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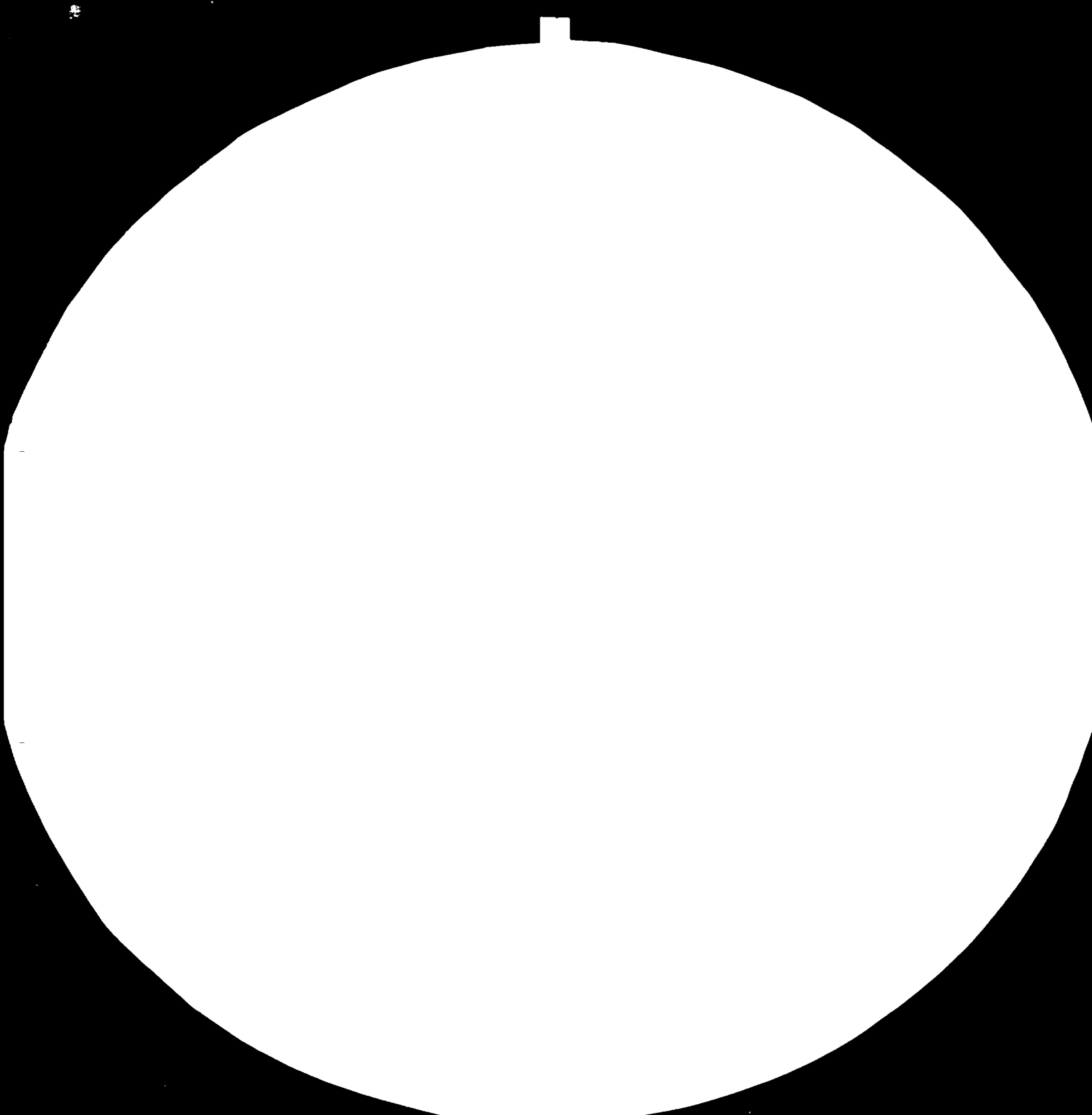

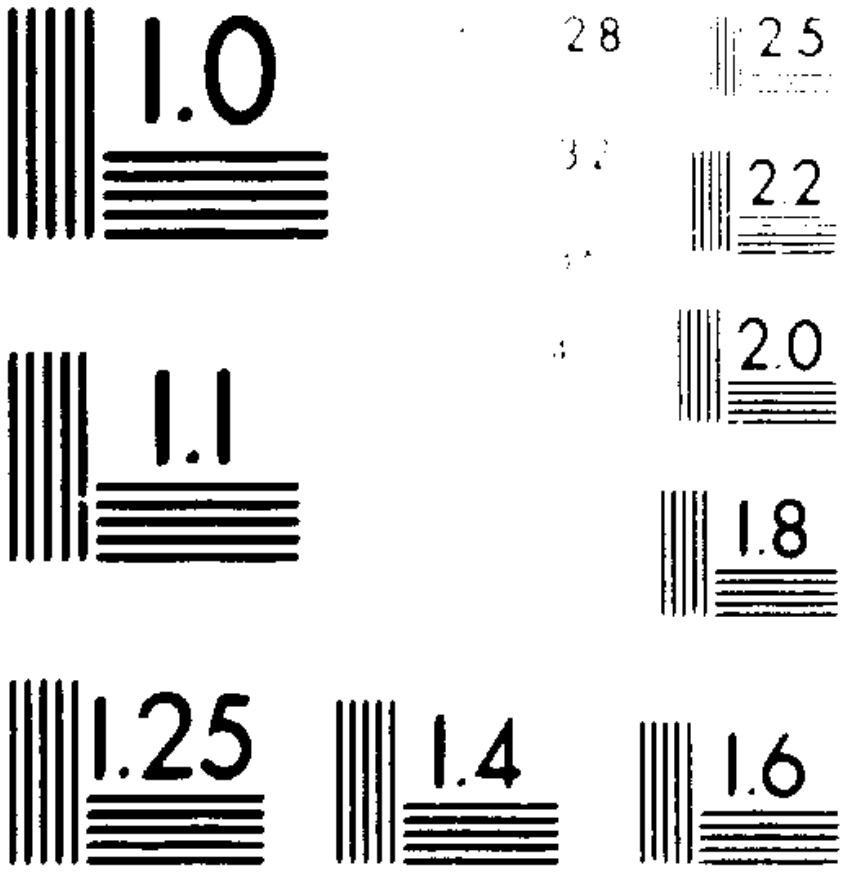

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STRENGTHENING THE ROYAL DRUGS RESEARCH LABORATORY

DP/NEP/80/003

NEPAL

Terminal report *

Prepared for His Majesty's Government of Nepal
by the United Nations Industrial Development Organization
acting as executing agency for the United Nations Development Programme

Based on the work of Ms.M.M. Cordes,
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United Nations Industrial Development Organization
Vienna

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Abbreviations

U S P	:	United States Pharmacopoeia
B P	:	British Pharmacopoeia
RDRL	:	Royal Drugs Research Laboratory
CDRI	:	Central Drugs Research Institute
HPPC	:	Herbs Production and Processing Company
Ned. Ph.	:	Nederlandse Pharmacopee

PART - I

SUMMARY AND RECOMMENDATIONS

1. SUMMARY

a. The microbiological aspects of pharmaceutical products, of their production and/or of their development are equally important as the chemical and physical aspects. The microbiological facility within RDRL can render a valuable contribution to the product development activities of RDRL and quality control of the production of pharmaceuticals, and through these activities will become more utilized and better integrated into the institutional framework.

b. Concerning the product development activities of RDRL contributions from microbiology could be rendered to the following subjects:

- in standardization: microbiological contamination.
- in stability studies: testing for sterility or resistance to microbial growth in the determination of optimum formulation, the most suitable container, the optimum storage conditions and the shelf-life.
- control procedures for the final production.

The participation of the microbiological laboratory in standardization could already start this fiscal year.

A guideline is given for the development of microbiological control procedures.

c. Concerning the quality control of the manufacture of pharmaceuticals, the following contributions are identified:

- microbiological control of raw materials, end products, water supply, environment and package materials;
- control of sanitation practices;
- propagation of hygienical attitudes.

d. It is expected that the section will have to handle much more samples in the future. Some measures have to be taken in anticipation, of which immediately needed:

- a constant and adequate electrical supply.
- a more spacious housing.
- a good supply of smaller items at the bench.
- the installation of an autoclave with higher capacity.

e. The methodology currently in use has been revised as far as possible. It is strongly advised to follow pharmacopoeial or standard methods for tests/analysis of pharmaceutical products.

The quality of the analysis results has to be given much attention. In broad lines this concept is explained and specified.

f. Preparatory work has been done for the establishment of the cultivation of axenic Entamoeba histolytica, the cause organism of amoebic dysentery, to extend the screening of plant extracts for antidysentric activity. Due to the late arrival of the indispensable materials and non-availability of amoeba the actual cultivation could not be initiated. Methodologies and media formulations were provided to the section.

g. The screening programme could be strengthened by the following measures:

- a centralised record keeping;
- the establishment of a data bank;
- the forming of a formal research group for this activity;
- the screening of a higher number of plants followed by
- a further testing of the active plant parts.

The centralised record keeping has been discussed and was agreed upon as well as its computerisation.

A guideline for the follow-up testing of active plant parts is presented.

h. Record keeping was found inadequate. The microbiological section should have records for methodology, samples, results of analysis, equipment, media, reagents, etc.. A guideline for the establishment of a laboratory manual and models for forms are presented.

i. The outfit of the section is modernized and extended; an air-conditioner was installed; the library is enriched with literature on microbiology and its applications.

2. RECOMMENDATIONS

a. Except for the screening of medicinal plants, the microbiological aspects of the research of RDRL should be enhanced.

b. More attention should be given to the microbiological quality control and the sanitary aspects of the production of medicines.

c. The section should become involved, whenever appropriate, in the establishment of the pilot plant, the new housing of HPPC and other production units to look into the sanitary aspects of production to encourage proper standards of hygiene.

d. The section should become more integrated into the research activities of RDRL by participation in the development of pharmaceutical products and the quality control of the production of pharmaceutical preparations.

e. The plants for which (chemical) standardization is planned could also be analysed for microbial contamination. This will render useful information for the general standardization of these materials.

f. It is desirable that a staff member be trained in the quality assessment of pharmaceutical products to a degree level. Such a training should be linked to a governmental control institute and a pharmaceutical company, to enhance both aspects of quality control.

g. A series stock cultures has to be acquired for quality control procedures according to the USP or BP. It is advised to obtain these directly from an official institute which collects standard strains.

h. The procedure for the sterility test should be carried out according to up-to-date standards as set out in an internal report.

i. Salmonella typhi should be eliminated from the laboratory.

j. The current space for the microbiological laboratory is not adequate. A more spacious housing is urgently required.

k. The laboratory should be sufficiently supplied with minor items needed for routine procedures.

l. The staff members must be urged to develop closer links in their work.

m. Contacts have to be established with clinical laboratories for the providing of clinical strains needed for secondary screening.

n. A higher number of plants could be screened. The factors limiting the primary screening of a higher number of plants should be identified and resolved.

o. A primary record keeping system regarding the screening of plants should be implemented. The record keeping in the section should be centralised and not kept by individuals only.

p. A formal research group could be formed consisting of the persons involved in screening and a co-ordinator, in order to integrate the investigations and to monitor the on-going activities. Quarterly reports should be given to the co-ordinator.

q. Pseudomonas aeruginosa could be included in the testplan for primary screening.

r. A staff member could be sent to CDRI, Lucknow, India for a short training in the cultivation of axenic E. histolytica and with the additional purpose to bring with him/her the axenic culture itself, as it is a long way to obtain this from a bacteria associated culture.

s. The cultivation of E. histolytica offers hazards to the laboratory personnel and special conditions for containment should be provided.

t. It is strongly advised to establish a manual with methodologies in use or to be used, along with a critical revision concerning the applicability of the methods in question.

u. New literature should be acquired regularly to keep informed of new developments in microbiology, especially with respect to methodology.

v. For the introduction of new methods a sufficiently long setting-up time is to be taken into account, because of a limited stock of media and other materials at RDRL as well as at the private enterprises. Some planning is therefore needed.

w. Much more information should be recorded than actually done. A record keeping system has to be available for the methodology (laboratory manual), incoming samples (separately for this section), results of analysis, equipment, media, reagents, etc. This is particularly useful for the expansion of the laboratory.

x. If more staff will be recruited for the microbiological section preference should be given to persons with at least a biological background. On the other hand the staff members of the section who have not received a formal education in microbiology should follow courses on a university level.

y. An incubator with temperatures in between 20 and 25°C is needed. This could be provided by lowering the room temperature or putting into use the refrigerated incubator.

z. The internal quality surveillance (see 3.2.3) should be continued and extended.

PART - II

BODY OF THE REPORT

1. MAIN DUTIES OF JOB DESCRIPTION

The assignment of the consultant was for a period of 6 months, split up in two parts, from 24 January until 12 March and from 7 May until 19 September 1983.

Duties, as stated in the job description, were as follows:

a. set up a facility for the microbiological screening of pharmaceuticals.

b. assist in the quality assessment of pharmaceutical preparation.

c. training local personnel in microbiological techniques and sensory evaluation.

d. perform such duties as will be assigned by the supervisors.

2. ACHIEVEMENTS

2.1 Screening facilities:

Screening work has been done at RDRL already for about 8 years. The screening comprises the antibacterial and antifungal activities of medicinal plants, selected for their traditional use in Ayurvedic and local medicine.

In the first meeting of the complete project team and the staff of RDRL on 31st of January 1983, it was mentioned by Dr. S.B. Malla that the screening of medicinal plants for anti-dysentric activity had a high priority. In this respect only a screening on activity against Shigella dysenteriae, the cause organism of bacterial dysentery, was included in the test plan. The occurance of amoebic dysentery is as frequent as, if not more than bacterial dysentery (Annex 2). It seems therefore desirable to establish a suitable test for screening against Entamoeba histolytica, the cause organism of amoebic dysentery.

Many of the objections to the use of in vitro tests for screening of potential amoebicides can now be overcome through use of axenic culture of the amoeba. This means that the amoeba are grown alone, without the presence of any other organisms, in a sterile medium. This is ideal for testing since the active compound(s) of the plants or drug act only on the amoeba. It is however also a more difficult method of culture, because the amoeba has very strict growth requirements.

The indispensable, but not available materials had to be imported which caused a considerable delay. In fact even at the end of August some items had not arrived. Another handicap for this culture method is that axenic growing E. histolytica is not available in Nepal. Obtaining this from a bacteria associated culture will take a very long time.

Alternatives were worked out for the axenic cultivation, but for whatever method some article had to be imported. It became thus clear by mid-July that within the consultants assignment no start could be made with the axenic cultivation of the amoeba.

Although a bacteria associated culture of E. histolytica was not the objective, it was thought to be at least a good start to familiarize with amoeba. Of the various possible media only one, the most simple medium, could be prepared with the limited resources. Unfortunately, stool samples positive for Entamoeba histolytica are not regularly available in the hospital.

A standardized record keeping for the plants collected for screening has been proposed jointly with the pharmacology expert and has been discussed with staff involved in this activity. The proposal was agreed upon as well as its computerisation. The form concerning the microbiological testing is included as Annex 3.

The methodology used for the preliminary general screening has been reviewed and subsequent recommendations made. Suggestions for an immediate follow-up screening of the active plant materials have been made and the requirements identified.

With regards to the microbiological screening of pharmaceutical preparations there have been discussions about testing formulations based on the work of Dr. O. Bojor, but no indication was given for microbiological testing.

2.2 Quality assessment of pharmaceutical preparations

At present the laboratory receives only occasionally samples for quality control. The current methodology has been revised as far as possible. The use of pharmacopoeial, other official and/or standardised methods is strongly advised. Apart from methodology other improvements have to be made in order to prepare the laboratory for the task of quality control work for the Drug Administration and for the development and production of pharmaceuticals. The consultant expects that in the future much more samples will have to be analysed by the section, although it is difficult to estimate

how fast the increase will be as the samples will come from different sources. Measures to enlarge the sample handling capacity of the section are proposed.

As it is her impression that the microbiological section could be strengthened through further integration within the institute, a detailed plan is worked out concerning this aspect. Microbiology can and has to play a (bigger) role in the development and subsequent (pilot) production of pharmaceutical preparations.

Much importance is given to monitoring the quality of the results of analysis by the introduction of various check procedures for proper operation of equipment, quality checking of media and reagents and validation of methods. The conditions reigning during the tests/analyses have to be optimal, known and recorded.

2.3 Training

It was mentioned that for the section more staff would be appointed, however only at the end of the consultants assignment the section was extended with one new staff member, not a microbiologist though. His training has taken a start, but only limited time could be dedicated as it coincided also with the arrival and subsequent installation of equipment. Trainees should however follow courses in microbiology on a university level. On the job training can not give them the fundamental understanding of the techniques and the interpretation of results.

2.4 Sensory evaluation

Regarding the introduction of sensory evaluation it was decided that this would be taken up in August, as at the start of the assignment the essential oil programme had not yet reached the stage in which sensory evaluation could be applied. But by then it had become clear that it would take a much longer time to reach that stage. With the intention to leave RDRL an opportunity to apply sensory evaluation techniques, whenever it will be appropriate, a short note about this subject was prepared with simple tests and practical suggestions about its preparations (Annex 9).

2.5 Organization of the laboratory

Attention was given to the organization of the laboratory. Suggestions for the keeping of records have been given together with samples for forms. Also safety aspects have been considered.

2.6 Constraints and limitations faced

The limited resources of the laboratory gave origin to a lot of difficulties in establishing new methods.

The equipment and materials ordered at the beginning of the assignment arrived at the end of it or thereafter due to long delivery times.

The consultants work was hampered furthermore by the fact that the counterpart left for a 6-month training period almost one month after the start of her assignment.

3. FINDINGS

3.1 Integration of the microbiological section into the research activities of RDRL

The microbiological section seems to serve more the outside world, e.g. Royal Drugs Ltd., the Drug Administration, the Public, then the research activities of RDRL itself. However there are several activities of RDRL to which the microbiological section can contribute to strengthen the research capacity of the institution as a whole. Apart from participation in the screening of medicinal plants or heading a new activity like fermentation, contributions can be made to the following activities:

Activity of RDRL	Contributions
Tissue culture	Control of infections
Product development	Standardization* Stability studies* Development of microbial control procedures and limits
Pilot production	Sanitary aspects Quality control*

Also assistance could be given to HPPC and other related production units with respect to sanitary aspects of production and microbiological quality control. Each of these activities are discussed below:

3.1.1 Tissue culture

The microbiological section could assist the tissue culture section at Godavari in the control of infections of the cultures.

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* Together with other sections.

3.1.2 Contributions to product development

With respect to the development of pharmaceutical preparations the microbiological section might contribute to the following activities:

- The determination of optimum formulations by evaluating the potential effect of factors, which can influence spoilage of the preparation.
- The determination of a suitable container/package design by evaluation of the potential effect of the package on the spoilage of the preparation.
- The determination of optimum storage conditions by assessing the effect of different storage conditions on the microbial deterioration.
- The determination of shelf-life (expiration date) by performing tests on completed formulations under similar conditions as the actual, which the product could encounter once leaving the factory.
- The development of control procedures and microbial limits. For the final manufacture of the preparation, procedures have to be developed to control the production. Microbial limits have to be set to accept or reject the end product and/or starting materials.

Four of these five contributions should not present any problem to the laboratory as simple methods can be applied to evaluate the effect of product formulation, container and storage conditions on the microbial population of the preparation. A sound experimental design is needed though. The last mentioned contribution may need some explanation, and is worked out in Annex 10.

3.1.3 Sanitary and quality control of (pilot) production of pharmaceuticals

The microbiological quality of pharmaceutical preparations is influenced by the environment in which they are manufactured and by the materials used in their formulations. With the exception of preparations which are terminally sterilised in the final container the microflora of the final product may represent the contaminants from the raw materials, from the equipment with which it was made, from the atmosphere, from the persons operating the process or from the final container into which it was packed.

It is the task of a microbiologist to look into all these aspects of the production be it on pilot scale or on a larger scale.

Regarding the sanitary aspects of pharmaceutical production the following aspects might be subject to the consideration of the microbiologist:

- Storage conditions for the starting materials, intermediate and final products, and packing materials.
- Equipment (in so far not specially designed for pharmaceutical products), e.g. ease of dismantling and cleaning, smooth surface etc.
- Building: finishing of the floors and walls, ventilation, sanitary convenience and washing facilities.
- Cleaning: general cleaning methods, sterilization and disinfection methods, waste disposal, control of infestation.
- Training in aspects of personal hygiene and microbiological understanding of the persons involved in production.

Regarding the quality assurance of the production, the microbiological section should monitor the production in the form of regular surveillance. This includes always the:

- examination of the microbiological attributes of the end product for compliance with standards agreed upon during the development of the product. The standard should mention the "point of control" as for some products this might matter. Quality assurance may also include:
- control of raw materials: during product development a limit* could be established for the maximum tolerated contamination of the raw materials. A treatment may be necessary, especially for those of botanical (or animal) origin to render them microbiologically acceptable;
- control of plant environment and water supply: microbial tests on plant environment samples, e.g. air, walls, can supply information about the level of sanitation and the potential for product contamination during processing. Tests on contact surfaces can check the efficiency of equipment clean up procedures;
- control of packing materials.

The degree of quality assurance can be increased by focussing attention on those ingredients, critical process steps and production periods where the potential hazard of contamination is greatest.

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* Instead of one limit, one could maintain two limits, dividing the raw materials in three classes according to their contamination: "Satisfactory" if below the lowest limit, "Unsatisfactory" if above the highest limit and "Doubtful" in between these two limits.

3.2 Current quality control task of the section

3.2.1 Present situation

Samples for quality assessment are submitted to the laboratory only on an irregular basis; usually these are originated by Royal Drugs Ltd. The following may illustrate the activities of the microbiological section in this field:

	Number of samples		
	1977/78	1978/79	1979/80
Sterility test	125	79	-
Other quality tests	5	-	3

The sterility test once applied on 125 samples a year, is now-a-days only occasionally done. The testing of non-sterile products is not often asked for, but this might be changing. During the last months some samples were tested for presence of coliforms and/or E.coli.

3.2.2 Immediate potential and requirements for further development

3.2.2.1 Handling capacity of samples

It is quite difficult to estimate the number of samples that are to be handled in the near future, because samples may come from within as well as from outside RDRL. The samples not originated from RDRL may come from at least three sources:

- samples coming from the Drug Administration should grow once the Drug Act will be implemented;
- Royal Drugs Ltd. will establish a microbiological laboratory and a sterility testing room at the plant site and this will terminate the submission of samples for control at RDRL. From then on samples of RD Ltd. will only be controlled by RDRL through the Drug Administration;
- some more samples may be coming from the public once the Drug Act will be implemented, as this will hopefully create some conscience about the quality of pharmaceuticals.

The number of samples from within RDRL depends at present entirely on the activity of the staff of the section itself. However if other activities are initiated (see 3.1) then the of samples will rise according to the joint programming with other sections.

In conclusion it is to be expected that the number of samples to be handled by the microbiological section will increase, but it is impossible to predict how fast. The section though should prepare itself for an increase of samples. A good start could be made with:

- connection of the laboratory to the generator for electrical supply without interruptions;
- providing a 220 V. supply for the autoclave and the steamer; their slow operation shortens the effective working day considerably;
- installation of the microbiological laboratory in a more spacious housing. This has been considered by the management, but is not yet possible as the building to which it will be transferred first has to be evacuated by Royal Drugs Ltd.;
- providing a good supply of smaller items at the bench, see Annex 11 for suggestions;
- creation of a laboratory manual in which the methods in use will be described and the methods that are expected to be applied in the near future. It would be convenient to know the plans of the Drug Administration about sampling for microbiological testing;
- installation of a modern model autoclave.

Another point has to be raised in this respect. A characteristic of microbiological analysis is the sequence of handlings set in a strict time frame. A workweek interrupted with a hol[illegible] is often lost for analysis. Some arrangement is therefore ne[illegible] the future.

3.2.2.2 Methodology

Taking into account the diversity of future samples for analysis, the laboratory should be prepared to have a variety of methods available. Official methods preferably backed by a pharmacopoeia, should be applied to products under regulatory action such as from the Drug Administration. For other samples more freedom is allowed as far as the methodology is concerned. Some standard references have been ordered.

The procedures applied to samples under regulatory action should be authorised by a pharmacopoeia. It is up to RDRL to decide which pharmacopoeia should be followed in this respect. Some pharmacopoeia allow more freedom to the investigator than others.

The microbiological control of non-sterile products has gained significant interest in the pharmaceutical world, as can be concluded from U.S.P. XX and B.P. 1980. The required tests, in the U.S.P. called microbial limit tests, are total microbial count, counts of index organisms (S. aureus, E. coli, Ps. aeruginosa, Salmonella) and sometimes moulds and yeasts. It depends on the intended use for the product which specific test would be required and is specified in the monograph on the product.

The required test organisms for the control tests required by the USP and BP should be obtained once the decision about which pharmacopoeia to follow has been made.

The microbial assay of antibiotics may be required in future. Serving this purpose Mr. P.M. Shakya, the microbiologist, went on a 6-month in-plant training under WHO fellowship at Hindustan Antibiotics Ltd., Poona, India.

As said before for samples not under regulatory action more freedom is allowed. The selection of the appropriate method should take into account:

i. the purpose of the analysis;

ii. the nature of the sample; and

iii. the prevalent conditions, such as: availability of staff, space and materials, number of samples to be analysed and the period of time allowed to obtain results.

Whatever the method used it is required to show its adequacy for the product under consideration.

3.2.2.3 Standards

For pharmacopoeial products the requirements for the individual product should be applied. As a matter of course the standard to apply should be from the same pharmacopoeia as the methodology used. With respect to standards for non-pharmacopoeial products referred is to Annex 10.

3.2.3 Internal quality surveillance

It is necessary to monitor the quality of the results of analysis by the introduction of various check procedures.

With regard to equipment: maintenance, periodic inspection and/or testing for proper operations is needed. Without the intention to give a complete programme, the following can be pointed out:

- the autoclave should be controlled for proper functioning. Biological indicators are used for periodic inspection, chemical indicators for daily use. Preference should be given to commercial products, which have been subjected to rigorous quality control. Chemical indicators have to be properly stored;
- incubators, should be controlled for proper operation: the temperature fluctuation should be known and improved;
- the laminar flow cabinet should be regularly checked for proper functioning. The filter is normally renewed after 3-5 years. New filters should therefore be available.

The quality of reagents, media etc. should never be taken for granted and is therefore checked when used. Such a control should be incorporated in the test procedure.

In the test procedures control tests should be incorporated for validation of the method: For example, sterility check of the media, growth of a control organism with the product in study, or whatever is appropriate.

3.3 Screening activities

3.3.1 Present state

The research into the antimicrobial activities of medicinal plants started about 8 years ago. By now around 60 plants have been screened. The activities are at present divided in general screening (antibacterial and antifungal) and antidysentric screening, carried out by two different staff members.

The test organisms used in the general screening are:

1. Staphylococcus aureus
2. Agromobacterium tumefaciens
3. Bacillus subtilis
4. Salmonella typhi
5. Escherichia coli
6. Shigella dysenteriae

7. Candida albicans
8. Saccharomyces cerevisiae
9. Cryptococcus neoformans

One plant pathogen is included (No. 2)

In early reports it is mentioned that 3-5 different extracts of one single plant part were tested. These extractions were made with ether, petroleum-ether, alcohol, water and 50% alcohol, either hot or cold or both (water). At present a 50% alcohol extract is favoured.

Three years ago a start was made with the screening of selected medicinal plants looking for antidysentry activity. A preliminary screening has been effectuated on 50% alcohol extracts of 11 plants. Shigella dysenteriae functioned as specific test organism, but it seems that also some other organisms were included for general testing if it was a "new" plant (part). The plants and their parts tested are given in Annex 1.

It was mentioned that Aegle marmelos had shown the strongest activity against Sh. dysenteriae followed by Punica granatum and Woodfordia floribunda.

According to the workplan for the current fiscal year (1983/84) two of the three active plants, i.e. Aegle marmelos and Woodfordia floribunda will be further screened; Punica granatum will be taken up for (chemical) standardisation and for the time being not further screened for antimicrobial activity.

There are some remarks to be made about the general procedure:

- It is strongly advised to eliminate Salmonella typhi from the test plan and also from the laboratory. Only small numbers of S. typhi are sufficient to provoke the outset of typhoid fever. It is too hazardous to work with this organism without taking special precautions, difficult to realise in a general laboratory. If a Salmonella has to be in the test plan, S. typhimurium could serve the purpose.
- The inclusion of Pseudomonas aeruginosa might be considered, because this organism has in recent years assumed the role of an important pathogen, although may be not (yet) in Nepal (Upadhyay, 1981). It is resistant to many antibacterial drugs.

- There is some argument possible, as pointed out by the UNIDO expert/pharmacologist in his first interim report (Jan.-April 1983), about the kind of extracts to be tested. His position is that "the initial testing of the material should be as near the reported use by people as feasible". This has the same application in the microbiological screening.
- About 300 grams plant material is extracted. The extract is then concentrated and 50 filter disks of 6 mm are then saturated with the extract. Although it is a qualitative test it would be useful to record to what volume the extract was concentrated.
- For a preliminary study of the antimicrobial properties much more plants could be screened. It is unknown which the limiting factor is for the screening of a higher number of plants. The test itself, once the extract is made, is a question of a couple of days and several extracts can be tested simultaneously.
- A centralised record keeping is highly desirable. A form for this purpose is included in Annex 8.

3.3.2 Extension of the antidysentery test

During this consultancy efforts have been made to initiate the cultivation of Entamoeba histolytica. However non availability of materials made this impossible within the assignment. It is expected that within short time the needed materials will all be at RDRL. Different media formulations are now available (Annex 6).

A handicap is that no axenic growing E. histolytica is available in Nepal. It is known that this culture exists at CDRI, Lucknow, India and for this reason and the fact that CDRI has a long experience in this field it is proposed that a staff member of the section be sent for a short training to CDRI (memo to Dr. Malla 15 July 1983). Upon return the trainee can thus carry back with him/her the axenic organism. Added to this proposal, it is recommended that the staff member takes with him/her samples of the test tubes bought for this culture to try them out at CDRI. It would also be helpful to test one of the selected media formulations, if this is not the same as at use in CDRI.

In the meanwhile the cultivation of E. histolytica associated with bacteria could be started. A suitable medium is prepared, the acquisition of a stool sample, in which the amoeba has been detected is needed.

3.3.3 Follow-up for active plant material

For an immediate follow-up of the active plant materials, the following steps are identified:

(a) A confirmation of the preliminary screening is carried out on fresh extracts from fresh plant materials.

(b) In collaboration with the chemist, assigned to this programme, an improvement of the yield of extract could be undertaken in appropriate cases.

(c) The selected extract(s) are then subjected to more extensive laboratory investigations of their antimicrobial properties. The breadth of spectrum of antimicrobial activity can be established against a wide variety of organisms and/or testing can be done against as large a range as possible of strains of one organism, depending on the final purpose of the screening. The organisms used should consist predominently of fresh clinical isolated organisms from several sources this in contrast to the preliminary screening where standard strains well adapted to laboratory conditions are used. An effective agent must have reproducible, reliable activity against a high proportion of the strains selected. In the case of screening for antidysentric activity, the test is carried out with strains of Shigella dysenteriae isolated from Nepalese patients. In a later phase the same applies for Entamoeba histolytica. A good collaboration between different clinical laboratories and RDRL is needed in this respect.

The method used in this stage should be the minimum inhibitory concentration (MIC) determination (Annex 5), which is a quantitative method. Testing can be done in comparison to the activity of a relevant established antimicrobial agent on the same organism. Control test must be carried out to show that the test organism will grow under the selected conditions. It is important to record all the test conditions. Approximate MIC values of drugs for most of the common species are given by Garrod, (1981).

(d) There are possibilities for further in vitro testing depending on desirable characteristics of the extract, but these can not yet be identified in this phase of the screening.

3.4 Fermentation

It was mentioned at the beginning of the consultancy that RDRL is compromised to set up fermentation work under the Sixth Five Year Plan, ending in 1985. In order to assist the RDRL in its work it was decided to include the purchase of fermentation equipment in the revised priority listing.

The selection of the fermenter type was hampered by the fact that it has not yet been decided, for which purpose this equipment will be used. The following subjects for fermentation work were mentioned:

(a) compressed yeast
(b) industrial alcohol
(c) citric acid
(d) vitamin B_{12}
(e) antibiotics (penicillin, streptomycine, tetracycline HCl)
(f) transformation of steroids
(g) production of enzymes (cellulase, protease)
(h) vitamin C.

The characteristic of the fermenter bought is that it is composed of modules, offering the advantage that these modules may be used also for non-fermenting purposes.

3.5 Safety measures

The following safety measures have been identified for immediate action:

(a) the door which connects the "analysis-room" with the corridor should have a key on the inside, only to be opened in case of emergency.

(b) pipette fillers should be used when pipetting pathogenic microorganisms.

(c) the washing facility in the analysis room should be used.

(d) all glassware that has been used for, or that has come into contact with bacterial cultures has to be decontaminated before washing.

(e) S. typhi should not be maintained in the laboratory.

(f) the cultivation of E. histolytica offers hazards to the laboratory personnel and special conditions for containment must be provided.

3.6 Record keeping

A record keeping system is highly desirable for the methodology, the incoming samples, results of analysis, but also for equipment, media, strains etc.. The consultant did not encounter records about methodology and equipment, other subjects may be recorded somewhere else. The following suggestions are made:

(a) Establishment of a laboratory manual for methodology. See Annex 3 for further details.

(b) Establishment of a sample register exclusively for the microbiological section. Every sample coming to the laboratory should be registered in this book. See Annex 4 for a sample form. A loose leaflet register should not be used for this purpose.

(c) Establishment of a card system for equipment. Information about available accessories should also be noted on this card and where these can be found. This becomes important for accessories that are shared with other sections.

(d) Maintenance of records reflecting dates and nature of any change, monitoring, inspection, repair, etc. regarding the above mentioned subjects.

3.7 Library

The library has been extended with literature in the field of microbiology. Also the U.S. Pharmacopoeia XX (1980) was procured. It is advised to acquire each new edition of the USP from now, as well as the next edition of the European Pharmacopoeia, and more literature about laboratory methods.

References:

1. United States Pharmacopoeia (1980). Twentieth revision. United States Pharmacopeial Convention, Inc. Rockville, USA.

2. British Pharmacopoeia (1980). Vol. II. London: HMSO.

3. Nederlands Pharmacopee (1978). 8th edition. The Netherlands.

4. Reeves D.S. et al, 1978. Laboratory methods in antimicrobial chemotherapy. Churchill Livingstone, Edinburg.

5. International Commission on Microbiological Specifications for Foods (ICMSF), 1982. Microorganisms in Foods 2. Sampling for microbiological analysis: Principles and specific applications. University of Toronto Press, Toronto, Canada.

6. Upadhyay M.P. et al, 1981. Antimicrobial sensitivity of bacteria isclated from corneal ulcer. J. Inst. Med. 3 (1), 33-40.

7. Garrod, L.P. et al (1981). Antibiotic and chemotherapy. Churchill Livingstone, Edinburgh.

ANNEX - I

List of plants screened for antidysentric activity

(mid 1980 - mid 1983)

Plant name	Part used
Oroxylum indicum	stem bark
Alstonia scholaris	stem bark
Woodfordia floribunda	flower
Aegle marmelos	fruit
Bombax malabaricum	flower
Melia azadirachta	root
Butea frondosa	seed
Trigonellia foenumgranecum	seed
Punica granatum	fruit bark
Calotropis gigantea	root powder
Bauhinia variegata	flower powder

ANNEX - 2

Incidence of dysentery according to hospital admissions

Disease	Admissions	% Total admissions	Deaths	% Total Death	case fatality rate in %
Dysentery (not specific)	240	0.29%	13	0.43	5.42
Shigelliosis	177	0.21%	9	0.30	5.08
Amoebiasis	522	0.63%	9	0.30	1.73

Source: In-patients morbidity and mortality statistics 1980-81.
Survey of 28 hospitals.
Epidemiological Bulletin 1981, 2
HMG Department of Health Services, Nepal.

ANNEX - 3

A guideline for the establishment of a laboratory manual

For the creation of a laboratory manual one starts with the review of the current practices, comparing these with the new available literature. A written description of the revised methodology is then made, including sample preparation, media, reagents, controls, standards and the corresponding literature. If the methodology is not familiar then it should be tried out first. Next comes the adoption of methodology expected to be useful for future samples.

With this information a provisional laboratory manual is assembled. As a model a description of the detection of E.coli in dry powders is included, and a model for visualization of methods, especially suitable for beginners.

It has to be established as a rule that changes in the adopted laboratory procedures are only allowed with the approval of the senior microbiologist. The nature and date of changes should be recorded and, if such a change becomes current practice, an according version of the new procedure should be made for the laboratory manual.

It is recommended to make written statements about desirable changes but not feasible at the time of writing the manual, in order to do so in future.

The laboratory manual should be available to all staff of the section.

Finally, it is essential to keep up-to-date.

(Model for Manual)

DETECTION OF E. COLI IN DRY POWDERS

This test is suitable for recently heated, dry and/or acid products.

Materials

(a) screw-cap containers
(b) pipettes of 1 ml (total flow)
(c) pipetter
(d) spatulas
(e) waterbath at 44°C
(f) incubator at 37°C
(g) tubes with a durham-tube, containing 5 ml MacConkey broth
(h) tubes with 5 ml peptone water 1%
(i) tubes with peptone water 0.1%
(j) plates of MacConkey agar
(k) Kovac's reagent
(l) nutrient broth
(m) E. coli culture

Procedure

I. Detection of E. coli

1. Enrichment

Place 5 g. in a sterile screw-capped container, add 50 ml of nutrient broth, shake, allow to stand for one hour and shake again. Loosen the cap and incubate at 37°C for 18-24 hours.

2. Primary test

Add 1.0 ml of the enrichment culture to a tube containing 5 ml of MacConkey broth. Incubate at 37± 1°C for 48 hours. If the contents of the tube show acid and gas carry out the secondary test.

3. Secondary test

Add 0.1 ml of the contents of the positive tubes to each of two tubes containing:

(a) 5 ml of MacConkey broth
(b) 5 ml of peptone water, 1%

Incubate in a water-bath at 44± 0.5°C for 24 hours.

Examine tube (a) for acid and gas and
tube (b) for indole.

4. Indole test

Add 0.5 ml of Kovac's reagent, shake well, and allow to stand for one minute; if a red colour is produced in the reagent layer indole is present.

5. Interpretation

E. coli is indicated as present if the secondary test is positive for acid and gas and indole production.

II. Control test

Add 1.0 ml of the enrichment culture and a volume of broth containing 10-50 *E. coli* prepared from a 24 hour culture in nutrient broth, to 5 ml of MacConkey's broth. Follow the primary and secondary test.

Validity of the test

The test is invalid if the results do not indicate that the control contains *E. coli*.

III. Report

E. coli (not) present in 0.1 g.

Note: To be able to report the presence of *E. coli* in 1 g. one could add 10 ml of the enrichment to 10 ml of double strength MacConkey broth.

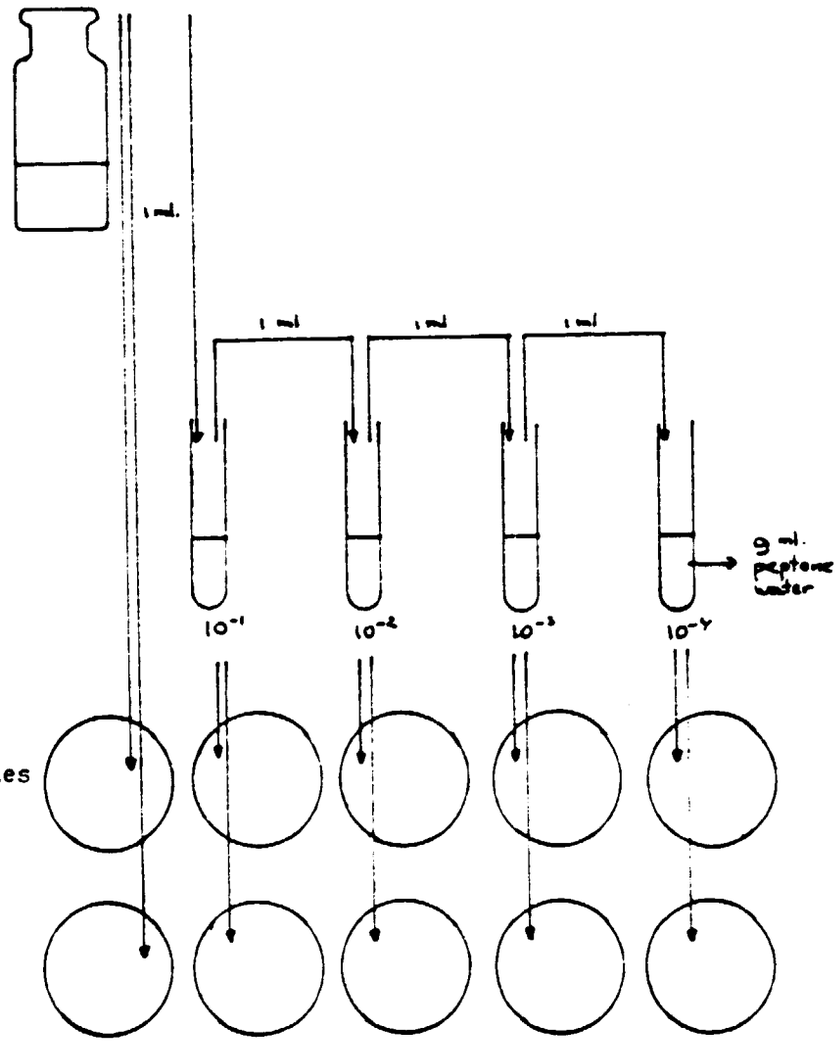
Reference: British Pharmacopoeia Vol. II, 1980.

(Model for Manual)

VISUALIZATION OF METHODS

PLATE COUNT

1. SAMPLE



2. DILUTION

3. PIPETTE
1 ml. into petri dishes

4. POUR 15 ml.
of the _____ agar

5. INCUBATE at _____ °C, _____ hr.

6. COUNT the plates with 30-300 colonies.

ANNEX - 4

MICROBIOLOGICAL SECTION

SAMPLE REGISTER

(model)

No.	Description	From	Date	Analysis/test	Method	Date	Results	Date Report

ANNEX - 5

The minimum inhibitory concentration value

The minimum inhibitory concentration (M.I.C.) involves a serial dilution of the extract in solid agar or broth media containing a culture of the organism. The lowest concentration of the agent that prevents visible growth after 18 to 24 hours of incubation is known as the minimal inhibitory concentration (MIC) and the lowest concentration that sterilizes the medium or results in a 99.9% decline in bacterial numbers is known as the minimal bactericidal concentration (MBC). The latter test is used only in special instances where very precise knowledge is required.

It is generally accepted that two-fold dilutions are adequate for the MIC determinations. For the laboratory method reference is made to Reeves D.S. et al, 1978. Laboratory methods in antimicrobial chemotherapy.

ANNEX - 6

Media formulations for cultivation of E.histolytica

I. For axenic cultivation

1. Diamond's TYI-S-33 and BI-S-33 Medium

Reference: Diamond, L.S. (1978) A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Roy. Soc. Trop. Med. and Hyg. 72 (4), 431-432.

2. Diamond's BI-S-33 modified

(a) Basic medium

Biosate	3 g
Glucose	1 g
Na Cl	0.2 g
K_2HPO_4	0.1 g
KH_2PO_4	0.06 g
L-cysteine hydrochloride	0.1 g
L-ascorbic acid	0.02 g
Ferric ammonium citrate (purified brown pearls)	0.00228 g
Glass distilled water to 87 ml	

The above ingredients are dissolved in order and made up to 87 ml with glass distilled water. The pH of the medium is adjusted to 6.8 by the addition of 1 N NaOH. If necessary filter through filter paper Whatman no.1. Sterilize for 15 minutes at 121°C. Cool to room temperature.

(b) Dissolve 0.01 g DL-a-lipoic acid in 10 ml ethanol absolute. From this take 0.01 ml and add 0.02805 g NCTC 135 medium and 0.0066 g $NaHCO_3$ and make up to 3 ml with glass distilled water. Filter these 3 ml through a membrane filter of 0.45 microns and add to the sterile basic medium.

(c) Add also 10 ml inactivated (30-50°C, 60 min.) foetal calf-serum to the basic medium.

(d) Dispense in tubes, 9 ml each.

Reference: Prof. Laarman, Department of Parasitology, Royal Institute for the Tropics, Amsterdam, The Netherlands.

3. Diamond's TP-S-1 medium

Reference: Diamond, L.S. (1968). Techniques of axenic cultivation of Entamoeba histolytica Schaudinn, 1903 and E. histolytica-like amoeba. J. Parasitol. 54 (5), 1047-1056.

4. Diamond's TPS-1 modified

(a) Basic medium:

Trypticase	BBL	10.0 g
Pannede, liver digest	Paines and Byrne	20.0 g
Glucose	Difco	5.0 g
L-cysteine HCl	BDH	1.3 g
Ascorbic acid	ICN	0.2 g
NaCl	Merck	5.0 g
KH_2PO_4	Merck	0.6 g
K_2HPO_4	BDH	1.0 g
Foetal calf-serum		100 ml
Glass distilled water		900 ml

The above ingredients are dissolved in order except the serum. The pH is adjusted to 7.0 and after this the serum is added. Sterilize through membrane filter.

(b) Add aseptically 10 ml NCTC 135 medium (Gibco)

(c) Dispense in tubes, 7 ml each.

Reference: Dr. F. van Knapen, Department of Parasitology National Institute for Public Health (R.I.V.) Bilthoven, The Netherlands.

II. For xenic cultivation

Modified Boeck and Drbohlav's medium:

Glassware and material used in the preparation should be sterile because the length of time the medium is sterilized may not be adequate to kill large numbers of bacteria in the solid egg base. Experience with the efficiency of one's autoclave will determine whether aseptic precautions are necessary.

(1) Modified Locke's Solution (Overlay)

Sodium chloride (NaCl) -	8.0 gm
Calcium chloride ($CaCl_2.2H_2O$) -	0.2 gm
Potassium chloride (KCL) -	0.2 gm
Magnesium chloride ($MgCl_2.6H_2O$) -	0.01 gm
Sodium phosphate (Na_2HPO_4) -	2.0 gm
Sodium bicarbonate ($NaHCO_3$) -	0.4 gm
Potassium phosphate (KH_2PO_4) -	0.3 gm
Distilled water -	1,000.0 ml

Add the above chemicals in the order listed to the distilled water. Mix each until dissolved. Boil 10 minutes. A precipitate will form. Cool to room temperature and filter through paper. Sterilize in an autoclave for 15 minutes at 121°C.

(2) Preparation of Egg Slants

1. Wash 4 fresh eggs and carefully break them in a 1,000 ml flask containing a few glass beads or into a blender. It is usually easier to break 1 egg at a time into a beaker and then transfer it to the flask through a small funnel. Any eggs that do not appear fresh should be discarded. A "blood spot" in a fresh egg does not harm the medium.

2. Add 50 ml of sterile Locke's solution and emulsify by shaking the flask.

3. Filter the mixture through gauze. Place under vacuum to draw out all small bubbles.

4. Dispense into sterile test tubes (15 x 125 mm) in such a quantity that there will be a short butt and a 4 to 5 cm slant.

5. The tubes are slanted inside the hot air oven at 80°C.

6. Allow the slants to cool and cover with Locke's solution so that the overlay forms a column 5 to 6 cm deep.

7. Sterilize in the autoclave for 15 minutes at 121°C. Allow the pressure to drop gradually.

8. Incubate at 37°C for 24 hours and check for sterility. If the fluid is clear the medium can be stored in the refrigerator for future use. Fresh medium is more satisfactory than stored medium, but, if necessary, it can be kept for a month.

(3) Inoculation

1. Just before use, add approx. 30 mg of sterile rice starch to each tube of medium. This may be done with a sterile wire loop (use 1 loopful).

2. Warm the tubes of medium to approx. 37°C.

3. Add 3-4 drops of a 0.2% solution of a sterile mixture of gentian violet (be careful: causes staining) and acriflavin to each tube.

4. Inoculate as follows:

   (a) Liquid or semi-solid material

   With a large bore pipette, add 0.5 ml of specimen to each tube of medium. Add as little air as possible. Mix the inoculum with the overlay, being careful not to introduce air bubbles.

   (b) Formed feces

   With an applicator stick, transfer a portion of specimen the size of a small pea to the tube of medium. Mix with the overlay by carefully rubbing the material against the side of the tube. Portions for inoculating are preferably selected from any mucus, blood, or abnormal appearing areas in the fecal materials, if present.

5. Incubate the tubes at 37°C for 48 hours. Examine.

6. With a sterile large bore pipette, transfer about half of the sediment to a tube of medium. The amoebae grow at the bottom of the culture or on the surface of the slant near the bottom. Add 3-4 drops of the sterile mixture of gentian violet and acriflavin (0.2%) and a loopfull of rice starch. Incubate at 37°C for 48 hours and examine.

Reference: Melvin, D.M. and M.M. Brooke. Laboratory procedures for the diagnosis of intestinal parasites. US Department of Health and Human Services. Publ. no. (CDC) 80-8282(1980).

ANNEX - 7

Literature collected for the cultivation of E.histolytica

1. Diamond, L.S. (1968) Techniques of axenic cultivation of Entamoeba histolytica Schaudinn, 1903 and E.histolytica-like amoeba. J. of Parasitol. 54(5), 1047-1056.

2. Diamond, L.S. and I.L. Bartgis, (1971) Axenic cultures for in vitro testing of drugs against Entamoeba histolytica. Archivos de Investigacion Medica, suppl. 1, 339-347.

3. Diamond, L.S. et al (1978). A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Tr. Roy. Soc. Trop. Med. and Hyg. 72(4), 431-432.

4. Diamond, L.S. (1960). Axenic cultivation of Entamoeba histolytica: progress and problems. Arch. Invest. Med. (Mex.)11 (Supl.1), 47-54.

5. Dutta, G.P. (1981). Experimental and clinical studies on amoebiasis. Tata-McGraw-Hill, New Delhi, India.

6. Gillin, F.D. and L.S. Diamond, (1980). Entamoeba histolytica and Entamoeba invadens: effect of temperature and oxygen tension on growth and survival. Exp. Parasit. 49, 328-338.

7. Gillin, F.D. et al (1982). Bruceatin, a potent amoebicide from a plant Brucea antidysenterica. Antimicrob. Agents Chemother. 22, (2), 342-345.

8. Nelson, E.C. and M.M. Jones, (1955). Some factors related to Entamoeba histolytica growth in rice products in a simple medium. Am.J.Trop. Med. and Hyg. [illegible], 322-332.

9. Nelson, E.C. and M.M Jones, (1964). Cultivation of Entamoeba histolytica in carbon dioxide-bicarbonate buffer system media. Am. J.Trop. Med. and Hyg. 13(5), 667-673.

10. Robinson, G.L. (1968). The laboratory diagnosis of human parasitic amoebae. Tr.Roy.Soc.Trop.Med. and Hyg. 62(2), 285-294.

11. Sepulveda, B.(1982). Progress in amoebiasis. Scand.I.Gastroenterol. Suppl. nr. 77, 153-164.

12. Different culture methods for Entamoeba histolytica: polixenic, monoxenic and axenic.

ANNEX - 6

Bioassay 2

Microbiological testing

1. Plant name: _____ Nepali name: _____

   Family: _____ Local name: _____

2. Plant part: _____ 3. Species No. _____

4. Collection No.: _____ 5. Date collected: _____

6. Extract No.: M/CC/DD,DD,DD/V/EE/TT

7. Date received: _____ 8. Dry weight equivalent _____

9. Solubility/Vehicle _____

10. Reported activity: _____

11. Appearance _____

12. Test type: _____ 13. Date commenced: _____

14. Active: Y, N, E _____

15. Comments:

16. Note book no. page no. _____

17. Carried out by: _____ Date _____ Signature _____

18. Comments and recommendation by Senior Microbiologist:

Copies to:

Project Director
Botanist
Chemist
Pharmacologist

Key to form microbiological testing

1. Plant name, both binomial and local.

2. Plant part, see appended list.

3. Species no., this should be allocated from the master list of plants SSS

4. Collection no. this is allocated by the botanist in the field and refers to his collection, this should also be recorded in the field note book.

5. Date collected. The date the specimen was collected not the dates of the collecting trip.

6. Extract No. M=Microbiological test SSS/CC/DD,DD,DD, as P1,P2,P3. EE extract number as P2. TT Microbiological test number.

7. Date received from chemist.

8. Dry weight equivalent from chemist's form.

9. Solubility/vehicle (if not sol. in water vehicle should be recorded here).

10. Activity reported in literature

11. Appearance of extract at time of microbiological testing.

12. Test type performed e.g. antibacterial - E. coli

13. Date test commenced

14. Active, Yes, No. Equivocal.

15. Comments

16. Note book ref. no. page no.

17. Name signature of person who carried out test and date.

18. Comment and recommendation by senior microbiologist.

ANNEX - 9

A short note about organoleptic testing techniques and how to apply it at RDRL

Organoleptic or sensory evaluation techniques make use of the human senses, e.g. taste and smell, as a "tool". In daily life we use our senses frequently, for example when consuming a meal. We even perform some kind of an evaluation when eating: how it tastes, about the way of preparation (too much of this or that ingredient) etc.

In fact, what we do in daily life is uplifted to a technique and as such will be carried out as a standard procedure to minise or control the effect that psychological errors and physical conditions of the person or environment can have on human judgement.

The first and simplest form of organoleptic evaluation is made at the bench by the research worker who develops the new product. Organoleptic evaluation is conducted on a more formal manner by laboratory panels. An experienced panel can guide product development and improvement, evaluate quality and assess product changes, for which no adequate instrumentation is available.

For example: herbal tooth paste could be evaluated against the product of "the competition" by a panel on characteristics such as appearance, colour, taste, smell and consistency; herbal medicines could be evaluated on appearance; essential oils on smell. The idea behind this is that, if the consumer does not like the developed products, then there will be little demand for it, how good it may be.

Some general remarks about the panelists, testing room and sample preparation

The panelists serving as the measuring instrument in organoleptic evaluation, need to be in good condition for testing. It is therefore that persons feeling not well should not participate, nor should they be in a hurry. It is better to perform testing in the morning than in the afternoon: the persons could feel tired. Only persons with a good disposition towards those tests should participate. The testing area should be a quite, comfortable environment. If possible, the room should be air-conditioned. Foreign odors should be kept from the testing room and smoking not permitted.

The presentation of the samples should be:

(a) uniform

(b) always at the same temperature

(c) coded, preferably with two or three digit numbers

(d) in randomized or balanced order.

The test is best performed on a limited number of samples in one session.

The test method

Basically there are three test types: difference tests, preference tests and descriptive tests. The following test plans are relatively simple:

(A) Difference tests.

(a) Triangle test:

Three samples are presented to the panelist, two of the samples are the same and one is different. The panelist is asked to identify the odd sample. The samples are presented in a triangular composition.

(345) (462)

(972)

This method is very useful to ensure that samples from different production lots are the same or to determine if a change in manufacturing results in a detectable difference in the product.

(b) Duo-trio test:

In this test also three samples are presented to the panelists, two identical and one different, but one sample serves as a reference (standard) and is labeled R. One of the coded sample is identical with R and the other is different. The panelist is asked to identify the odd sample.

(566)

(R)

(103)

The duo-trio test has the same applications as the triangle test, but is easier for the panelist.

(B) Preference test.

The simple paired comparison is the easiest test under the preference tests. A pair of coded samples is presented for comparison and the panelists is asked which one he/she prefers.

Analyzing the data

The results of these tests must be treated by statistical methods, to compare them with those that would be obtained by chance alone. The table below includes the statistical tables for the above mentioned methods. The numbers in this table are the minimum correct answers needed to conclude that the samples are different at the selected level of significance. If the number of correct answers is less than required, then the conclusion is that there is no detectable difference at that level of significance. Statistical chart 1 is used for the triangle test; chart 2A for the paired comparison-preference test and chart 2B for the duo-trio test.

The presented forms serve as models and may be utilized for the tests (see page 39 and 40 of this report).

Reference: Larmond, E. (1977). Laboratory methods for sensory evaluation of food. Canada Department of Agriculture, Ottawa, Canada.

DUO-TRIO TEST

Name: _____ Product: _____

Of the three samples the one marked "R" is the reference sample. Of the other two samples, one is identical with R and the other is different. The question is which of the coded samples is different from "R"?

Samples	Mark odd sample
566	_____
103	_____

Comments: _____

_____

_____

_____

_____

****

TRIANGLE TEST

Name: _____ Product: _____

Two of the three samples are identical, the third is different.

(A) Smell the samples in the order indicated and identify the odd sample.

Samples	Mark odd sample
645	_____
972	_____
482	_____

(B) Indicate the degree of difference between the duplicate samples and odd sample.

Slight _____

Moderate _____

Much _____

Extreme _____

PAIRED COMPARISON

Name: _____ Product: _____

Smell the samples in the following order: 385 269

Which of these two samples do you prefer? _____

Comments: _____

_____

_____

_____

_____

PAIRED COMPARISON

Name: _____ Product: _____

Evaluate these two samples for the indicated characteristics. Mark which sample you prefer for each characteristic. Per characteristic start always with the left one.

	385	269
General appearance	_____	_____
Colour	_____	_____
Smell	_____	_____
Taste	_____	_____
Consistency	_____	_____

STATISTICAL CHART :

Triangle test, difference analysis

Number of tasters	Number of correct answers necessary to establish level of significance			Number of tasters	Number of correct answers necessary to establish level of significance		
	•	••	•••		•	••	•••
	5%	1%	0.1%		5%	1%	0.1%
7	5	6	7	57	27	29	31
8	6	7	8	58	27	29	32
9	6	7	8	59	27	30	32
10	7	8	9	60	28	30	33
11	7	8	9	61	28	30	33
12	8	9	10	62	28	31	33
13	8	9	10	63	29	31	34
14	9	10	11	64	29	32	34
15	9	10	12	65	30	32	35
16	10	11	12	66	30	32	35
17	10	11	13	67	30	33	36
18	10	12	13	68	31	33	36
19	11	12	14	69	31	34	36
20	11	13	14	70	32	34	37
21	12	13	15	71	32	34	37
22	12	14	15	72	32	35	38
23	13	14	16	73	33	35	38
24	13	14	16	74	33	36	39
25	13	15	17	75	34	36	39
26	14	15	17	76	34	36	39
27	14	16	18	77	34	37	40
28	15	16	18	78	35	37	40
29	15	17	19	79	35	38	41
30	16	17	19	80	35	38	41
31	16	18	19	81	36	38	41
32	16	18	20	82	36	39	42
33	17	19	20	83	37	39	42
34	17	19	21	84	37	40	43
35	18	19	21	85	37	40	43
36	18	20	22	86	38	40	44
37	18	20	22	87	38	41	44
38	19	21	23	88	39	41	44
39	19	21	23	89	39	42	45
40	20	22	24	90	39	42	45
41	20	22	24	91	40	42	46
42	21	22	25	92	40	43	46
43	21	23	25	93	40	43	46
44	21	23	25	94	41	44	47
45	22	24	26	95	41	44	47
46	22	24	26	96	42	44	48
47	23	25	27	97	42	45	48
48	23	25	27	98	42	45	49
49	23	25	28	99	43	46	49
50	24	26	28	100	43	46	49
51	24	26	29	200	80	84	89
52	25	27	29	300	117	122	127
53	25	27	29	400	152	158	165
54	25	27	30	500	188	194	202
55	26	28	30	1000	363	372	383
56	26	28	31	2000	709	722	737

STATISTICAL CHART 2

Two-sample test

Number of judgments	A: Minimum agreeing judgments necessary to establish significant differentiation			B: Minimum correct answers necessary to establish significant differentiation		
	Probability level			Probability level		
	5%	1%	0.1%	5%	1%	0.1%
5	....	....	....	5	....	....
6	....	....	....	6	....	....
7	7	....	....	7	7	....
8	8	8	....	7	8	....
9	8	9	....	8	9	....
10	9	10	....	9	10	10
11	10	11	11	9	10	11
12	10	11	12	10	11	12
13	11	12	13	10	12	13
14	12	13	14	11	12	13
15	12	13	14	12	13	14
16	13	14	15	12	14	15
17	13	15	16	13	14	16
18	14	15	17	13	15	16
19	15	16	17	14	15	17
20	15	17	18	15	16	18
21	16	17	19	15	17	18
22	17	18	19	16	17	19
23	17	19	20	16	18	20
24	18	19	21	17	19	20
25	18	20	21	18	19	21
26	19	20	22	18	20	22
27	20	21	23	19	20	22
28	20	22	23	19	21	23
29	21	22	24	20	22	24
30	21	23	25	20	22	24
31	22	24	25	21	23	25
32	23	24	26	22	24	26
33	23	25	27	22	24	26
34	24	25	27	23	25	27
35	24	26	28	23	25	27
36	25	27	29	24	26	28
37	25	27	29	24	27	29
38	26	28	30	25	27	29
39	27	28	31	26	28	30
40	27	29	31	26	28	31
41	28	30	32	27	29	31
42	28	30	32	27	29	32
43	29	31	33	28	30	32
44	29	31	34	28	31	33
45	30	32	34	29	31	34
46	31	33	35	30	32	34
47	31	33	36	30	32	35
48	32	34	36	31	33	36
49	32	34	37	31	34	36
50	33	35	37	32	34	37
60	39	41	44	37	40	43
70	44	47	50	43	46	49
80	50	52	56	48	51	55
90	55	58	61	54	57	61
100	61	64	67	59	63	66

ANNEX - 10

A guideline for the development of microbiological control procedures

A design for a microbiological control procedure for non-sterile pharmaceutical preparation could include the following:

(a) A statement of the microorganisms of concern and/or their toxins

For this a thorough knowledge of the normal kind and numbers of microorganisms in the raw materials and end-products, produced under good sanitary conditions, is needed. The significance of certain microorganisms should be evaluated in terms of the processing conditions, the use of the product, the nature of the product and the potential hazard to the user. In this respect the microbiological laboratory should already receive samples of plant material collected for (chemical) standardization to acquire knowledge about the contamination level of raw materials.

(b) The methods for their detection and quantification

Many methods may be equally good, but it must be established that reliable results with the chosen method can be obtained. Generally, it is preferable to use a method that has been subjected to collaborative study, especially if the parameter is being determined in the laboratory for the first time or if a product has not been analysed previously. It is also extremely important to include procedures for validating the method.

(c) The microbiological limits

In establishing microbiological limits, already existing criteria may be adopted. For a good understanding one should keep in mind that the application of microbiological criteria falls in three categories:

- microbiological standards: which may be mandatory, incorporated in a law or regulation. Nepal may adopt standards from other countries or create its own.
- microbiological specifications: these may be incorporated in a code of practice, which is of an advisory nature.
- microbiological guidelines: which may be used where no standard and no specifications for the particular product exist.

When no pharmacopoeial specifications are available for developed preparations, RDRL itself has to establish the limits. For this purpose the following may serve as a guideline (Ned.Ph.):

Category

I. Parenteral solutions, eye products and preparations used for body cavities normally free from micro-organisms:

Sterile.

II. Preparations used topically:

less than 100 micro-organisms per g or ml, among whom
no Enterobacteriaceae
no Ps. aeruginosa
no S. aureus

III. Preparations used orally:

less than 1000 to 10000 aerobic micro-organisms per g/ml
less than 100 yeast and moulds per g/ml
no E.coli per g/ml
in some cases: no Salmonella per g/ml
no more than 100 other Enterobacteriaceae per g/ml
no S.aureus per g/ml
no Ps. aeruginosa per g/ml

Products belonging to category III because of their use, may be treated as belonging to category II if they are susceptible to microbial growth (e.g. sirups, drops).

The upper limit, mentioned for products of category III is used when by no means the contamination can be reduced during preparation, the lower limit is used for all other products.

(d) A sampling plan defining the number of samples to be withdrawn and the size of sample unit

Without a satisfactory sampling plan, whatever method will be inadequate to appraise the microbiological quality of a product.

Existing sampling plans may serve as a guideline and/or pharmacopoeial requirements for similar products. For the principles of sampling and sampling plans referred is to ICMSF(1982).

ANNEX - 11

List of equipment and other materials provided by NEP/80/003

1. Inverted microscope
2. Trinocular microscope
   (photographic equipment ordered by Pharmacology)
3. Stereo Zoom microscope
4. Electronical balance (capacity 2000 grams)
5. Analytical balance (capacity 160 grams)
6. Magnetic stirrer/hotplate
7. Pipette washer
8. pH meter
9. Haemacytometer
10. Membrane filter holders
11. Spin mix
12. Fermentor with spare parts and provided with the necessary equipment to allow controlled parallel experiments; includes modules for control of pH and temperature and monitoring of dissolved oxygen
13. Vacuum pressure pump
14. Pressure vessel
15. Disposable filter units
16. Tubing for pressure and vacuum
17. Airconditioner
18. Ampules for the freeze dryer, ordered by Pharmacology
19. Borosilicate bottles and tubes
20. Media
21. Chemicals.

ANNEX - 12

Desirable items for the laboratory

1. Bottles of several sizes
2. Measuring cylinders, preferably of polypropylene
3. Graduated pipettes:
   - bacteriological type
   - serological type
4. Pipette fillers
5. Racks, baskets, trays } preferably of polypropylene
6. Disinfectant jars }
7. Steamer (may be made locally)
8. Autoclavable plastic boxes for test tubes
9. Pressure cooker for fast decontamination of materials
10. Different sizes petri-dishes
11. Indicators for correct autoclaving
12. Rubber stoppers
13. Blender for sample preparation.

ANNEX - 13

Addresses

1. Gibco Pacific Ltd. (NCTC 135 medium)
   Flat A8, P.C. Yip Building 62-70
   Texaco Road
   New Territories
   Hong Kong.

2. Becton-Dickinson and Co. (Trypticase, Bicarbonate)
   International Sales Division
   Paramus, New Jersey 07652
   USA.

3. American Type Culture Collection (ATCC)
   12301 Parklawn Drive
   Rockville, Maryland 20852
   U.S.A.

4. National Collection of Type Cultures (NCTC)
   Central Public Health Laboratory
   London N.W. 9
   England.