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REPORT ON LABORATORY TECHNIQUES COURSE ON QUALITY ASSURANCE FOR ACCREDITATION AT THE ALFRED JORGENSEN LABORATORY A/S, COPENHAGEN-DENMARK

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TRAINING AND EXPOSURE IN GOOD LABORATORY PRACTISE

AIM: To obtain better knowledge on quality assurance for accreditation.

SPONSORS: Danish International Development Agency, DANIDA.

DURATION: 9th OCTOBER - 6th NOVEMBER 2000.

SUBJECTS:

1. To follow the analysis of food, feed brewery and pharmaceutical products samples in the microbiological laboratory.

2. Read and compare Nordic methods(NMKL) at AJL to International Standards Organisation(I.S.O) methods used in F.R.I. laboratory.

3. To study the quality assurance required for accreditation for the microbiology laboratory.

4. To examine two freeze dried mxtures (ampoules) of micro-organisms according to NMKL methods and compare results with known content.

5. Two day working visit to a microbiological laboratory in a Danish Food Control Region at Køge, Tigervej 39, 4600, Køge.

6. Discussion and review of visit.

1.MEDIA

1.1 MEDIA PREPARATION

Culture media and chemicals

All culture media and reagents used in the media room were made from bacteriological grade chemicals. Every consignment of culture media and chemicals are examined physically for 1.Abnormality 2. Leakage 3.Clogging e.t.c. Registration of goods provide the following: name of manufacturer, name of product, date received, quantity, batch number, expiry date. Dates (received, open, expiry date, initial) must be written on the bottle/container.

The culture media and chemicals are stored in accordance wth the manufacturers instruction, otherwise stored at room temperature in a dry and dark place. Ready to use prepared media(broth, agar) are kept at 4--6^{0}C in a refrigerator. Tightly capped bottles and tubes can be kept at room temperature in a dark and dry place. Bacteriological dye solutions are kept in a fridge 3_12 months. Antibiotic solutions should not be kept for more than 3 months.

Preparation and Dispensing

Culture media are prepared from

1. Individual chemicals
2. Dehydrated powder.

Media prepared from the individual chemicals/dehydrated powder in the laboratory have caution cards placed on them. The cards read QUARANTINE - substrates may not be used before substrate control is approved and AWAITING RELEASE: Substrates may not be used before approval respectively.

Culture media prepared(in the laboratory) have the following information entered/registered on a file or note book:

- production date
- substrate type
- internal batch number(by which it is possible to go back tothe real
- batch number)
- quantity of dehydrated powder weighed.
- volume of water added
- number and volumes of tubes/bottles respectively.
- autoclaving condition(storage life)
- pH demand and measured pH.
- colour of the substrate after sterilization at (20 ^{0}C–25 ^{0}C).

The substrate data sheets are marked with red or green coloure to indicate whether supplements are to be added or not.

Growth Promotion

All substrates prepared in the laboratory are quarantined for growth tests before released for microbiological analysis. Known concentrations of relevant cultures are inoculated into samples of specific substrates and incubated at the appropriate temperature and time and recovery evaluated. Occasionally the substrates are used before the growth tests are completed but the results of the analyses are not released until growth promotion test results are confirmed.

Sterility Testing

For every substrate used a plate each was poured from the test substrate in the bottle and incubated alongside the inoculated Petri dishes to check for sterility. Opaque broths are tested by incubating them at the appropriate temperature and time and inoculating a non-selective agar with a loopful of the broth to test for colony appearance or growth.

2. EQUIPMENT

2.1.Autoclaves

Separate autoclaves are used for decontamination of disposable materials and sterilization of substrates. Records of autoclave operations, including temperature and time run cycles are maintained. Monitoring is done by print of pressure and temperature and recording of maximum temperatures attained.

Each month two vials of *Bacillus Stearothermophilus*_ATCC7653 are placed in the autoclave(top and bottom) to check for sterility after a full cycle.

Servicing and safety checks are conducted on the autoclaves annually but are drained and cleaned if visually dirty.

2.2 Incubators

The various incubators are at set at different temperatures and the internal temperatures checked daily with calibrated thermometers immersed in glycerol and placed at different locations. For a small incubator, temperature measurement are made from three different points (top, centre, bottom).Measurements are made from 5 6 different points in big incubators. The control thermometers are also calibrated monthly with thermocouple.

Graphs of limits of acceptance of temperatures recorded in the various incubators are plotted against time(over 24 hours) daily on the computer and used as checks to note any abnormality. Records of the graphs are also kept as internal control/audit checks.

Corrective measures may be taken if any incubator is not the set temperature. Entries made are counter-checked by another person for a period of e.g one week. Proper loading of the incubators are strictly adhere to. Stacks of Petri dishes are not more than 5-10 plates high. The incubators are thoroughly cleaned and disinfected internally every month and recorded in the incubator log book.

2.3 pH meter

The pH meters are calibrated daily using two different buffers(4.01 and 7.00). The buffers are stored in the dark e.g canisters and are marked with expiry date and date opened. pH in substrates are adjusted using 4N HCl and 10N NaOH(could be other concentrations e.g 6.87).

The electrodes are cleaned after each use and stored in 3mol/L KCl solution. Thorough cleaning of the electrodes are done monthly by using pepsin solution. The KCl level is checked and topped-up if necessary. Every procedure for maintenance or cleaning is recorded and signed in the appropriate book(e.g pH log book).

2.4 Balances

Balances are verified daily by checking against a standard(calibrated) weight e.g 100g.The weights are protected from dust, kept dry and handled with glove during use.

3 SAMPLE HANDLING AND IDENTIFICATION

Samples received from clients are first checked physically and laboratory numbers given to them. The laboratory numbers are then entered on to the clients request forms with date and initials. The above information is for some clients counter-checked by another officer/person

The following entries are made on the laboratory data sheet:
laboratory number

- sample identification number text from the client
- number of samples
- identity of organisation/person making the request
- date received
- nature of sample

- condition of sample on receipt
- conditions under which samples are stored in the laboratory
- lists of tests requireda
- and any other relevant information about the samples

4. AMPOULE EXAMINATION

Two Ampoules/ vials with the following codes, 2000:1 and 2000:2 respectively were provided by AJL and the following analysis/test conducted in their laboratory according to the Nordic methods(NMKL)/standards.

Ampoule:2000:1

Aerobic counts	25 C	NMKL 86
Coliform counts	37 C	NMKL 44
Thermotolerant coliform	44 C	(presumptive E.coli) NMKL 125
Clostridium perfringens		NMKL 95
Staphylococcus aureus		NMKL66
Salmonella		NMKL 71

Ampoule: 2000: 2

Aerobic counts	25 C	NMKL 86
Coliform bacteria count	37 C	NMKL 44
Enterobacteriaceae		NMKL 144
Enterococcus		NMKL 68
Bacillus cereus		NMKL 67
Yeast		NMKL 98

4.1 Preparation of Ampoules samples

The ampoules which are kept at $-18\ ^{0}C$ were brought out and left on the bench to thaw for about 5 mins. It was reconstituted with 1 ml of SPO and the mixture

transferred aseptically to 100ml SPOwith a sterile pasteur pipette. The ampoules were rinsed three(3) times with 1ml SPO.each time bringing the final volume to 104ml with the same pasteur pipette.

The samples were mixed thoroughly clockwise and anti-clockwise by shaking and not allowing it to touch the lid of the bottle. The samples were now ready for microbiological analysis.In preparing the samples it must be noted that,care must be taken to avoid spillages in order not to contaminate yourself and the laboratory environment since it is spiked with different types of pathogenic organisms. It may also affect your results or counts.

4.1.1.Aerobic counts

1ml of the sample is diluted in 10 folds in 9ml SPO four to five times and 1ml aliquote pipetted into petri dishes. Molten plate count agar(PCA) AT 45 ^{0}C is poured into the plate but not directly on the sample. The petri dish is rotated in the shaped of 8 or shaked on the bench to and fro for about 1minute to mix. It is then allowed to set and incubated at 25 ^{0}C for 3days. Colonies developed are counted after incubation(state incubator's number and temperature) umder a colony counter and the counts expressed as number of colonies counted x10 to the degree of the dilution(exponential) and reported as the colony forming unit per ml/g of the sample. Normally plates of between 10-100 colonies are selected and counted.

4.1.2 Coliform at 37 ^{0}C

After the appropriate dilutions are done and inoculated into petri dihes,molten Tryptone Soya agar(non-selective) is poured on it,mixed well and allowed to set and stand incubated on the bench for 1-2 hours to resuscitate stressed bacteria before violet red bile lactose is poured on to it at a ratio of at least 1:2 respectively and allowed to set and then incubated at 37 ^{0}C for 24hours, ±3. Colonies developed after incubation(dark red colonies of about 1-2mm with hallows around them are counted). Five(5) suspected typical colonies are subcultured into Brilliant green bile lactose broth equipped with durham tubes. It is incubated at 37 ^{0}C for 24hours. Coliform

bacteria produced gas(i.e a positive reaction). Calculate the population density from the colony count and the degree of dilution and report as such.

4.1.3 Coliform at 44 ^{0}C/Presumptive *E.coli/E. coli*

Bacteria growing in typical dark red colonies on violet red bile lactose agar at 44 ^{0}C - 24hours, ±3hours. The principle behind this is the same as that described above.

Presumptive *E. coli* are thermotolerant coliform bacteria which are indole positive at 44 ^{0}C

E. coli are thermotolerant coliform bacteria which in the 1M VIC test gives the reaction + + − −.

sample

↓

Diluted and plated on TSA/VRBL and incubated at 44 ^{0}C for 24hours.

(Poured TSA 1-2hours before pouring VRBL)

↓

Five(5) suspected colonies incubated into (preheated) LTLSB with durham tubes at 44 ^{0}C_24hours in the water bath.

↓

0.3—0.5 ml Kovacs reagent is added to the LTLSB tubes which produces gas after incubation.

↓

Indole positive, red ring appears on top of broth after 30secs.-1min. in addition to the production of gas.

↓

Calculate the population density from the colony count and the degree of dilution and report as such.

4.1.4 *Clostridium perfringens*

Sample is serially diluted 10−1 → 10−2 and plated at TSC plates which have been coated with 5ml TSA to avoid the black clostridia colonies from growing at the bottom of the petri dishes which usually spread out.

↓

Inoculated plates are inverted and incubated at 37 C anaerobically at 37 C for 24hours ± 3hours.

↓

Characteristic colonies which appear black on TSC and between 10-100 are counted/enumerated.

↓

5-10 suspected colonies are streaked on blood agar and incubated anaerobically at 37 C_24hours,± 3hours which shows haemolytic reaction around the colonies.

↓

Pure single colonies appearing(Pure culture)

↓ ↓

Stab inoculated into motility medium

incubated anaerobically at 37 C 24hours, ± 3hours.

Inoculate.Lactose fermenting medium with durham tubes.

↓

Motile organisms will grow through the medium.Non motile will grow only along the stab.(motility can also be examined by inoculating in a liquid medium B.H.I incubated anaerobically 4-6 hours and examined by phase contrast microscopy.

↓ Positive reaction

Gas production in the durham tubes.Colour of the medium also from red to yellow.(fermentation of lactose)

4.1.5 *Staphylococcus aureus*

Sample which was preheatd already was serially out with SPO(1ml to 9ml, vortex and transferred) to the next tube(1ml) and plated on BP with tellmite egg yolk emulsion supplements.

↓

0.1 ml of suitable dilution were transferred into petri dishes and spread out on BP.

↓

It was incubated for 24-48hours at 37 C

↓

Five suspected characteristic grossy colonies were plated on Blood agar at 37 C for 24hours.

↓

The pure cultures were inoculated into thamed coagulase medium, incubated at 37 C and checked every 2hours for clotting. It clotted after just 2hours.

↓

The number of coagulase positive colonies were enumerated and reported as such.

4.1.6 *Salmonella*

25ml of sample

↓ Pre enrichment

225ml of BPW incubated at 37 C for overnight.

↓ Enrichment

0.1ml of sample transferred to 10 RV incubated in a water bath at 42 C 18-24hours.

↓

It was plated on an Ramback and xLD, incubated at 37 C for 24hours.

↓

Five suspected colonies wre plated out on TSA, incubated at 37 C for 24hours.

↓

Pure isolated colonies obtained.

↓

Serological test

Colonies were emulsified with a drop of salmonella polyvalent O and H antiserum and checked for agglutination/gluboles.

↓ ↓

A colony was also emulsified with saline solutions to check for auto agglutination(negative control)

Agglutinated colonies are confirmed as salmonella species.

N.B: *Salmonella adrabraka* was inoculated alongside as a positive control since it was not isolated in the temperate countries. Negative controls (blank broths and agars) were also run alongside.

4.1.7 Enterobacteriaceae

Samples are pretreated as usual and 1ml aliqoute of appropriate dilutions are plated-out

↓

Voilet Red Bile Lactose agar is poured onto it and mixed. After solidification, another layer of the same medium is poured on it to set.It is then incubated at 37C for 24hours.

↓

Dark red colonies developed after incubation are enumerated. Normally plates with between15 -150 colonies having a diameter of >0.5mm are counted.

↓

5 suspected colonies are streaked out on TSA and incubated at 37 C 24hours.

↓

Pure single colonies are streaked on oxidase test strips and allowed 30

seconds to changed positive → deep blue violet. Negative → no changed or changed later afer 1 minute or more.

↓

Calculate the population density of the Enterobacteriaceae from the percentage of oxidase negative colonies on the counted plate.

Pseudomonas aeroginosa which is oxidase positive was used as the negative control.

4.1.8 Enterococcus

Samples are pretreated as usual and appropraite dilutions plated-out into the petri-dishes.TSA is first poured onto it mixed well and allowed to set on the bench for about 1-2 hours (Pre-incubation)

↓

An overlayer of Slanetz and Bartley agar is on it to a ratio of 1:2 respectively.

↓

Plates are inverted and incubated at 44 C for 48hours.

↓

Faintly coloured colonies are enumerated/counted and subcultured on TSA at 37 C for 24hours.

↓

Catalase test were performed on pure isolaed colonies which were catalase negative.

↓ ↓

Five suspected colonies were subcultured into Brain Heart Infusion broth with 6.5% NaCl and incubated at 37 C and checked for growth after 24hours..

Five suspected colonies subcultured subcultured into Brain Heart Infusion broth with pH of 9.6 and incubated at 37 C for 24hours.

There was profuse growth which confirms enterococcus.

Catalase Test(Control experiment)

Enterococcus feacalis negative

Staphylococcus aureus positive

4.1.9 *Bacillus cereus*

Sample is pretreated as usual and decimal dilutions made. 0.1ml or 1ml aliquotes are plated out on Bacillus cereus agar. It is incubated at 37 C for 48hours.

↓

Characteristic blue large colonies appearing after incubation are enumerated.

↓

Five suspected colonies are subcultured on Blood agar incubated at 37 C for 24hours.

↓

Haemolytic zones appear around the characteristic greyish white colonies.Double haemolytic zones appears if incubated further for 48hours at 37 C.

↓

Phase contrast microscope is used to examined for sporulation, since Bacillus cereus produce spores on Blood agar after 48hours incubation at 37 C.

↓

Confirmed colonies are counted/enumerated as such.

4.1-10 Yeast

40ml of the sample is weighed into 360ml of 0.1% peptone water. It is homogenised and serially diluted and plated out on DG 18 agar. It is incubated at 25 C for 5 days.

↓

Colonies appearing are enumerated.

↓

Microscopy and further identification test such as using API 32 D is used to identify the type of yeast.

↓

Confirmed colonies can be expressed as the colonies counted multiplied by 10 to the exponential of the dilution and expressed as cfu/ml.

LISTERIA MONOCYTOGENES

The above test were performed on party-packed cold smoked salmon fish ready to service.

25g of the sample.
normally the sectional areas are cut and weighed aseptically into.

↓

225ml of UVM 1 broth with supplement(SR 14 2E OXOID) incubated at 30 C for 24hours.

↓

0.1ml is subcultured into 10ml UVM II incubated at 30 C for 24hours..

↓

It is streaked out on O xford and Palcam selective agar plates, incubated at 37 C for 24-48hours.

↓

Suspected colonies appearing are restreaked on Blood agar incubated at 37 C for 24hours.

↓

The plates are checked for β-Haemolysis.

↓

Pure single colonies prepared for identification are runon the Accuprobe machine.(which prints out whether *Listeria* positive or negative in about 5 minutes).

DETECTION OF LACTIC ACID BACTERIA , OTHER BACTERIA AND WILD YEAST IN BREWERY.

Method; About 100ml beer was filtered through 0.45μ pore size(using the millipore filter apparatus) The filter is then placed aseptically on pre-poured dried Universal beer agar(UBA) agar plates and incubated anaerobically at 25 C for 3days...

For aerobic counts the filter is placed on yeast water agar at 25 C for 3days. Colonies developed after incubation are counted and expressed as the number of colony forming unit per 100ml of beer.

For yeast the filter incubated on wort agar with actidione/cycloheximide to suppress the growth of bacteria.

Malt yeast glucose peptone(MYGP) with copper sulphate is used to isolate wild yeast. Lysine media can also be used.

Colonies developed are purified and examined under the stereo microscope or using the ordinary light microscope(x10 objective).

FOR BACTERIA

(I) Colonial morphology (shape, colour, edge, pigmentation e.t.c)
(ii) Cell morphology (rods or cocci, arrangement of cells)
(iii)Gram reaction (3% KOH)
(iv) Catalase test (3% H_2O_2)
(V)Gas production
(vi) Growth at different temperatures
(vii)Assimilation of sugars(API)

FOR YEAST

(I)Colonial morphology (as for bacteria)
(ii) Cell morphology (oval, budding, vacoules, hyphae or pseudo hyphae)
(iiI) Gas production
(iv) Growth at different temperatures
(v) Assimilation of sugars(API)

During our visit two yeast contaminants and a bacteria from a brewery and candy

industries were identified.

STERILISED CANNED FOODS

Food packed in air-tight cans/containers keeping quality of which has been approved by bactericidal treatments.

Slightly acidic products with pH above 4.5

- incubated at 30 C for 14days.
- incubated at 55 C for 14days(tropical conditions)

Products with low pH 4.5 and below

- incubated at 25 C for 14days

Temperatures in the incubators must not deviate ± 2 C. Enough time must be allowed for good air circulation between cans.

After incubation the cans are inspected for
(i) bulging and other defects.It is then opened for examination of contents
(ii) microbiologically(direct plating)
(iii) chemically
(iv) lacquer, e.t.c
Reporting
(i) Type, labeilling and size of lot.
(ii)number of cans incubated, incubation temperature and time.
(iii)number of defective cans after incubation and character of defects
(iv)probable percentage of defective cans in lot.
Refer to standard sampling chart.

SUBSTRATE CONTROL

(a) All substrates used were written down in the laboratory sample data sheet(name and type of substrate)

(i) When the substrate were made, with date and initials.

(ii) When substrate was poured, with date and initials

(iii)When substrate was used, date and initials.

(b) All corresponding supplements added to the substrates were noted with

(i) Supplements name

(ii)Supplements batch/lot number

(iii)Expiry date of supplements.

(iv)If possible when supplement was first opened and used

(c) pH of substrates are noted and recorded before usage.

(d)Blank poured plates or tubes of substrates used are incubated and run parallel to test as negative controls

(e)Relevant organisms are also inoculated with substrates and incubated at the same temperature and time to run parallel to test as positive control.

(f) Prepared substrates are kept at 2-8 C in the refrigerator ready for use

(g) Poured plates of substrates are kept in polythene bags on the laboratory working bench ready for use

(h) Melted substrates are kept at 45 C in the water bath for at least 1hour prior to usage.

A.P.I

Three different micro-organisms overnight cultures of

(i) Escherichia coli

(ii) Enterobacter aerogenes

(iii) Salmonella enteritidis

which are reference strains at AJL were prepared to 0.5 Mcfarland and inoculated into A.P.I 20E strip with a pasteur pipette. Inoculation is carefully done into the small capsules to avoid air bubbles from ONPG capsule to OX capsule. All the capsules were sealed with sterile paraffin with the exception of CIT, VP and GEL.

The plastic trays (A.P.I) were carefully labelled with the strains and moistened and the inoculated strips placed into it and covered. It was incubated at 37 C for 24hours.

After incubation, certain reagents were added to GLU, CIT, VP and GEL in safety cabinet and the reaction read according to the A.P.I manual/instructtions.

The positive and negative reaction figures were added up and the sum in each block fed to the computer with the A.P.I PLUS programme on the accompanying sheet with any other relevant information.

The computer is able to tell which micro-organism to its significant taxanomy and tell you whether it is a good identification or any additional complimentary test to be done to confirm that particular micro-organism.

It also tells you the percentage identification. All this infotmation is printed out for the A.P.I 20E from bio Merieux was used.

N.B: Find attached a sample data sheet.

TWO DAY WORKING VISIT TO THE MICROBIOLOGY LABORATORY IN KØGE(DANISH FOOD CONTROL) TIGERVEJ 39, 4600 31ST OCTOBER-1ST NOVEMBER, 2000.

We visited a public accredited food reference laboratory in Køge which is about 40Km from Copenhagen. The laboratories are housed in two big blocks with an additional block as the administration. We were welcomed and briefed by Berit Behbahani, the vertenarian on the quality assurance/control systems in place and conducted around the laboratories.

We understudied how samples are collected or received, numbered and analysed using a computerised system. We also learnt about the maintenance, calibration and performance verification of laboratory equipment including limits of acceptance.
On media, we were showed how they are received, numbered and tested for growth

support and how colours are used to distinguish media received but not tested, number of substrates and media in use. Colours are also used on prepared substrates to differentiate between those that can readily be used without or with supplement. We also understudied the technicians analysing food samples with division of task at every stage.

Laboratory wastes such disposable wares are put in paper boxes and incinerated whiles glassware are kept in disinfectant in a safety cabinet, autoclaved at 121 C for about 1hour before washed.
There were small containment laboratories in each block for different purposes

CONCLUSION

Most of the procedures in food testing we were familiar with but we were exposed to the use of different media and new techniques. We were also exposed to the use of modern equipment such as the Delta dilutor, _80 C Freezer e.t.c. We had the opportunity to learn internal quality control practises in the microbiology laboratory e.g Growth promotion e.t.c. We evaluated and compared the Norfic methods(NMKL) to the ISO methods and worked on 2 freeze dried vials of mixture of micro-organisms and compared results with known content i.e "Calibrating the technician".
Samples delivered to the laboratory were promptly worked on and results dispatched immediately. One major observation was the division of work amongst staff which enhanced efficiency which we wish to recommend to Food Research Microbiology Laboratory. On the whole, the training was intensive, interesting and very short. Future follow-up of such opportunities would be very much beneficial, both to the technicians and the institute.

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