Group Research and Development Centre

SOUTHAMPTON ENGLAND

BRITISH – AMERICAN TOBACCO COMPANY LIMITED

A. 7-9-'74

THE ISOLATION OF LISTERIA MONOCYTOGENES FROM TOBACCO - METHOD DEVELOPMENT

LABORATORY REPORT NO. L.465-R 30.4.1974

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Group Research & Development Centre,
British-American Tobacco Co. Ltd.,
SOUTHAMPTON.

PCS/RA/61A

30th April, 1974

THE ISOLATION OF LISTERIA MONOCYTOGENES FROM TOBACCO - METHOD DEVELOPMENT

(Laboratory Report No. L.465-R)

SUMMARY

A procedure, reported in the literature, for the isolation of the pathogenic bacterium *Listeria monocytogenes* from human and veterinary clinical material has been modified for use with fermented tobacco.

With a reduced level of one of the selective agents, trypaflavin, the test *Listeria monocytogenes* strain was detected at a level approximately equivalent to 25 cells per gram in the presence of 10^8 per gram indigenous tobacco bacteria.

The procedure adopted gave substantially better recovery of the test strain in the presence of tobacco than did two methods reported in the literature, one of which was described as satisfactory for use with fermented vegetable material (silage). It is suggested that the procedure be used to survey a variety of air-cured fermented tobaccos for the presence of *Listeria monocytogenes*.

INTRODUCTION

A comprehensive review of the bacteriology and significance of *Listeria monocytogenes* is provided by Gray and Killinger (1).

Listeria monocytogenes was first described, in detail, in 1926 by Murray, Swann and Webb as the causative organism of a septicaemic disease in the rabbit characterised by the appearance in the circulation of large numbers of mononuclear leucocytes. The species name is derived from this feature of the condition.

At the present time the genus *Listeria* is considered to contain three additional species: *L. grayi*, *L. denitrificans* and *L. murrayi*, which may be differentiated from *L. monocytogenes* and from each other according to the criteria of Welshimer and Meredith (2) given in Table 1. These species, unlike *L. monocytogenes*, are not considered to be pathogenic to man or other animals. The pathogenicity of *L. monocytogenes* to man and a wide variety of other animal species is now well recognised. The first isolation from a human subject is credited to Nyfelt in 1929 since which time some 18 forms of human listeriosis have been described. Included are encephalitis, septicaemia, pneumonia and endocarditis. The gravid uterus appears to be particularly susceptible and *L. monocytogenes* is associated with habitual abortion. Serological evidence suggests the involvement of the organism in cases of mental retardation in children. Mair (3) draws attention to the elusive character of *L. monocytogenes* and considers that the figures for notifications of listeriosis, both world wide and in the U.K. (see Table in text) support the view that "listeriosis is not so much a rare disease as

a rarely recognised one". Mair considers that the figures emphasise the difficulty that many workers have experienced in attempting to confirm diagnoses of listeric syndromes, the only valid method of which is to isolate and characterise the causative organism.

	Notifications of Cases of Human Listeriosis (from Mair (3))	
Period	World Wide	U.K.
1930-1955	< 300	(1945-1955) 1
1956-1960	1200	12
1961-1965		99
1966 Jan-June		14

A variety of animal species are subject to listeric infection.

Listeria monocytogenes has been isolated from at least 17 avian species from all continents with the exception of Africa and Antarctica. Outbreaks of septicaemia in gosling and chicken flocks can assume economically significant proportions with mortality rates up to 40% within a few hours of onset (1).

Among aquatic species *Listeria monocytogenes* causes a disease in rainbow trout with up to 50% mortality rates and has been recovered from crustaceans and the stream water forming their habitat (1).

Listeria monocytogenes has been isolated from at least 37 mammalian species including domesticated and feral ruminants and monogastric animals (1). In ruminants listeriosis is typified by an encephalitis, in monogastric animals a septicaemia is the most common manifestation. Listeric abortion in sheep and cattle is well known. In a number of

countries it has been shown that ruminants fed on silage, i.e. fermented vegetable material, contract listeriosis more often than those fed on other foodstuffs. In Iceland the disease known as 'Votheysveiki', or silage sickness, of which *Listeria monocytogenes* is the causative organism, has been a serious problem since the beginning of the century. Gray (4) has established a definite epidemiological relationship between oat silage infected with *Listeria monocytogenes* and listeric encephalitis in sheep fed on this material, the same serotype of *Listeria monocytogenes* (type 4b) being isolated from the silage and from the brains of mice inoculated with silage extract. Gray considered that the upper respiratory tract and/or migration along the trigeminal nerve are possible infection routes, both vulnerable during the ingestion of silage. In a separate investigation Gray (4) found the same *Listeria monocytogenes* serotype (type 1) in corn silage and in the aborted foetuses of cattle fed this material. Blenden and co-workers (5) established that 'good' quality silage (pH 4.2) would not support the growth of *Listeria monocytogenes*. 'Poor' quality silage (pH 6.3) supported growth of *Listeria monocytogenes* to the extent of a 200 fold increase within 24 hours. Reversal of the pH values in the respective silage qualities also reversed their abilities to support the growth of *Listeria monocytogenes*. Blenden considered that rodents and avian carriers were possible sources of the organism in ensiled materials but Welshimer (6) has demonstrated that *Listeria monocytogenes* can survive in moist fertile soil for 295 days, and that it can be recovered from dead corn stalks held in fields over winter (7) suggesting that the organism occurs naturally on materials harvested for ensilement.

The observations that *Listeria monocytogenes* can occur on vegetation under natural conditions or by virtue of contamination from animals, can survive in soil for long periods, can grow under certain conditions in fermenting vegetable materials together with the evidence for its pathogenicity to man, raises the question of its survival and growth during tobacco leaf fermentations, e.g. those employed during the production of air-cured fermented leaf. The question is particularly pertinent in view of the findings in some samples of such leaf of large populations of diphtheroid bacteria (8). Diphtheroid bacteria are a heterogeneous collection of bacteria, commonly regarded as being of no medical significance but which share morphological and cultural features with *Listeria monocytogenes* which make them almost indistinguishable from each other. Herzberg (9) has questioned the desirability of discarding cultures of diphtheroid bacteria without first establishing whether hitherto unrecognised strains of *Listeria* are present.

It was considered that a method for isolating and rapidly and accurately characterising *Listeria monocytogenes* from fermented tobaccos should be developed.

The literature records a number of methods for the isolation of *Listeria monocytogenes* from a variety of materials. Two basic approaches have been made:

I <u>The 'Cold Enrichment' Procedure</u>

The procedure is described diagrammatically below. Under Henry's oblique illumination (10) *Listeria* colonies appear as low convex, entire, bright blue green to light green in colour with a glistening appearance

EXAMINATION FOR LISTERIA MONOCYTOGENES USING THE COLD ENRICHMENT PROCEDURE

Test material held at 4°C

↓

At desired intervals (weekly, monthly) subculture to agar plate

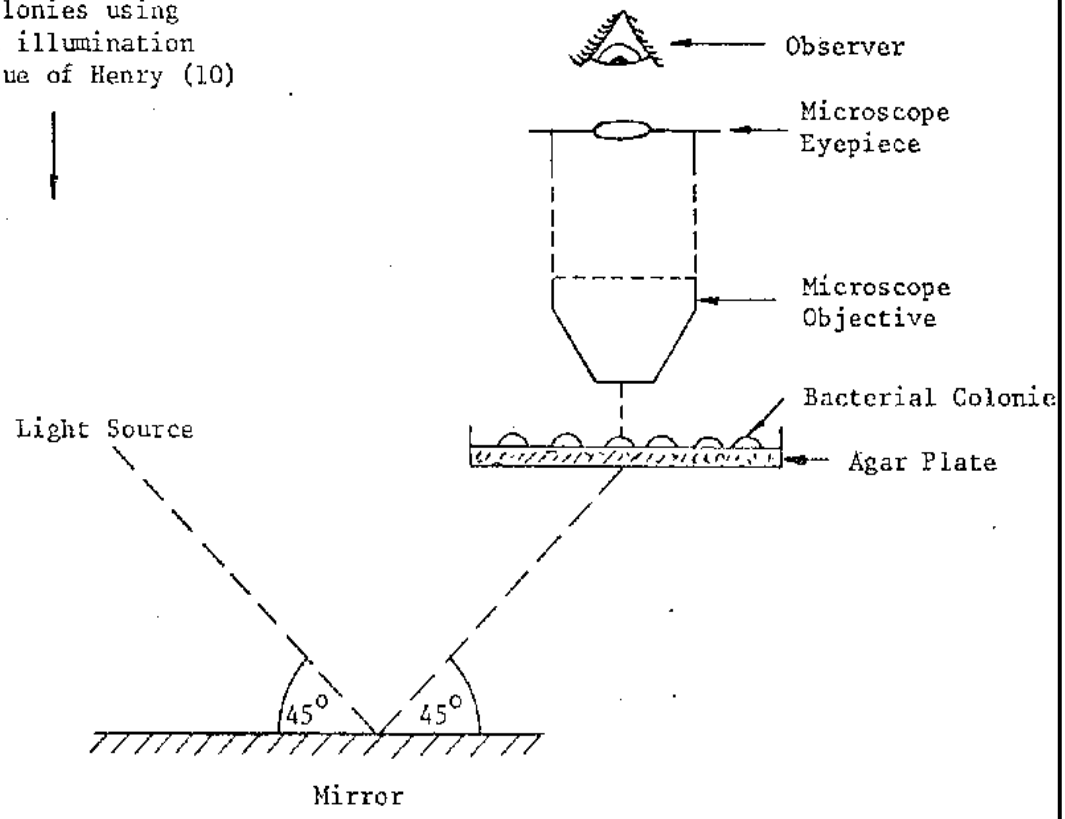
↓

Incubate plate at 37°C for minimum 24 hours

↓

View colonies using oblique illumination technique of Henry (10)

↓



and are readily differentiated from the colonies of 'other' bacteria. The cold enrichment method was first used by Gray (11) who, in 1948, confirmed clinical diagnoses of ovine listeriosis by isolating *L. monocytogenes* from the brains of sheep 5 weeks to 3 months following refrigeration of the material at 4°C.

Since that time a number of workers have used this approach in attempts to isolate *Listeria monocytogenes* from a variety of materials, but it is apparent that the length of holding time required for detection is inconveniently long in many instances. Errebo-Larsen (12) found that 31% of the faeces samples he examined required several months incubation before showing positive results. Ralovich et al. (13, 14) used a 4 week period for the isolation of *Listeria monocytogenes* from the faeces of slaughter house workers and freshly slaughtered swine with subculture to a selective medium for 2 days.

Dijkstra (15) demonstrated the survival of *L. monocytogenes* in materials held at 5°C as follows:- naturally infected bovine brain tissue greater than 6 years, bovine faeces greater than 5 years, milk greater than 2 years and silage 6 years. Bojsen-Møller (16) used 4°C incubation to isolate *L. monocytogenes* from the mixed microbial flora of human faeces and found that the holding period required was up to 14 weeks, with 22% of his samples requiring more than 12 weeks incubation before the first isolation.

In this investigation Bojsen-Møller showed that, following incubation at 4°C the number of *L. monocytogenes* cells which could be repeatedly detected was lower by factors of 10 and 100 than the number of cells detectable after incubation at 10°C and 37°C respectively.

II The Use of Culture Media Selective for *Listeria monocytogenes*

This approach has been used by numerous workers to suppress the growth of unwanted bacteria in samples with mixed microbial floras, thus permitting the relatively unhindered growth of those *Listeria* cells present. Although sometimes used following cold enrichment, the main attraction of the approach is that inconveniently long cold enrichment periods may be dispensed with and results obtained within days rather than months. Compounds reported to have some value as selective agents for *Listeria* include polymixin B (16, 17); nalidixic acid (13, 14, 15, 18); potassium thiocyanate, thallous acetate, colomycin, chloramphenicol and furacin (19); sodium thioglycollate (13); nalidixic acid with trypaflavin (neutral acriflavin) (14) and with other acridine dyes or potassium thiocyanate (20); methylene blue in combination with polymixin B and nalidixic acid (21). Ralovich et al. (14) showed that a combination of nalidixic acid and trypaflavin gave a much higher proportion of *L. monocytogenes* isolations from human faeces and swabs of slaughtered swine than did the use of nalidixic acid alone. They found that used alone nalidixic acid permitted the growth of a variety of gram positive cocci (often present on tobacco samples) which interfered with the growth and recognition of *L. monocytogenes*. This observation agrees with that of Behrens and Tahon-Castel (18) who found that most streptococci and some staphylococci were not inhibited on a nalidixic acid medium. In view of the success achieved by Ralovich and his co-workers in isolating *L. monocytogenes* from organic material known to contain large populations of a variety of bacterial types, it was decided to evaluate their nalidixic

acid trypaflavin combination for its selective qualities to *L. monocytogenes* in the presence of tobacco. Since no information is available on the extent to which *L. monocytogenes* occurs on tobacco naturally, it was decided to use a known strain of *L. monocytogenes* to inoculate samples of tobacco for recovery studies. The *L. monocytogenes* strain used was No. 10357 of the National Collection of Type Cultures. The tobacco was air-cured, fermented Indonesian leaf supplied through the courtesy of H. Wintermans Sigarenfabriken, Holland.

RESULTS

Initial experiments were made with pure cultures of the stock *Listeria monocytogenes* strain to establish the feasibility of using the 'spread plate' or 'drop plate' methods (22) for enumerating viable *Listeria* cells. Tables 2 and 3 give the results of comparisons between these methods and the pour plate technique normally used for counting viable bacteria in Group R. & D. Centre (23). It proved difficult to prepare glass pipettes with a constant orifice diameter and tests were made using 18 S.W.G. hypodermic syringe needles calibrated to the correct specification (see Methods Appendix A). The comparisons showed that both 'spread plate' and 'drop plate' methods gave results comparable to the pour plate count.

A suggestion by Gray (1) that salts solutions were deleterious to *Listeria* cells prompted the comparison in Table 4 between the standard Ringer solution diluent used in this laboratory and the recommended 0.1% w/v peptone water. The suggestion was not confirmed in respect of our *Listeria* strain and Ringer solution was retained as the standard diluent in our trials.

In order to assess the toxicity of the selective agents to the test *Listeria monocytogenes* strain, the materials were separately titrated against the test organism. Table 5 shows the results of a nalidixic acid (Sigma Chemical Co., St. Louis, U.S.A.) titration. No material difference in the recovery of *Listeria* cells was observed with nalidixic acid levels twice that used by Ralovich et al. (13). Titrations were carried out similarly using trypaflavin (neutral acriflavin, No. A-8126 Sigma Chemical Co., St. Louis, U.S.A.). The results in Tables 6a and 6b show that recovery of the *Listeria monocytogenes* test strain was reduced as the concentration of trypaflavin was increased.

Comparison of the recovery rates of the test strain when trypaflavin was added to the base medium before and after heat sterilisation (121°C for 15 minutes) is made in Figure 1 from the data in Tables 6a and 6b. With the medium containing heated trypaflavin the proportion of *Listeria* cells recovered dropped, with increasing trypaflavin concentration, to a progressively lesser extent than that with the medium containing unheated trypaflavin. The maximum difference in recovery was 25% and because this could have been a consequence of heating it was decided to add the trypaflavin, as a filter sterilised solution, to the heat sterilised base in order to avoid variations in the selective quality of the medium.

Following the recommendation of Ralovich et al. (14) a trial was conducted to assess the value of adding sterile horse serum (HS3, Oxoid Ltd., London) at the rate 5% v/v to the trypaflavin medium, as an extra nutrient source. The effect of this on *Listeria* recovery is shown in Table 7

and Figure 1. In the presence of horse serum trypaflavin appeared substantially more inhibitory to the test strain. This was surprising in view of the wide use of horse serum for the enrichment of culture media used for the isolation of fastidious bacteria, particularly those of medical significance. In a trial set up concurrently to assess the selective properties of various levels of trypaflavin in combination with the standard nalidixic acid level to the *Listeria* test strain in the presence of tobacco bacteria, serum was added as an extra nutrient source. The results in Table 8 show that *Listeria* recoveries were very similar to those found in the first serum/trypaflavin trial with the same marked 'fall off' in recovery at greater than 4 µg/ml of trypaflavin. Inhibition of tobacco bacteria was satisfactory when the test and control (no trypaflavin) plates at a one in ten dilution of tobacco macerate were compared. Although encouraging in demonstrating that substantial inhibition of tobacco bacteria could be obtained with minimal inhibition of the test *Listeria* strain on an agar medium containing 4 µg/ml trypaflavin, it remained necessary to test the ability of nalidixic acid-trypaflavin combinations, in liquid media, to encourage the selective growth of small numbers of *Listeria* cells in the presence of air-cured fermented tobacco and the bacteria indigenous to it.

A series of trials was therefore conducted in which tobacco and *Listeria* cells were inoculated to tryptose-nalidixic acid-trypaflavin enrichment broth to give known final concentrations of *Listeria* cells, broth nutrients and selective agents (nalidixic acid 50 µg/ml, trypaflavin as specified in each trial). After incubation for 24 hours growth of

Listeria at each original inoculum level was assessed by plating aliquots to tryptose serum agar (control medium) and/or tryptose nalidixic acid trypaflavin (4 µg/ml) agar. The first enrichment trial is summarised in Table 9. Contact for 24 hours with 16 µg/ml of trypaflavin did not have the deleterious effect on the recovery of *Listeria* cells that might have been expected from the results in a previous trial with this level in agar (Table 6b). This was probably due to the tobacco reducing the selective properties of the trypaflavin. Recoveries on the selective agar, expressed as a percentage of the recovery on the tryptose serum agar following 4 µg/ml trypaflavin enrichment, are lower than on the appropriate control plates nevertheless, at 4 and 16 µg/ml trypaflavin the recoveries are of the same order and represent an increase of 1000 fold over the number of *Listeria* cells inoculated originally.

In subsequent trials (Tables 10 and 11) the growth of 'other' bacteria, i.e. those derived from the tobacco was followed in addition to that of the inoculated *Listeria* cells.

In both trials enrichment factors in the absence of tobacco were not less than 10^6. In the presence of tobacco *Listeria* enrichment varied. In one trial (Table 10) factors of 10^4 and 10^6 were recorded when respectively, the selective and non-selective agars were used, representing a 100 fold difference in the original *Listeria* inoculum level detected on these two media. In the second trial recovery was made from all *Listeria* inoculum levels on both plating media with less than 10 fold differences between the selective and non-selective agars and represented an enrichment factor of between 10^6 and 10^{10}. In both

trials the number of tobacco bacteria per ml of macerate were known and comparison with the number of 'other' bacteria at the conclusion of the enrichment procedure gives a measure of the extent to which those unwanted types have grown and therefore an indication of the selective quality of the medium. Tobacco, i.e. 'other', bacteria increased by factors of 10^3 (Table 10) and 10^1 (Table 11). Thus in these trials enrichment of *Listeria* over tobacco bacteria occurred by factors of 10^2 (Table 10) and at least 10^6 (Table 11) indicating that the procedure was selective to *Listeria*.

The detection level for *Listeria* cells varied. In one trial (Table 10) the detection level was equivalent to 2500 cells per gram when the selective agar was used compared to 25 with the non-selective agar. In the other trial (Table 11) the difference between recoveries on selective and non-selective agars were much smaller and *Listeria* were detected from an inoculum equivalent to 0.75 cells per gram of tobacco.

From the averages quoted in Tables 10 and 11 at a 1 in 10^5 dilution level for 'other' bacteria, it can be seen that the selectivity of trypaflavin agar at 4 µg/ml is sometimes marginal. Increasing the trypaflavin in order to reduce the number of tobacco bacteria recovered would almost certainly result in a decrease in recovery of *Listeria* (Table 6b). However, with *Listeria* enrichment of the orders seen in the above trials this may not present a problem. In the trials described the objective was the maximum recovery of *Listeria* and higher trypaflavin levels in the recovery agar have not been used. It would seem prudent to use both tryptose agar, for maximum recovery of *Listeria* and trypaflavin agar, at a minimum level of 4 µg/ml, to provide some protection against the possibility of overgrowth of *Listeria* by tobacco bacteria.

The recovery of *Listeria* cells from a level of 0.015 cells per ml of mixed enrichment medium and tobacco macerate (Table 11) was surprising since the calculated total of *Listeria* cells in this system (total volume 20 ml) was only 0.3 cells. Since at least one whole viable cell in the total system would be necessary for growth, i.e. enrichment, to take place the calculated figure must be out by at least a factor of 3. This level of error stems from difficulties in estimating accurately very small populations of bacteria with a dilution plating technique.

In any event the detection of less than 10 cells per 10 ml of standard tobacco macerate, i.e. detection of 25 cells per gram of tobacco, in the presence of a mixed population of tobacco bacteria in excess of 10^8 per gram is considered to represent adequate performance for a selective enrichment procedure.

A feature of the trypaflavin medium is its yellow colour. To check the extent to which this might interfere with the accurate differentiation of *Listeria* and non-*Listeria* colonies two observers picked off colonies classified, according to their appearance under Henry's oblique illumination, as 'definitely *Listeria*' and 'probably not listeria'. Each isolate was examined for somatic ('O') antigens specific to *Listeria* using a commercially prepared *Listeria monocytogenes* Poly O antiserum ('Bacto' *Listeria* antiserum Poly O 2302 Difco Labs., Detroit, Michigan). The results are given in Table 12. The predictions of both observers concerning colonies sampled independently, from both the selective and non-selective agars, were all correct. All colonies chosen as *Listeria* went to the same titre (23) with the *Listeria* antiserum as did the control culture

and all those selected as 'probably not *Listeria*' showed no reaction with the antiserum. It was concluded that problems in recognition of *Listeria* colonies by this method were minimal.

Although recognition of *Listeria*-like colonies presented no difficulties, the removal of such colonies from the agar media was sometimes complicated by the proximity of non-*Listeria* colonies. An attempt to boost the enrichment of *Listeria* cells at the expense of other bacteria was made using a double enrichment procedure. After 24 hours at 37°C 1 ml of the original tobacco/enrichment broth/*Listeria* suspension was subcultured to a second 10 ml volume of sterile enrichment broth for a further incubation period.

Table 13 shows the results of such a trial. In the absence of tobacco enrichment factors between 10^6 and 10^{10} times the starting level for *Listeria* cells were obtained with recovery from an inoculum calculated at 0.03 cells per ml of enrichment broth, i.e. similar to that found in a previous trial (Table 11). In the presence of tobacco the results were disappointing. No *Listeria* colonies were detected in a confluent growth of tobacco bacteria. It is possible that the double enrichment procedure, with the levels of selective agents employed, gives those tobacco bacteria less sensitive to the agents sufficient time to produce populations large enough to swamp the *Listeria*. Improved performance might be obtained in a double enrichment procedure using different levels of trypaflavin at each stage, i.e. applying the argument used for increasing the trypaflavin concentration in the selective agar medium that, given a good first stage enrichment of *Listeria* one could afford to sacrifice

a greater proportion of these in the interests of greater selectivity against the tobacco bacteria than has been permitted so far.

It has been noticed in these trials and in the preparation of *Listeria* broth cultures, that despite substantial differences in inoculum levels there appears to be a growth 'ceiling' in the region of 10^7–10^8 cells per ml. The factors contributing to this are not understood but Tyrell (24) has suggested that an inherent autolytic factor may be present in many *Listeria* strains and has demonstrated with a number of these that from an initial level of 10^7 cells per ml optical density rose during incubation to a maximum at 8 hours and then declined due to autolysis, by 20-80% of this value over a 48 hour period. It is possible that such a mechanism contributes to our observed 'ceilings'.

Tables 14 and 15 show the results of comparisons made between our own and two enrichment procedures from the literature. Despierres (21) has used an enrichment both containing polymixin, nalidixic acid and methylene blue followed by subculture to polymixin agar. The procedure was designed to isolate *Listeria monocytogenes* selectively from human clinical material and faeces with the inhibition, in particular, of *Streptococcus faecalis*. The procedure of Kramer and Jones (19) using a thallous acetate - nalidixic acid enrichment broth followed by subculture to thallous acetate-nalidixic acid agar was reported to be suitable for the isolation of *Listeria* from a variety of materials including soil, manure and silage (at pH 9.0).

In the Despierre medium under pure culture conditions there appeared to be a loss of *Listeria* cells while in the presence of tobacco enrichment

was at least 10^2 times worse than with our procedure. Since this medium was designed to isolate *Listeria* from human clinical material its performance, in unmodified form, with tobacco is perhaps not surprising. The performance of the Kramer and Jones medium (Table 15) was however, considered disappointing in view of the reported successful isolation of *Listeria* from materials much closer in type to tobacco than clinical material. Unfortunately the level of enrichment found using this medium was grossly inferior to our own and recovery of *Listeria* from tobacco suspensions was not demonstrated. It was found necessary to omit the sodium chloride from the nutrient broth base described by Kramer and Jones to avoid precipitation on the addition of thallous acetate. Attempts to clarify the exact manner of preparation with one of the authors have not been successful.

DISCUSSION

A combination of nalidixic acid and trypaflavin at 50 and 4 µg/ml respectively final concentration in a commercial tryptose broth base has been used successfully to enrich *Listeria* cells in the presence of air-cured, fermented tobacco and the organisms indigenous to it. The performance of this medium was shown to be substantially better than two procedures described in the literature. Many compounds used as selective, i.e. inhibitory, agents in a microbiological culture medium will, above a certain concentration, progressively inhibit the microbial type(s) that it is desired to isolate. Thus a compromise has to be made between the levels of recovery of the desired type and 'other' bacteria. Because differentiation of *Listeria* from 'other' colonies presented little difficulty with the oblique light technique used,

it was decided to use levels of selective agents biased towards the maximum recovery of *Listeria* rather than the maximum suppression of other bacteria. On this basis the level of trypaflavin chosen was 4 µg/ml which is considerably lower than that recommended in the literature (14) (25). Improved performance, in terms of greater suppression of tobacco bacteria, probably could be obtained by raising the level of trypaflavin. The inevitable concomitant reduction in recovery of *Listeria* probably could be accepted in most cases in view of the *Listeria* enrichment factors observed in our trials.

Ralovich (25) refers to the necessity to titrate each batch of nalidixic acid and trypaflavin against the basal medium but has not, as far as we are aware, published such information. It is therefore not possible to assess the degree of selectivity offered by his medium. Recently Ortel (26) has used a nalidixic acid (40 µg/ml) agar and a nalidixic acid-trypaflavin (45 µg/ml) agar to isolate *Listeria* from clinical material. The latter medium always gave greater recoveries of *Listeria* and almost complete inhibition of faecal streptococci. It is interesting that Ortel reports that *Listeria* is inhibited by trypaflavin concentrations greater than 50 µg/ml medium but again, no titration trials are reported.

The variation between the levels of trypaflavin recommended by different workers in the literature and ourselves is probably a result of:

a. Variations in the effects of the materials under examination for *Listeria* on the selective properties of trypaflavin.

b. Variations in the selective, i.e. inhibitory properties of trypaflavins of different manufacture.

It would therefore seem necessary to titrate each selective compound against the micro-organism which it is desired to isolate from a given substrate, in the presence of that substrate and the microbial flora indigenous to it.

In summary, therefore, the test *Listeria monocytogenes* strain was successfully enriched in the presence of fermented tobacco and recovered on media which permitted its recognition by a simple illumination technique. The procedure finally adopted was:

1. 10 ml of tobacco macerate in Ringer Solution (1:25 w/v) was added to 10 ml of enrichment broth prepared at double strength to give final constituent concentrations as follows:

	grams/litre
'Bacto' tryptose broth (0062-01 Difco Labs. Detroit, Michigan)	26.0
Nalidixic acid (N8878 Sigma Chemicals, St. Louis, U.S.A.)	0.05 (50 µg/ml)
Trypaflavin (Neutral acriflavin No. A-8126 Sigma Chemicals)	0.004 (4 µg/ml)

(Added as filter sterilised solution following heat sterilisation of other components)

2. Incubate 37°C for 24 hours.
3. Subculture to:
   a. Tryptose agar ('Bacto' tryptose broth + 1.5% w/v 'Oxoid' agar No. 1 Oxoid Ltd., London).

b. Tryptose-nalidixic acid-trypaflavin agar. Nalidixic acid and trypaflavin levels as enrichment broth.

4. Incubate 37°C for a minimum of 24 hours.

5. Examine using Henry's oblique illumination. *Listeria* colonies present a distinct bright blue green to light green glistening appearance. The addition of horse serum appeared to enhance the inhibitory quality of trypaflavin but because this effect would need to be investigated and controlled and because no obvious advantage accrued from using it in the non-selective medium, its use was discontinued.

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APPENDIX

METHODS

An overnight broth culture of the stock *Listeria monocytogenes* strain was decimally diluted (23) in Ringer solution. Aliquots of dilutions between 10^{-5} and 10^{-9} inclusive were tested by each technique as follows.

(i) Pour Plate Method

1 ml of the appropriate dilution was pipetted into a sterile petri dish. Approximately 15 ml of molten tryptose agar ('Bacto', Difco Labs., Detroit, Michigan) cooled to 45°C was added and mixed with the inoculum.

(ii) Surface Spread Plate Method

0.2 ml of the appropriate culture dilution was pipetted onto the surface of a prepared dried tryptose agar plate and spread over the surface of the agar using a sterile glass 'hockey' stick (23).

(iii) Drop Plate Method

0.02 ml of the appropriate dilution was inoculated to the surface of a prepared, dried tryptose agar plate and allowed to dry in undisturbed. For each dilution examined a number of replicate drops were pipetted to separate areas of the same plate. The pipettes used were glass 'Pasteur' pipettes nominally of orifice diameter 0.036" to deliver 0.02 ml water (drop weight 20 ± 1 mg) but this proved difficult to control in preparation necessitating the calculation of a factor for each pipette. Alternative 'pipettes' were constructed from 18 S.W.G. hypodermic syringe needles, cut at right angles to the bore, to deliver water drops to the appropriate specification.

All cultures were incubated at 37°C for a minimum of 24 hours before counting colonies. In the pour plate and spread plate methods dilutions giving between 30 and 300 colonies per plate were selected for counting where possible. In the drop plate method dilutions were selected for counting where the colonies in each drop were discrete. Where possible two adjacent dilutions in the series were compared to check the validity of each other.

The number of viable *L. monocytogenes* cells in the original culture was calculated as follows:-

Average number of colonies from replicates of the chosen dilution x reciprocal of the dilution x reciprocal of the replicate inoculum volume = Number of cells per ml original culture.

FIG. 1 L 465 - R

RECOVERY OF LISTERIA IN PRESENCE OF TRYPAFLAVIN EFFECT OF HEAT STERILISATION AND ADDITION OF HORSE SERUM

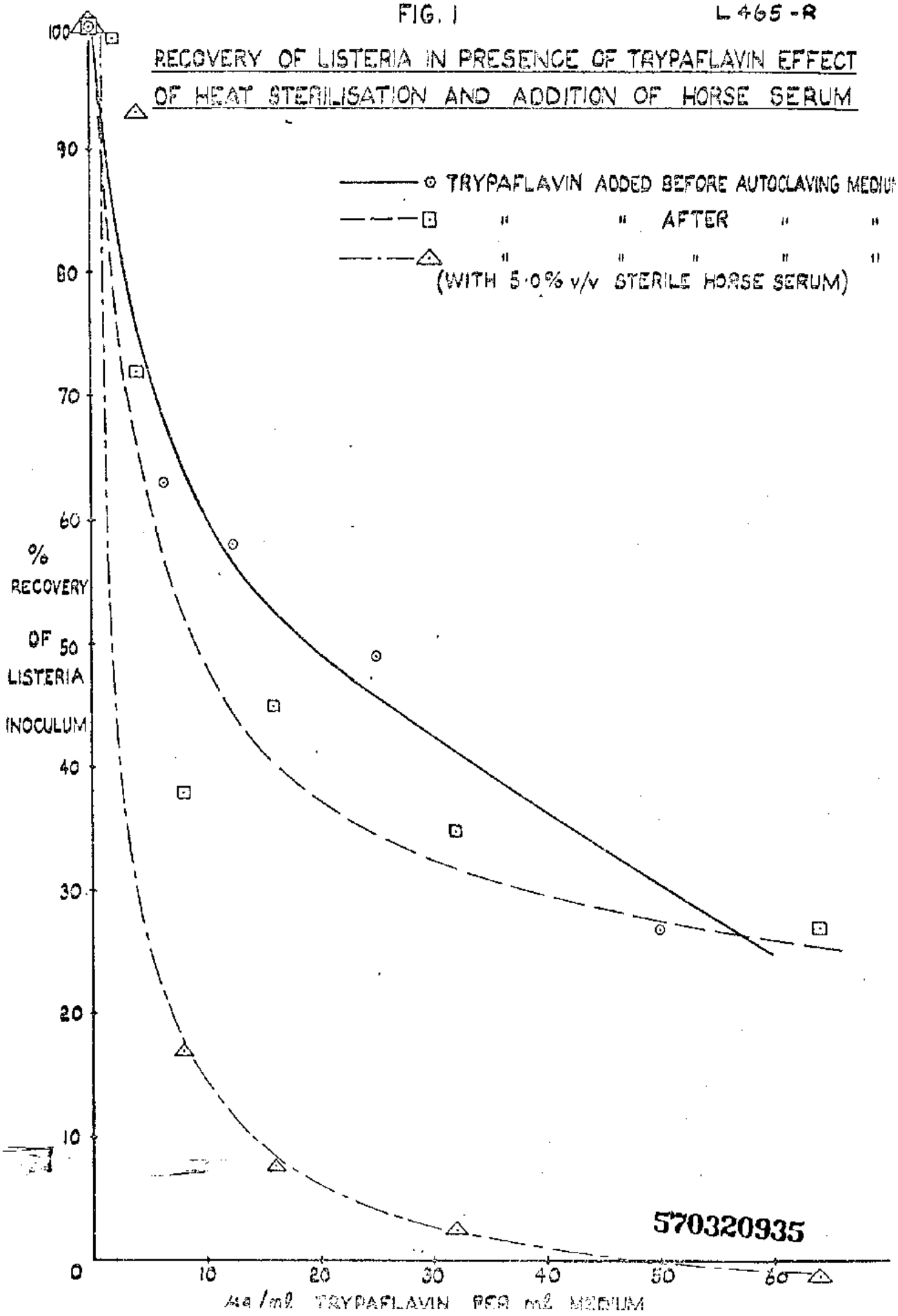

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TABLE 1

CHARACTERISTICS OF SPECIES OF LISTERIA

Listeria Group Characteristics	Gram positive rods, varying from cocco-bacilliary to filamentous forms. Occur in pairs, singly, occasionally in 'palisades'. Aerobic or facultative anaerobic growth, optimum temperature for growth 37°C, good growth at 4°C. Motile at 22-25°C rarely at 37°C. Catalase positive, cytochrome oxidase negative.			
Species Characteristics	*L. monocytogenes*	*L. grayi*	*L. denitrificans*	*L. murrayi*
Methyl Red	+	+	+	+
Voges Proskauer	+	+	-	+
Acid from Carbohydrates[1]				
Glucose	+	+	+	+
Lactose	V/D	+	+	+
Maltose	+	+	+	+
Salicin	+	+	+	+
Trehalose	+	+	+	+
Levulose	+	+	+	+
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Inulin	-	-	-	-
L-Arabinose	-	-	+	-
Glycogen	-	-	+	-
Mannitol	-	+	-	+
Xylose	-	-	+	-
Nitrate Reduction	-	-	+	+

1. + = Acid formed within 48 hours; - = No acid at 21 days;
V = Variable; D = acidification after several days.
For other tests, + = positive, - = negative.

TABLE 2

COMPARISON OF POUR PLATE, SURFACE SPREAD PLATE AND DROP PLATE COUNTING TECHNIQUES FOR THE ENUMERATION OF *LISTERIA MONOCYTOGENES* IN PURE CULTURE. (SEE APPENDIX FOR METHODS AND CALCULATION)

Culture Dilution Examined	Method of Count		
	Pour Plate (1.0 ml)	Surface Spread Plate (0.2 ml)	Drop Plate (0.02 ml)
10^{-5}	TNTC*	580, 576, 634	TNTC
10^{-6}	324,** 324, 320	57, 68, 64	6, 13, 10, 7, 11, 16, 13, 13, 17, 17
10^{-7}	33, 28 36	9, 6, 1	4, 5, 2, 1, 2, 0, 1, 0, 3, 1, 0, 0
10^{-8}	5, 1, 3	0, 0, 1	No colonies present
10^{-9}	1, 0, 0	0, 0, 0	
Listeria per ml original culture	3.22×10^8	3.00×10^8	3.69×10^8

* TNTC = Too numerous to count.

** Number of colonies per replicate test.

TABLE 3

COMPARISON OF DROP PLATE COUNT, USING A MODIFIED PIPETTE, WITH POUR PLATE COUNT FOR THE ENUMERATION OF *LISTERIA MONOCYTOGENES* IN PURE CULTURE. (SEE APPENDIX FOR METHODS AND CALCULATION)

Expt	Culture Dilution Examined	Method of Counting		
		Pour Plate (1.0 ml)	Drop Plate (0.02 ml) (Needle A)	Drop Plate (0.02 ml) (Needle B)
1	10^{-4}	TNTC*	TNTC	TNTC
	-5		126, 114, 122	96, 101, 112
	-6	528,** 464	23, 15, 13	12, 7, 10
	-7	40, 43	1, 1, 0	
	-8	4, 3	0, 1, 0	0, 0, 0
	-9	0, 0	0, 0, 0	
	Listeria per ml original culture	4.15×10^8	6.05×10^8	5.15×10^8
		Pour Plate	Drop Plate (Needle C)	Drop Plate (Needle D)
2	10^{-4}	TNTC	TNTC	TNTC
	-5			
	-6	554, 586	11, 13, 12	17, 15, 17
	-7	58, 63	3, 1, 2	2, 2, 3
	-8	10, 8	0, 0, 0	0, 0, 0
	-9	1, 1		
	Listeria per ml original culture	6.05×10^8	6.0×10^8	8.25×10^8

* As notes Table 2
**

TABLE 4

COMPARISON OF DILUENTS ON RECOVERY OF *LISTERIA MONOCYTOGENES* FROM PURE CULTURE SUSPENSION

	Diluent	
	Ringer Solution	0.1% Peptone Water
Recovery from 10^{-6} dilution of *Listeria Monocytogenes* culture	154 colonies ≡ 3.08×10^8 viable cells per ml original suspension	85 colonies ≡ 1.70×10^8 viable cells per ml original suspension

TABLE 5

TITRATION OF NALIDIXIC ACID IN TRYPTOSE AGAR AGAINST A *LISTERIA MONOCYTOGENES* PURE CULTURE SUSPENSION

Nalidixic acid level in base agar[1], µg/ml	0	25	50	75	100
Number of *Listeria monocytogenes* colonies, average of six replicate drops of 0.02 ml of a 10^{-5} culture dilution	Avg = 18.5 ≡ 9.25×10^7 cells per ml original suspension	19.0 ≡ 9.5×10^7	20.8 ≡ 1.04×10^8	20.8 ≡ 1.04×10^8	19.8 ≡ 9.9×10^7

1. 'Bacto' Tryptose agar, Difco Laboratories, Detroit, Michigan.

TABLE 6a

TITRATION OF TRYPAFLAVIN AGAINST *LISTERIA MONOCYTOGENES* PURE CULTURE SUSPENSION
(TRYPAFLAVIN ADDED TO MEDIUM BEFORE HEAT STERILISATION)

Trypaflavin level in base agar[1], µg/ml	0	6.25	12.5	25	50	100	200
Number of *Listeria monocytogenes* colonies, average of six replicate drops of 0.02 ml of 10^{-5} culture dilution	18.5 ≡ 9.25×10^7 cells/ml original suspension	11.7 ≡ 5.85×10^7	10.8 ≡ 5.40×10^7	9.1 ≡ 4.55×10^7	5.0 ≡ 2.5×10^7	0	0
% Recovery	100	63	58	49	27	<1	<1

1. 'Bacto' Tryptose agar, Difco Laboratories, Detroit, Michigan.

TABLE 6b

TITRATION OF TRYPAFLAVIN AGAINST *LISTERIA MONOCYTOGENES* IN PURE CULTURE SUSPENSION
(TRYPAFLAVIN ADDED TO MEDIUM AFTER HEAT STERILISATION)

Trypaflavin level in base agar[1], µg/ml		0	2	4	8	16	32	64
Number of *Listeria monocytogenes* colonies, average of six replicate drops of 0.02 ml of culture dilution:-	10^{-4}	TNTC[2]	TNTC	TNTC	TNTC	TNTC	TNTC	63.8 ≡ 3.19×10^7
	10^{-5}	22.5 ≡ 1.13×10^8 cells per ml original suspension	22.2 ≡ 1.11×10^8	16.2 ≡ 8.10×10^7	8.5 ≡ 4.25×10^7	10.2 ≡ 5.08×10^7	7.8 ≡ 3.9×10^7	3.5
% Recovery		100	98.7	72	37.8	45	35	28[3]

1. As Table 6a.
2. TNTC = (Colonies) 'Too numerous to count'.
3. Based on 10^{-4} figures.

TABLE 7

TITRATION OF TRYPAFLAVIN AGAINST *LISTERIA MONOCYTOGENES* PURE CULTURE SUSPENSION IN PRESENCE OF HORSE SERUM (TRYPAFLAVIN AND HORSE SERUM ADDED AFTER HEAT STERILISATION)

Additions to tryptose agar base	Trypaflavin µg/ml	0	0	2	4	8	16	32	64
	Horse serum ml per 100 ml base	0	5	5	5	5	5	5	5
Number of *Listeria monocytogenes* colonies, average of six replicate drops of 0.02 ml of culture dilution:-	10^{-2}								Avg 5.3 $= 2.65 \times 10^4$
	10^{-3}					Avg 59.8 $= 2.99 \times 10^6$	27.6 $= 1.38 \times 10^6$	9.3 $= 4.65 \times 10^5$	0.5
	10^{-4}	41.6 $= 2.08 \times 10^7$ per ml original suspension	35.1 $= 1.76 \times 10^7$	37.0 $= 1.85 \times 10^7$	32.8 $= 1.64 \times 10^7$	7.1	2.2	0.5	
	10^{-5}	4.1	6.0	5.0	4.0				
% Recovery		*Listeria* suspension control	100	105	93	17	7.8	2.6	0.15

TABLE 8

TITRATION OF TRYPAFLAVIN AGAINST *LISTERIA MONOCYTOGENES* TOBACCO SUSPENSION IN THE PRESENCE OF NALIDIXIC ACID AT 50 µg/ml

	Tobacco Macerate Dilution and Colony Type Isolated		Agar Recovery Medium			
			Tryptose Serum 50 µg per ml Nalidixic Acid (TSN)	TSN + 4 µg per ml Trypaflavin	TSN + 16 µg per ml Trypaflavin	TSN + 50 µg per ml Trypaflavin
Number of bacterial colonies, average of six replicate drops of 0.02 ml at given dilutions of tobacco macerate inoculated with *Listeria monocytogenes* suspension	10^{-1}	*L. monocytogenes*	TNTC[2]	TNTC	TNTC	TNTC
		Others	TNTC	0.3	0.2	0
	10^{-2}	*L. monocytogenes*	TNTC	TNTC	TNTC	Avg 30.3 = 1.52×10^5
		Others	Avg 7.7	0.6	0.3	0
	10^{-3}	*L. monocytogenes*	TNTC	TNTC	Avg 24.0 = 1.2×10^6	2.3
		Others	Avg 7.2	0	0	0
	10^{-4}	*L. monocytogenes*	Avg 22 = 1.1×10^7 per ml original suspension	Avg 19.7 = 9.85×10^6	2	0
		Others	1	0	0	0
	10^{-5}	*L. monocytogenes*	Avg 3.3	Avg 3.3	0	0
		Others	0	0	0	0
	% Recovery of Listeria		100 (Control, no trypaflavin)	90	11	1.4

NOTES: 1. Air-cured fermented Indonesian leaf.
2. TNTC = Too numerous to count. Colonies coalesced could not be separated.

TABLE 9

RECOVERY OF *LISTERIA MONOCYTOGENES* FROM TOBACCO MACERATE USING TRYPTOSE-NALIDIXIC ACID ENRICHMENT MEDIUM WITH VARIOUS LEVELS OF TRYPAFLAVIN

Enrichment broth/ tobacco macerate combination		Tryptose-nalidixic acid (50 µg/ml) + tobacco (1:50 w/v) + trypaflavin 30°C for 24 hours					
Trypaflavin level (µg/ml)		4		16		50	
Subculture agar		TS[1]	TNST4[2]	TS	TNST4	TS	TNST4
Number *Listeria monocytogenes* cells inoculated per ml broth before incubation		Number *Listeria monocytogenes* cells per ml broth after enrichment (% recovery of *Listeria* cells)					
	4.25×10^6						
	4.25×10^5						
	4.25×10^4	*Listeria monocytogenes* colonies too numerous to count, growth confluent					
	4.25×10^3						
	4.25×10^2						
	42.5					850 (21%)	650 (16%)
	4.25	4.1×10^3 (100%)	3.1×10^3 (75.6%)	4.75×10^3 (116%)	3.2×10^3 (78%)	750 (18%)	400 (10%)

Notes: 1. TS = Tryptose serum agar.
2. TNST4 = Tryptose nalidixic acid (50 µg/ml) - trypaflavin (4 µg/ml) - horse serum (5% v/v) - agar.

TABLE 10

RECOVERY OF *LISTERIA MONOCYTOGENES* USING TRYPTOSE NALIDIXIC ACID (50 µg/ml)-TRYPAFLAVIN (4 µg/ml) BROTH TO SHOW THE EFFECT OF TOBACCO

Enrichment Broth		Tryptose nalidixic acid (50 µg/ml) – trypaflavin (4 µg/ml)								Tryptose nalidixic acid – trypaflavin + tobacco macerate 1:50 w/v[8]									
Listeria cells per ml broth		50		5		0.5		0.05			50		5		0.5		0.05		
Subculture medium (agar)		TS[1]	TNT4[2]	TS	TNT4	TS	TNT4	TS	TNT4		TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4	
Recovery of bacteria, Number of colonies, average of three replicate drops of 0.02 ml at culture dilutions	10^{-4}	TNTC[3]	TNTC	NT[4]		Avg 7.7 = 3.86 x 10^6	10.3 = 5.17 x 10^6	21 = 1.05 x 10^7	18 = 9.0 x 10^6	L[5]	Avg 6.3 = 3.15 x 10^6	7.7 = 3.85 x 10^6	4 = 2.0 x 10^6	0	1.3 = 6.5 x 10^5	0	0	0	
										O[8]	CONF[6]	CONF	CONF	CONF	CONF	CONF	CONF	CONF	
	10^{-5}	Avg 18 = 9 x 10^7 cells/ml original suspension	17.3 = 8.67 x 10^7	NT		Avg 1.3	0.6	2.3	2.3	L	0.3 = 1.5 x 10^6	0.3 = 1.5 x 10^6	0.3 = 1.5 x 10^6	0	0	0	0	0	
										O	13.6 = 6.83 x 10^7	14.7 = 7.33 x 10^7	23 = 1.15 x 10^8	16.3 = 8.83 x 10^7	15.7 = 7.83 x 10^7	13 = 6.5 x 10^7	NR[7]	NR	
	10^{-6}	1.7	1.7	NT		0	0	0	0	L	0	0.	0	0	0	0	0	0	
										O	0	1.0	1.6	0	0.3	0.7	NR	NR	

NOTES: 1. TS = Tryptose serum agar (Difco Labs., Detroit, Michigan).
2. TNT4 = Tryptose nalidixic acid (50 µg/ml) – Trypaflavin (4 µg/ml) agar.
3. TNTC = Too numerous to count, colonies coalesced.
4. NT = Not tested.
5. L = Listeria. O = Other bacteria.
6. CONF = Confluent growth.
7. NR = Not recorded.
8. Tobacco macerate contained 2.08 x 10^5 'Tobacco' bacteria per ml = 1.04 x 10^7 per gram.

TABLE II

RECOVERY OF *LISTERIA MONOCYTOGENES*, USING TRYPTOSE NALIDIXIC ACID (50 µg/ml) – TRYPAFLAVIN (5 µg/ml) MEDIA, FROM PURE CULTURE AND MIXED TOBACCO MACERATE/CULTURE SUSPENSIONS

Enrichment broth		Tryptose Nalidixic acid (50 µg/ml) + trypaflavin (5 µg/ml)								Tryptose nalidixic acid – trypaflavin + tobacco 1:50 w/v[3] (± 0.1 g per test)									
Listeria cells per ml broth		15		1.5		0.15		0.015		15		1.5		0.15		0.015		0 (Tobacco suspension control)	
Subculture agar		TS[1]	TNT4[2]	TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4	TS	TNT4
Recovery of bacteria: Number of colonies average of three replicate drops of 0.03 ml ex culture dilutions–	10^{-1}	Confluent growth of *Listeria*, not countable.								L	Confluent mixed growth of *Listeria* and other bacteria, not countable.							–	–
										B								Avg 62.7 ± 4.3 × 10^7	43 ± 2.75 × 10^7
	10^{-3}	Avg 61.3 ± 2.84 × 10^8 cells per ml original suspension	49.3 ± 2.7 × 10^8	22 ± 1.4 × 10^8	25.4 ± 1.84 × 10^8	8.3 ± 5.33 × 10^7	6.7 ± 4.27 × 10^7	6.7 ± 4.27 × 10^7	4.7 ± 2.99 × 10^7	L	14.7 ± 1.3 × 10^8	20.3 ± 1.3 × 10^8	15 ± 1.02 × 10^8	12.3 ± 7.89 × 10^7	14.3 ± 1.17 × 10^8	7.3 ± 4.69 × 10^7	10.3 ± 1.24 × 10^8	22 ± 1.41 × 10^8	–	–
										B	9.7 ± 6.9 × 10^7	0.3	10 ± 6.4 × 10^7	2.3 ± 1.47 × 10^7	6.3 ± 5.33 × 10^7	3.7 ± 3.41 × 10^7	9 ± 3.7× 10^7	1.6	Avg 11 ± 7.04 × 10^7	8.3 ± 5.33 × 10^7
	10^{-4}	5 ± 3.2 × 10^8	4.3 ± 2.77 × 10^8	3.7 ± 2.13 × 10^8	2.67 ± 1.7 × 10^8	1.0	1.3	<0.3	<0.3	L	3.0	3.0	2.3	<1.0	2.0	0.3	3.3	0.7	–	–
										B	0.3	0.3	0.3	<0.3	1.3	0.7	1.3	0.7	1.0	1.3

NOTES: 1. TS = Tryptose serum (5% v/v) agar.
2. TNT4 = Tryptose nalidixic acid (50 µg/ml) – trypaflavin (5 µg/ml) agar.
3. Tobacco macerate contained 2.04 × 10^8 enhanced bacteria per ml ± 1.03 × 10^8/g tobacco.

TABLE 12

SEROLOGICAL EXAMINATION OF COLONIES ISOLATED FROM MIXED TOBACCO MACERATE/*LISTERIA* SUSPENSION AND CLASSIFIED ACCORDING TO HENRY'S OBLIQUE ILLUMINATION TECHNIQUE

Colony classification according to Henry's method	Observer	Number of Tests	Titre against Poly O antiserum[1] (containing antibodies to *Listeria monocytogenes* somatic antigens 1 and 4)
Listeria	A	10	All tests:- 640 (Titre against test strain of *Listeria monocytogenes* prior to inoculating tobacco = 640)
	B	10	All tests:- 640
Probably non-*Listeria*	A	5	All tests:- 0
	B	5	All tests:- 0

1. 'Bacto' Poly O (2302) *Listeria monocytogenes* antiserum Difco Labs., Detroit, Michigan.

TABLE 13

RECOVERY OF *LISTERIA MONOCYTOGENES* USING TRYPTOSE NALIDIXIC ACID (50 ug/ml) TRYPAFLAVIN (4 ug/ml) BROTH IN A DOUBLE ENRICHMENT PROCEDURE

		Recovery of Bacteria following Double Enrichment (2 x 24 hours at 37°C) in TNT4[1] Broth to Show the Effect of Tobacco			
Tobacco		Absent		Present (1:50 w/v)	
Subculture medium (agar) for 2nd enrichment broth		Tryptose Serum Agar	TNT4 Agar[1]	Tryptose Serum Agar	TNT4 Agar
Number listeria cells inoculated per ml of 1st enrichment broth	300	1.6×10^8	2.0×10^8	Confluent growth of tobacco bacteria, no *Listeria* recovered	
	30	1.6×10^8	1.7×10^8	*Listeria* not recovered	
	3	2.4×10^8	3.0×10^8	*Listeria* not recovered	
	0.3	1.3×10^8	9.3×10^7	*Listeria* not recovered	
	0.03	1.4×10^8	1.5×10^8	*Listeria* not recovered	

1. TNT4 = Tryptose-nalidixic acid (50 ug/ml) - Trypaflavin (4 ug/ml).

TABLE 14

COMPARISON OF ENRICHMENT PROCEDURES FOR *LISTERIA MONOCYTOGENES* USING PURE CULTURE SUSPENSIONS AND MIXED TOBACCO-CULTURE SUSPENSION

Enrichment broth		P-MB[1]	TNT4[2]	P-MB		TNT4	
Tobacco		0	0	1:50 w/v		1:50 w/v	
Listeria monocytogenes cells per ml		3.15×10^4	3.15×10^4	1.26×10^4		1.26×10^4	
Recovery medium (agar)		Tryptose	Tryptose	Tryptose	PN[3]	Tryptose	TNT4
Dilution of selective broth and number colonies of *Listeria monocytogenes* average of six replicate drops of 0.02 ml	10^{-2}	Avg 0.8 = 4.17×10^3 cells/ml original suspension	TNTC[4]	TNTC	TNTC	TNTC	TNTC
	10^{-3}	0, 0, 0, 0, 0, 0	TNTC	Avg 12.2 = 6.08×10^5	Avg 11.3 = 5.67×10^5	TNTC	TNTC
	10^{-4}		TNTC			TNTC	TNTC
	10^{-5}		Avg 12.2 = 6.08×10^7			Avg 22.8 = 1.14×10^8	Avg 11.3 = 5.7×10^7
	10^{-6}		Avg 1.0			Avg 3.5	Avg 1.5

NOTES: 1. Polymixin-Methylene Blue Broth (21)
2. Tryptose-Nalidixic Acid (50 µg/ml) - Trypaflavin (4 µg/ml) Broth
3. Polymixin-Nutrient Agar (21).
4. TNTC = 'Too numerous to count'. Colonies coalesced.

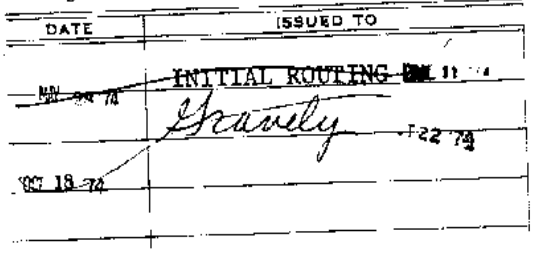
Incubation 37°C for 24 hours before subculture following recommendation for P-MB Broth

TABLE 15

COMPARISON OF ENRICHMENT PROCEDURES FOR *LISTERIA MONOCYTOGENES* USING PURE CULTURE SUSPENSION AND MIXED TOBACCO-CULTURE SUSPENSION

Enrichment broth ($30^{o}C$[1] 48 hours)	Thallous acetate (0.2% w/v) – nalidixic acid (40 µg/ml)				Tryptose-nalidixic acid (50 µg/ml) – trypaflavin (4 µg/ml)			
Number of *Listeria* inoculated per ml of enrichment broth	4		4		5		5	
Tobacco	0		1:50 w/v		0		1:50 w/v	
Subculture medium (agar)	Tryptose agar	Thallous acetate – nalidixic acid agar	Tryptose agar	Thallous acetate – nalidixic acid agar	Tryptose agar	Thallous acetate – nalidixic acid agar	Tryptose agar	Thallous acetate – nalidixic acid agar
Maximum dilution of enrichment broth from which *Listeria* isolated	10^{-1}	*Listeria* colonies not detected at any dilution			10^{-5}	10^{-6}	10^{-5}	10^{-5}
					Sample colonies confirmed serologically as *Listeria monocytogenes*		Sample colonies confirmed serologically as *Listeria monocytogenes*	

Note 1. Incubation $30^{o}C$ for 48 hours following recommendation for thallous acetate-nalidixic acid broth (19).



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BAT, Southampton, P. C. Stauber.
THE ISOLATION OF LISTERIA MONO-
CYTOGENES FROM TOBACCO - METHOD
DEVELOPMENT.
Proj. Job 49. 4-30-74.