morphological, epidemiological, and pathological aspects of trichomonal infestation (Trussell, 1947; Whittington, 1957; Burch, Rees, and Reardon, 1959a; Frost and Honigberg, 1962; Niekerk, 1963; Honigberg and King, 1964; Nielsen, Ludvik, and Nielsen, 1966).

There was no effective therapy for *Trichomonas* infection until the discovery of metronidazole (Flagyl®) by Durel, Roiron, Siboulet, and Borel (1960). This great therapeutic advance led to the need for improved methods of demonstrating *T. vaginalis* in secretions. The simplest and most rapid method is direct microscopy of unstained wet preparations in which the actively moving organism is readily recognized. However, since the development of suitable media it has been found that culture is the most sensitive method (Whittington, 1957; Burch, Rees, and Reardon, 1959b; Petri, 1962; Lowe, 1965; Hess, 1969).

Routine culture of *T. vaginalis* has been carried out in the Neisseria Department of the Statens Seruminstitut, Copenhagen, since 1962, and a method of testing the sensitivity to metronidazole has been developed concurrently.

Material and methods

TRANSPORT OF SAMPLES

Secretion for investigation for *T. vaginalis* is taken with a charcoal impregnated swab from the urethra and/or vagina in women and from the urethra in men, preferably after prostate massage. If the investigation is to be carried out on a urine sample this is centrifuged and the deposit transferred to a swab. The swab is placed in a modified Stuart's medium and transported to the laboratory in the same way as samples to be investigated for gonococci (Reyn, Korner, and Bentzon, 1960). If it is not possible to dispatch the sample immediately, it should be stored at either 4° or 36°C., as the survival time of *T. vaginalis* is longer at these temperatures than at room temperature. However, the storage temperature is of no practical importance when the transport time is less than 24 hrs (Nielsen, 1969).

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Address for reprints: Dr. Ruth Nielsen, Neisseria Department, Statens Seruminstitut, Amager Boulevard 80, DK-2300 Copenhagen S, Denmark.
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MICROSCOPY AND CULTURE

On receipt of the samples in the laboratory the swabs are warmed to 36°C. First the culture medium (Diamond's substrate (Diamond, 1957)) is inoculated by means of the swabs, and then the remainder of the secretion is rubbed on to a drop of saline on a slide and examined at once under the microscope (direct microscopy). Phase-contrast microscopy is used at a magnification of $\times 320$. The cultures are examined after incubation at 36°C. for 3 and 6 days, using microscopy of wet preparations.

DETERMINATION OF SENSITIVITY TO METRONIDAZOLE *IN VITRO*

Two-fold dilutions of metronidazole in Diamond's medium without addition of agar are used in the sensitivity determinations. The concentrations of metronidazole used range from 0·25 to 8 μg./ml. and occasionally up to 32 μg./ml. Two series of tubes with metronidazole dilutions and two control tubes are set up for each strain. When a new batch of medium is used, two strains with known sensitivity to metronidazole are included as controls. Each tube in the dilution series is inoculated with 0·5 ml. of a 24-hr culture; for the strains studied this corresponds to about $2{\cdot}5 \times 10^5$ cells (Nielsen, 1969). The tubes are incubated at 36°C. and examined after 48 hrs incubation.

As a control for the presence of viable organisms, subcultures in medium without metronidazole are made from those tubes in which microscopy reveals from one to fifty motile *T. vaginalis* per 50 fields. These control tubes are read after 72 and 96 hrs' incubation. The sensitivity of the individual strain to metronidazole is recorded as the minimum inhibitory concentration (MIC) of metronidazole at which no living *T. vaginalis* are found after 48 hrs' incubation and subsequent subculture as above. The MIC is read for each of the two dilution series, and the final result is expressed as the geometric mean of these two values.

Results

NUMBER OF SAMPLES

During the 10-year period 1962-1971, there was a marked increase in the number of samples sent in

for investigation for *T. vaginalis*, from 1,100 in 1962 to almost 19,000 in 1971. In recent years the number of samples from men has increased; in 1971 these comprised 11 per cent. of the total. Over 90 per cent. of the samples originated from the Greater Copenhagen area.

Fig. 1 shows the monthly variations in the number of samples for the years 1963-1969; the figures for the even years have been omitted, as these graphs were found to be parallel to those shown. There is an increase in the number of samples submitted during the autumn, but the percentage of samples from which *T. vaginalis* was cultured remained the same throughout the year.

Fig. 2 shows the age distribution of 4,750 women in whom *T. vaginalis* was demonstrated either by direct microscopy or by culture in the three years 1968 to 1970. The Figure also shows the age distribution for over 7,000 women who had gonorrhoea in 1970 (Lind, 1973). The graphs for the two groups differ; the mean age of patients with gonorrhoea is considerably lower than that of patients with trichomoniasis. Of the women with trichomoniasis, 32 per cent. were under 25 years of age and another 32 per cent. were 40 years old or above, while for gonorrhoea about 85 per cent. were under 25 years of age and only 2 per cent. 40 years or more. There was no variation in the age distribution within the three years during which the 4,750 cases of trichomoniasis were diagnosed.

CULTURES

The results are given in Table I. During the first years *T. vaginalis* was found in about 25 per cent. of the samples, slightly less than half being detected only on culture. The Table shows that the

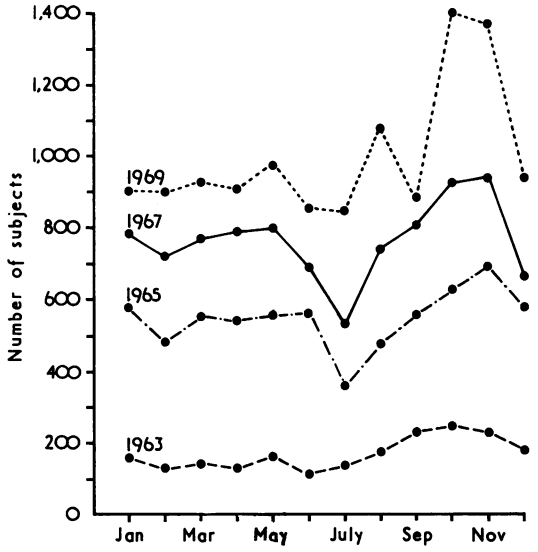

FIG. 1 *Number of specimens received for examination for* T. vaginalis, 1963-1969

TABLE I *Demonstration of* T. vaginalis *by direct microscopy and supplementary culture*

Year	*Number of specimens per year*	*Percentage of* T. vaginalis *positive*		
		Direct microscopy	*Culture only*	*Total*
1962	1,100	12·9	10·9	23·8
1963	1,995	12·8	12·8	25·6
1964	5,092	13·9	12·6	26·5
1965	6,572	10·3	10·1	20·4
1966	7,314	10·8	7·8	18·6
1967	9,113	10·2	8·3	18·5
1968	9,244	10·6	6·2	16·8
1969	11,951	8·6	5·7	14·3
1970	14,910	8·4	5·8	14·2
1971	18,937	7·8	5·1	12·9

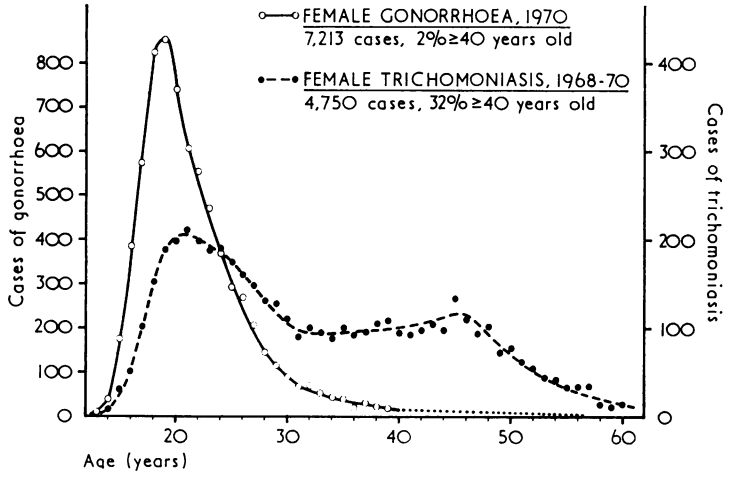

FIG. 2 *Number of women with trichomoniasis* (1968-70) *and with gonorrhoea* (1970), *by age groups*

number of positive samples decreases as the total number of samples increases. As the majority of samples are sent in without any clinical information, it has not been possible to divide them into diagnostic samples and those taken as a test of cure after metronidazole therapy.

Another factor of importance is that the number of samples from men has increased so much during recent years that this will affect not only the relation between the positive findings obtained by microscopy and those found by culture, but also the total percentage of positive findings. On an average only 1 to 2 per cent. of samples from men were found to be positive, and only about one in six of these were discovered by direct microscopy.

PRESENCE OF YEASTS

In cultures of samples taken for demonstration of *T. vaginalis* there is often a growth of yeast cells. Table II shows the incidence of yeast cells in samples which proved to be positive on culture and in those which proved to be negative for *T. vaginalis*. It can be seen that yeast cells were much more common in samples which were negative for *T. vaginalis*.

TABLE II *Incidence of yeast cells in specimens received for demonstration of* T. vaginalis

Year	T. vaginalis *positive*		T. vaginalis *negative*	
	Number	*Per cent. yeast*	*Number*	*Per cent. yeast*
1965	1,345	0·2	5,227	10·7
1966	1,365	1·8	5,949	14·2
1967	1,690	2·7	7,423	16·3
1968	1,550	3·2	7,694	18·9
1969	1,707	4·0	10,244	22·4
1970	2,111	4·7	12,799	20·1
1971	2,448	4·9	16,489	20·2

SENSITIVITY TO METRONIDAZOLE *IN VITRO*

The results of the determination of sensitivity of 93 strains of *T. vaginalis* are presented in Fig. 3. These results are derived from the first determination for each of the strains. The MIC ranged from 0·25 to 16 μg. metronidazole/ml. medium.

The trichomonicidal concentrations were between 0·25 and 4 μg./ml. in 93 per cent. of the strains. These values correspond to the serum concentrations obtained during oral metronidazole therapy. (Durel and others, 1960; Jennison, Stenton, and Watt, 1961; Kane, McFadzean, Squires, King, and Nicol, 1961). Six strains showed a reduced sensitivity to metronidazole; the MIC in four of the strains was 8 μg./ml., and in two it was 16 μg./ml.

In order to test the reproducibility of the method, the sensitivity determination was repeated twice or more on 63 of the 93 strains. An analysis of variance

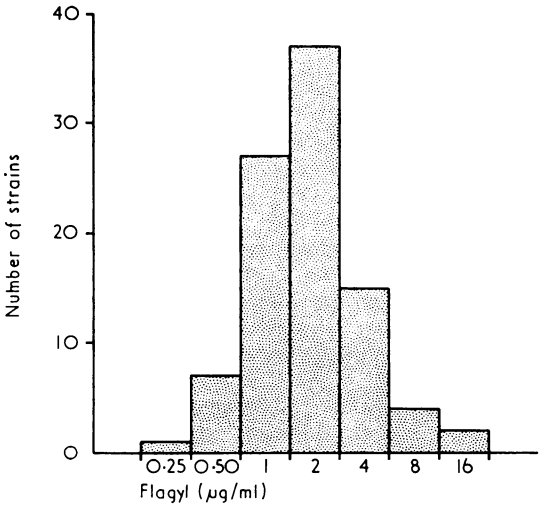

FIG. 3 *Trichomonicidal levels of Flagyl* (μg./ml.) *for* 93 *strains of* T. vaginalis

was carried out by M. W. Bentzon, Department of Biostatistics, Statens Seruminstitut, and the standard deviation was calculated to be half a dilution step. In practice this means that, on repeated determination on the same strain, there may be a difference of one dilution step, but rarely more. The variance is therefore sufficiently small to make it theoretically possible for sensitivity determinations to help identify strains isolated from two different patients.

Discussion

T. vaginalis infestation is stated to occur in about 25 per cent. of all women (Trussell, 1947; Burch and others, 1959a). The highest incidence is found in women attending VD clinics, and in those who congregate in environments in which promiscuity is accepted. Among patients attending the Dermato-venereological Department of the Marselisborg Hospital, Aarhus, Denmark, direct microscopy revealed trichomoniasis in 47 per cent. of samples from 292 women (Bundgaard, 1965). In other studies of women with vaginal discharge and/or vaginitis, *T. vaginalis* has been found in between 23 and 49 per cent. of samples, while in contrast the organism was found in only 1 to 15 per cent. of women without symptoms (Kucera, 1957). Apart from known contacts of infected women, the incidence in men is lower (from 2 to 30 per cent.) being highest in a group of patients with non-specific urethritis (Bauer, 1957). Among male consorts of women with trichomoniasis, the organism has been found in from 18 to 76 per cent. of cases (King, 1964).

The percentage of samples in which it was possible

to demonstrate *T. vaginalis* fell from 26·5 to 12·9 per cent. between 1964 and 1971. The most probable explanation of this decrease is that an increasing proportion were follow-up specimens from patients who had been treated with metronidazole, and an increasing number of samples were from men.

There was a significant difference between the incidence of yeasts in the samples which were *T. vaginalis* positive and those which were negative (Table II), and with some reservations this might be taken as evidence that many of these latter samples had been taken as tests of cure after metronidazole therapy (Beveridge, 1964; Clark and George, 1966). However, the incidence of yeast cells in the samples which were negative for *T. vaginalis* is of the same order as that reported in other groups, whether or not these consisted of women with or without genital infection (Mårdh, Stormby, and Weström, 1971). The difference observed in the present study would suggest that yeast cells do not thrive in the presence of *T. vaginalis*, or in the conditions which encourage infestation with *T. vaginalis*.

It has been debated whether trichomoniasis should be considered a venereal disease. It is generally accepted that *T. vaginalis* may be transferred by direct sexual contact, but it is not possible to exclude other modes of infection. Thus, it has been demonstrated that *T. vaginalis* can survive for up to 45 minutes in vaginal secretion placed on toilet seats (Whittington, 1957), and it is also possible that drops of infected urine sprayed up onto the toilet seat during defaecation may act as a source of infection (Burgess, 1963). Finally, the literature contains reports of newborn babies which had been infected during delivery, and of small girls infected by means of wash cloths and suchlike (Littlewood and Kohler, 1966; Blattner, 1967). It is noteworthy that the graphs of the age distributions (Fig. 2) for women with gonorrhoea and trichmoniasis are quite different (Lind, 1973); the occurrence of numerous cases of trichomoniasis in the years around the menopause is particularly remarkable.

Metronidazole is still the treatment of choice in trichomoniasis, giving a cure rate of about 90 per cent. It is generally non-toxic, and side-effects are rare. It has no action against gonococci, Döderlein's bacilli, yeasts, and a number of dermatophytes (Cosar, Ganter, and Julou, 1961). The possibility that metronidazole might have a therapeutic or masking effect in syphilis was investigated by Wilkinson, Rodin, McFadzean, and Squires (1967), who found that undiluted serum from eight patients receiving treatment with metronidazole was capable of immobilizing *Treponema pallidum* (Nichols pathogenic strain) *in vitro*. The serum concentration of metronidazole was 5·0 μg./ml. or more in six of these cases. They treated one patient suffering from primary syphilis with the dose of metronidazole normally employed in trichomoniasis (200 mg t.i.d for 7 days), but found that there was no evidence of healing of the lesion, and dark-field microscopy continued to reveal motile *Treponema pallidum*. In contrast, Davies (1967) found that high doses of metronidazole (2-4 g. daily for up to 9 days) caused local healing and disappearance of the treponemes in six patients with secondary syphilis (condylomata lata). In three of these patients the serum concentrations of metronidazole were found to be 44·1, 61·5, and 72·5 μg./ml., while the serum concentrations in patients treated with standard doses of metronidazole are in the region of 5 μg./ml. (Jennison and others, 1961; Kane and others, 1961). The authors concluded that it was improbable that metronidazole used in standard doses would be capable of masking syphilis. However, as the metronidazole content of the serum might invalidate the *Treponema pallidum* immobilization test, it is inadvisable to administer metronidazole therapy to patients in whom syphilis is suspected.

Holm and Mobacken (1972) tested the effect of metronidazole in different concentrations on *Treponema pallidum* (Nichols) *in vitro* and *in vivo* in rabbits. They demonstrated immobilization of spirochaetes at concentrations of 10 μg./ml. and higher, but they found no effect below 1 μg./ml. As this immobilization is not complement-dependent, it cannot be mistaken for a specific TPI antibody reaction. Experiments in rabbits with syphilitic orchitis failed to disclose any effect of parenteral metronidazole on the spirochaetes. The highest dose administered was about 2·5 times that usually given to patients.

Sensitivity determinations in 93 strains of *T. vaginalis* revealed that the MIC in 93 per cent. ranged from 0·25 to 4 μg./ml. These values correspond to those which are reached in the serum during oral treatment with standard doses of metronidazole, *i.e.* 200 mg. three times a day for 7 days. In this connection it may be mentioned that the sensitivity to metronidazole demonstrated here is not influenced by any possible trichomonicidal action of the serum itself (Forsgren, 1972), as the serum used as part of the Diamond's medium in the sensitivity determinations was heat-inactivated (Diamond, 1957).

The MIC for six strains was higher than 4 μg.ml.; four strains required 8 μg. metronidazole/ml., and two required 16 μg./ml. The two patients from whom these last two strains were isolated had both been treated repeatedly with standard doses of metronidazole with only transient effect or none. It was

possible to obtain clinical information for two of the other patients with less sensitive strains. One patient was treated repeatedly with a transient effect or none, and the other was cured.

Highly resistant strains have thus not so far been observed, but less sensitive strains were encountered which gave rise to therapeutic problems when metronidazole was used in standard dosage. In such rare cases sensitivity determinations would provide valuable guidance for future therapy.

Summary

The results are reported of diagnostic examination of secretions for *Trichomonas vaginalis*, and a method of determining the sensitivity of the organisms to metronidazole is described. The factors of importance in the transport of samples to the laboratory, and in microscopy and culture are mentioned in brief.

During the 10-year period 1962-71, the annual number of samples received increased from 1,100 to almost 19,000, and there was a decrease from about 25 to 13 in the percentage of samples in which *T. vaginalis* was demonstrated.

The age distribution of women with trichomoniasis, which was unchanged throughout the 3 year period 1968-1970, was found to be quite different from that of women with gonorrhoea. Many cases of trichomoniasis were observed at the time of the menopause.

In 93 per cent. of the total of 93 strains of *T. vaginalis* studied, the minimum inhibitory concentration of metronidazole ranged from 0·25 to 4 μg./ml. The MIC in four strains was 8 μg./ml. and in two strains 16 μg./ml. No strain examined was found to be highly resistant.

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Trichomonas vaginalis

II. Recherches de laboratoire dans la trichomonase

SOMMAIRE

On rapporte les résultats de l'examen des sécrétions en vue du diagnostic du *Trichomonas vaginalis* et on décrit une méthode pour déterminer la sensibilité de l'organisme au métronidazole. On mentionne brièvement des facteurs ayant une importance dans le transport des échantillons au laboratoire ainsi que dans l'examen microscopique et dans la culture.

Pendant la période de 10 ans 1962-1971, le nombre des échantillons reçus annuellement est passé de 1.100 à presque 19.000 et il y eut une diminution d'autour de 25 à 13 dans le pourcentage des échantillons dans lesquels le *T. vaginalis* fut mis en évidence.

La distribution selon l'âge des femmes atteintes de trichomonase qui resta inchangée tout au cours d'une période de 3 ans, 1968-1970, a été trouvée complètement différente de celle concernant les femmes gonococciques. Beaucoup de cas de trichomonase furent observés à l'époque de la ménopause.

Dans 93 pour cent d'un total de 93 souches étudiées de *T. vaginalis*, la concentration minima inhibitrice (C.M.I.) de métronidazole alla de 0,25 à 4 μg./ml. La C.M.I. fut à 8 μg/ml pour quatre souches et à 16 μg/ml pour deux souches. Aucune des souches examinées ne fut trouvée hautement résistante.