ANIMAL PRODUCTION AND HEALTH SECTION
JOINT FAO/IAEA DIVISION
VIENNA, AUSTRIA

FAO/IAEA/SIDA

GUIDELINES FOR SERO-MONITORING OF CATTLE CONDUCTED BY PARC

OAU/IBAR/PARC

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1) INTRODUCTION

This booklet has been prepared by the Animal Production and Health Section of the Joint FAO/IAEA Division in Vienna in cooperation with the Animal Production and Health Division of FAO, Rome, together with the EEC and PARC co-ordinators from Nairobi, Kenya. It is designed to give guidelines to those involved in the serological monitoring of antibodies to rinderpest virus in cattle in Africa.

The Pan African Rinderpest Campaign (PARC) is attempting initially to control but eventually to eradicate the disease from the African continent by vaccination and by the control of cattle movement. The task will be carried out by national teams and will generally involve the mass vaccination of the cattle population in a country; in certain areas strategic vaccination along borders is to be adopted. OAU/IBAR/PARC is responsible for international campaign co-ordination and EEC is the major donor for the campaign.

To assist in evaluating the success of national vaccination programmes it is proposed to establish a laboratory or laboratories in each country where serological monitoring of antibodies to rinderpest can be carried out using an Enzyme Linked Immunosorbent Assay (ELISA) to determine the level of immunity in the national herd or along border zones.

Numbers of animals will be blood sampled annually and the serum tested for the presence of specific antibodies to rinderpest virus. From the results of this sample an estimate will be made of the number of animals in the population which carry these antibodies, and hence the level of vaccination achieved in the population. (It should be noted that these antibodies could have been induced either through vaccination or natural infection but in either case these animals will be immune.)

This information will be used by the national co-ordinators of each country to assess the effectiveness of their vaccination campaigns. Carried out on a district basis it will even allow evaluation of individual vaccination teams.

The objective of this booklet is to give guidelines to those involved in this sero-monitoring exercise. In particular when and where to sample, what data to collect with the samples, what equipment is needed and how to process the results. These guidelines, although based on sound epidemiological principles, are designed to take into account the different conditions that exist in the countries involved in PARC. They are therefore intended to be practical and workable, although each country may have to make adjustments to suit its own particular situation.

The actual testing will be carried out in national or regional laboratories using ELISA kits supplied from the FAO/IAEA Agricultural Laboratory, Austria, in conjunction with the Institute for Animal Health, UK. Protocols for the test itself are covered in a separate manual.

It is hoped that by following these guidelines and using standardised kits in the testing laboratories a uniform approach to sero-monitoring will be obtained throughout the countries involved in PARC. This will enable meaningful comparisons to be made both within countries and on a country-to-country basis.

2) REQUIREMENTS

a) Equipment

This booklet is concerned only with the actual collection of the sera, its transport to the laboratory and the presentation of the results. It is not concerned with the testing of the sera in the laboratory. Hence the equipment listed below is limited to that required for serum sampling. It is not possible to give numbers for each item as this will depend on the size of the national herd and hence the number of animals to be sampled.

List of Equipment Needs for Blood Sampling Teams

ITEM
Vacutainers (10ml draw). (Approx. 25 000)
Vacutainer needles and holders.
Vacuum pump and tubing. (for recharging vacutainers; this need only be adopted if new vacutainers not available)
Sterile bijoux bottles.
Cold box for transport of sera and/or portable refrigerator.
Sterile pipettes and bulbs.
Blunt forceps.
Chest freezer* (–20°C).
Centrifuge* (for spinning vacutainers).
Clip boards.

* These items will probably remain in the base laboratory.

Field sheets.
Labels.
Boxes for storage of sera.
Water-proof pens and markers.
Transport.
Camping equipment.

This equipment can be obtained from any laboratory supplier i.e. Gallenkamp Ltd. The total cost is approximately 34 000 ECU excluding the price of the vehicles.

b) Running Costs

It is accepted that funds for this equipment will be discussed during the preparatory phase of National PARC projects; funds may also be committed for the purchase of a suitable vehicle (four wheel drive); for spares and vehicle running costs.

c) Sampling Teams

It is vital that separate serum sampling teams are established. These should be entirely self sufficient and **not** linked to vaccination teams. Sampling of animals at the time of vaccination will produce biased results and in that they relate to previous campaigns, not provide the national co-ordinators with information of any real use. However, sampling 3 weeks or more post-vaccination will be of use in assessing the efficiency of vaccination teams.

d) Serum Banks

A decision will need to be made regarding the establishment of a serum bank. Further details of this are given in this booklet; however if the establishment of a serum bank is considered necessary funds may be requested for the purchase of suitable cold storage facilities, storage containers and data handling equipment.

e) Incentives to Owners

In some countries it may be necessary to provide incentives to animal owners to allow serum collection. Most appropriate is the supply and administration of drugs for animal treatment. If this course of action is chosen then adequate funds should be allocated from national resources. However, this option should be adopted with caution as it may lead to a request for incentives whenever collaboration from livestock owners is requested.

3) SAMPLING PROCEDURES

In this section guidelines will be given on when, where and how many samples are to be taken. In many ways this is the most important aspect of the whole procedure and will determine the value of the final results. In drawing up these guidelines a compromise has to be reached between the ideal and reality. Suitable epidemiological principles have been employed allowing mathematical manipulation of the results to estimate immunity levels in the population as a whole, whilst being applicable to conditions in African countries.

It should be understood that the primary reason for carrying out monitoring of the vaccination campaign is to provide national co-ordinators with a quality control/trouble shooting system that can be used to improve the effectiveness of campaigns and identify problems as and when they occur. Sampling procedures have been designed with this objective in mind.

In considering sampling procedures, two distinct types of cattle management exist in Africa and a separate approach to sampling of the animals is needed for each. These two husbandry types are:

a) Sedentary Cattle Farming: the cattle are based around a farm or smallholding and although they will graze in the surrounding areas, the herd as such is stationary and remains in the locale. This type of husbandry is more common in East Africa and in humid tropical countries. It involves most government farms and these herds are sampled more easily.

b) Nomadic Cattle Rearing: the cattle are not kept in one locale but move over large distances on a seasonal basis in search of food and water. This is most common in West and Central Africa and involves the movement of cattle herds through regions and across national bounderies. Nomadic herds are more difficult to sample but are most important for national evaluation of vaccination cover.

Both of these systems may be found in one country; thus two sampling approaches will be required.

It is **essential** that sampling is carried out **after** the vaccination teams have been into the area and not at the same time. Sampling at the same time as vaccination will determine the level of vaccination achieved the previous year and only in animals presented for vaccination. Such information is of limited value to national co-ordinators.

Samples should be collected no earlier than three weeks after a vaccination team has been in the area, to allow for the development of antibodies following vaccination. **Samples should be collected, if at all possible, within two months of a vaccination team leaving an area,** after this the cattle population in an area may have significantly changed, and vaccination teams may have been disbanded or the personnel changed so that attribution of praise or blame will be impossible.

Any survey over regions of even modest size will have to be carried out using some form of cluster sampling procedure: simple random sampling will not be possible. Tables for the number of samples to be collected and for the subsequent accuracy of prevalences estimated from the samples usually assume that the data was collected using simple random sampling but this will not be the case. Thus at some stage such factors as the intra-cluster correlation and design effect will need to be determined to establish the accuracy of the prevalences estimated from the sample. If the guidelines given in this booklet are followed for the first year of the survey, an acceptable and useful indication of the prevalence of antibodies can be determined. The transfer of these results to the PARC epidemiological team in Nairobi will then allow a full evaluation of the results. Subsequent sampling procedures can then be altered if necessary to give the desired degree of accuracy.

It is not the intention of this booklet to give complex statistical formulae for the analysis of results. For such, reference should be made to standard epidemiological textbooks (see reference list) or to the PARC epidemiological team. It is expected that if the samples are collected in the manner described in this booklet the results will be meaningful and obvious, without recourse to complex mathematical manipulation.

4) SAMPLING APPROACHES

a) Sedentary Herds

The country must be divided up into small units with sub-populations of animals. Most countries will already be divided up into "Districts" for administrative purposes and these districts can serve as units for sampling. In nearly all cases a similar partitioning of the country will have been carried out for the vaccination campaign and these partitions can be equally adopted as sampling units without introducing an unacceptable bias in the results.

For any given District (or unit) specific numbers of animals will be sampled at randomly selected sites. In each District (or unit) **SIX** sites will need to be selected from the total number of sites identified. Sites will be determined according to livestock distribution, geographical factors and the way in which the vaccination teams have been operating so as to represent the District as a whole and cover all vaccination teams operating in the District. Actual sites can be a village, dip site, or any other suitable location where animals congregate. **Sites where the animals are known to have been more carefully vaccinated than normal or conversly areas where vaccination has not been carried out, must not be specifically chosen.** The total number of sites in each District will vary, depending on the size of the District but this will not alter the sampling approach.

Each site in the District is allocated a number and, using a random number table (one is to be found at the back of this manual), **six** of the sites identified for sampling. These six sites should be sampled in a two week period if at all possible. Sampling should not be carried out within three weeks of vaccination but should be completed within two months of vaccination. Thus these sampling teams follow-on behind the vaccination teams by about a month.

At each site 40 serum samples should be collected. These 40 must be selected from the following age groups:

10 from animals under one year old
10 from animals between one and two years old
10 from animals two to three years old
10 from animals over three years old.

The age of an animal can be determined by questioning the owner and by an examination of the animal's dentition.

The actual sampling is unlikely to be random but this will not affect the validity of the results as the sampling sites themselves were randomly selected. True random selection at a sampling site is unlikely to be possible.

Defined data will need to be collected at the sampling site and with each serum sample taken. This will be dealt with later in the booklet.

b) Nomadic Herds

Nomadic herds represent a high risk group for the vaccination campaign because they are more difficult to locate and can transmit disease over large areas. Thus, although the difficulties which face vaccination teams will also face the sampling teams, every effort must be made to sample serum from these herds.

In many cases these herds are large (over 200 animals) and tend to gather in specific places such as water holes. This can help sampling. In some areas nomadic herds can cross national borders and this will need to be taken into consideration when sero-monitoring. However, the PARC may, by means of border harmonisation meetings, overcome many of the difficulties likely to be encountered in frontier areas.

An estimate based on the 1985 FAO Animal Production Year Book gives the following numbers of nomadic animals:

West Africa — 13 million
Central Africa — 10 million
East Africa — 7 million (Ethiopia and Sudan excluded)
Ethiopia and Sudan — 20 million.

Thus these nomadic herds represent a significant number of animals and the sampling procedures must take this into account. In the case of these animals the word "herd" is used to refer to a collection of animals travelling together, and these may well be owned by several people.

Once again, blood sampling should follow on behind the vaccination teams. However, as with the vaccination of these animals, blood sampling will probably not be possible throughout the year; it should probably be carried towards the end of the dry season when cattle populations concentrate around permanent water sources.

As with sedentary herd areas, the country should be divided into areas; this again will probably be at District level. Within the District the number of herds will need to be identified. This can be done with reference to local livestock offices, vaccination team records and if necessary an examination of the area prior to sampling. The number of herds to be actually sampled depends on the total number of herds estimated to be located in the District. The following table indicates the number of herds to be sampled for any given total number of herds in a District. It also shows the total number of animals that will thus be sampled in the District.

Estimated No. of herds in a District	No. of herds to be sampled	No. of animals to be sampled (40/herd)
10	9	360
20	16	640
50	31	1240
100	46	1840
200	59	2360
500	72	2880

As with the sampling site for sedentary farms 40 animals from each herd should be sampled, 10 from each of the following age groups i.e.

10 from animals under one year old
10 from animals between one and two years old
10 from animals two to three years old
10 from animals over three years old.

The age can be determined by questioning the owner and by examination of the dentition.

5) SAMPLE COLLECTION AND TRANSFER TO TESTING LABORATORY

Ideally each serum collection team will be provided with a centrifuge, generator for operating this centrifuge, and a portable refrigerator. The samples, collected in a 10 ml vacutainer, should be kept at ambient temperature, except in the very hottest of areas, or at +4°C until a clot forms (usually overnight). The clot should then be removed using a pair of blunt forceps and the sample centrifuged before decanting it into a sterile bijoux bottle.

Although for ELISA testing it is not important that the samples remain sterile, for other tests to be carried out on this sera this may be essential and thus a sterile approach to sera collection and storage is to be highly recommended. The teams will therefore need sterile pipettes and bottles for this part of the process.

The serum should then be held in the refrigerator until it is transported to the local Veterinary Investigation Centre or Regional Office. Here it should be stored at –20°C until it is transported to the testing laboratory. It should be at the laboratory within one month of the collection and all relevant documentation should accompany the samples.

Clearly local conditions can necessitate alternative approaches to this. The samples may be stored in the field in the vacutainers (once the clot is removed) and the centrifugation may be done at local Veterinary Investigation Centres. Ice boxes could be used to transport the samples back to the laboratory avoiding the need for portable refrigerators, but the samples must arrive at the laboratory on the same day as they were taken. If no power supply is available 20 ml of blood may be collected in a 20 ml vacutainer, in which it is

allowed to clot at ambient temperature for a few hours, and then is kept at +4°C overnight. If the tubes are kept flat during this time then the clot will stick to the rubber bung which can then be carefully removed so pulling out the clot. The serum is then held at +4°C for a day in order to allow the remaining red cells to settle before decanting into a sterile bijoux bottle.

The essential features of whatever system is adopted are:

a) that between 5 to 10 ml of blood is collected from each animal;
b) that the samples are sterile on arrival at the testing laboratory;
c) that each sample is clearly labelled;
d) that adequate information accompanies the sample.

Suitable boxes are required for transporting the sera to the testing laboratory and for storing it there. Putting serum samples into plastic bags is not recommended, since all the sera have to be sorted out at the laboratory. Preference is to be given to the use of cardboard boxes with suitable dividers for holding serum bottles separately. Such boxes should be obtained in bulk before the seromonitoring begins.

Collection and Recording of Information

Basic information must be collected by survey teams at the time of bleeding. This information is vital if the results of the seromonitoring are to be of any real value. Survey teams should approach this part with as much care as the actual bleeding and allow sufficient time for its proper completion. The details suggested below are the **minimum** that should be collected. If the establishment of a serum bank is a planned objective, further information will be needed to give any real value to the serum bank. Suggestions are also given on labelling of samples and accompanying documents. These are details which have been found to work in practice. Seromonitoring, if carried out correctly, will generate a large number of samples and a great deal of paperwork accompanying the samples. Unless an organised approach with regard to sampling and documentation is adopted, chaos will ensue. There are many appoaches to this and everyone can think up new ideas and alternatives. However it would be most useful if the methods suggested here are adopted by all the teams throughout PARC. Other systems exist and ours may not be the ideal, however it does work.

An unlabelled sample or a sample with no accompanying information is useless and a waste of valuable resources

Two categories of information are required:

a) a description of individual animals that are bled;
b) a description of the group (herd or herds) of animals at the sampling site.

Field recording sheets should be used to ensure that all required information is collected. It is important that these sheets are fully and accurately completed by the survey teams. These sheets must be provided by the testing laboratories and adequate funds should be allocated for them.

An example of the record sheet is given in Figures 1 and 2. The information should be on either side of a single sheet if at all possible.

Considering the various headings on side one (Figure 1):

SITE CODE

Region — each region should be allocated a code letter.

District — each district within a region should be allocated a two digit code number.

Site — each site within a given district should be allocated a number (1—6).

(For example — the code D/07/2 would indicate the second sampling site in district number 7 in region D.)

SAMPLE NUMBER

The serial number allocated to the blood sample by the field team. In each district samples should be numbered serially, starting at 1.

SEX

Enter "M" for entire male, "C" for castrated male and "F" for female.

AGE

Enter the age given by owner/herder. If in doubt check the dentition.

Figure 1.

PAN-AFRICA RINDERPEST CAMPAIGN
RINDERPEST SERO-MONITORING

code

REGION			DATE		
DISTRICT			SITE CODE		
PLACE NAME			ADDRESS OF LOCAL CONTACT		
MAP REF.					

DETAILS OF SAMPLED CATTLE:-

MILK		SETTLED		COMMON GRAZING	
MEAT		TRANSHUMANCE		ENCLOSED GRAZING	
DUAL PURPOSE		NOMADIC		ZERO GRAZING	
OTHER		OTHER		OTHER	

BREED:-

HISTORY OF RINDERPEST VACCINATION:-

DATES OF LAST TWO VACCINATIONS:-

ESTIMATE OF PROPORTION OF CATTLE AT SITE WITH PUNCHED EAR

0%	25%	50%	75%	100%
1	1	1	1	1

REMARKS:-

FOR LABORATORY USE:-
LABORATORY NUMBERS FROM:-
TO:- LAB. FILE REF. NO.:-

Figure 2.

PAN-AFRICAN RINDERPEST CAMPAIGN
RINDERPEST SERO-MONITORING

CODE REG. DIST. SITE

Sample No.	Sex	<1	1-2	2-3	>3	Punched	Sample No.	Sex	<1	1-2	2-3	>3	Punched

Collected by:- Signature:- Date:-

EAR PUNCHED/BRANDMARK

Enter "Y" if ear punched or animal branded with the particular pattern being used by the vaccination teams (in many countries a clover leaf pattern). "N" if not ear punched/branded.

On side two of the sheet (Figure 2):

MAP REF.

Give, if possible a longitude and latitude reference.

LOCAL CONTACT

Name and address of chief or veterinary assistant or other local contact.

DETAILS OF CATTLE AT SITE

Enter x in appropriate box(es).

HISTORY OF RINDERPEST VACCINATION

Give brief details of vaccination history — date of last vaccination, vaccination carried out in the past few years (eg none, calves only, annual etc.).

ESTIMATE OF PROPORTION OF ANIMALS AT SITE WITH EARS PUNCHED WITH THE PARC CLOVER LEAF OR NATIONAL MARK

Put "x" on scale to indicate your estimate of the percentage of ear punched/otherwise marked animals.

The sera, together with the data sheets will be held at a local site (Veterinary Investigation Centre, Regional Veterinary Office etc.) until transported to the testing laboratory. At the testing laboratory the sample details should be entered into the laboratory accession ledger — an example of this is given in Figure 3. Many alternatives to this can be devised but this should be adhered to, if at all possible.

LABORATORY NUMBER — sera should be numbered consecutively. This is strongly recommended for ease of testing. This number will not be the same as the field number of the sample.

FIELD NUMBER — the sample number allocated by the field team.

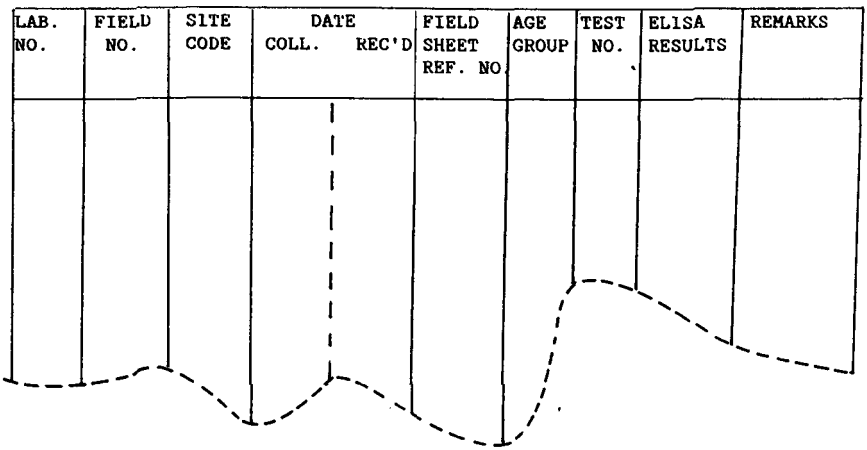
SITE CODE

DATE SAMPLED/DATE RECEIVED (at laboratory)

FIELD SHEET REFERENCE NUMBER — the reference number for the laboratory file in which the field sheet is filled.

Figure 3.

Format for Laboratory Ledger



AGE GROUPS — 1 = <1 year of age
2 = Between 1 and 2 years of age
3 = Between 2 and 3 years of age
4 = >3 years of age.

TEST NUMBER — the serial number allocated to the test.

ELISA RESULT — recorded as positive or negative. It would be helpful if the optical density reading is also given.

REMARKS — such as vaccination history, positive/negative cut off point, etc.

It is hoped that, as the sero-monitoring develops, most of the testing laboratories will be equipped with simple micro-computers connected to the ELISA readers. These will allow direct collection of the results from the reader and will allow storage of all information relevant to the sample. They would also allow data manipulation and for presentation of results to national co-ordinators. Suitable software is being developed at the FAO/IAEA Agricultural Laboratory, Seibersdorf. In all cases back-up storage is imperative in case records are accidentally destroyed.

6) SUBMISSION OF RESULTS TO NATIONAL RINDERPEST CO-ORDINATORS

The laboratory results should be submitted to the National Co-ordinator immediately after completion of testing of each batch of sera. If necessary the National Co-ordinators can then act immediately to adjust his vaccination strategy. This testing is an attempt at ongoing evaluation of the vaccination campaign, so that if areas are being missed or vaccination teams are not being successful, action can be taken immediately in the present vaccination year and not deferred until the following years' campaign. The National Co-ordinators are unlikely to want either raw data from the reader or data that has undergone a series of complex mathematical manipulations. The following format is strongly recommended:

SITE	SITE CODE	DATE	AGE GROUPS 1234	TOTAL
Give name and map reference			For each age group give the number positive divided by the total number tested in the group	

7) STATISTICS

Estimates of the size of District populations may be available. The size of populations of animals in each age group can be derived using estimates of herd composition (either obtained as an ancillary activity of PARC, or from existing data from research centers/ projects).

Using these data, estimates of the number of animals by age group and district can thus be obtained.

It must be remembered that serum sampling can only produce an ESTIMATE of the proportion of sero-positive animals in a population. This estimate can be given more meaning and depth if a confidence interval (C.I.) is calculated and used to derive the lower confidence level (LCL) and upper confidence level (UCL). The 95% C.I. is acceptable.

C.I. 95% = 1.96 × standard error (approx. 2)
LCL = p – C.I. where p = proportion of animals positive in ample
UCL = p + C.I.

When the 95% C.I. is used then one can be 95% confident that the true proportion of sero-positive animals in the sampled population lies within the range LCL to UCL.

Reporting the results of sampling in the form of statistics (i.e. the proportion of positive animals in the sample) AND giving the LCL and UCL is more meaningful than reporting the data alone.

It is important to note that calculation of the standard error when cluster sampling has been used (as in this exercise), requires the use of appropriate formula, as the use of the formula for simple random sampling will produce an under-estimate of the value of the standard error.

These statistical studies can be carried out in each country and the results conveyed to the FAO/PARC epidemiological team in Nairobi, Kenya. On the other hand, raw data can be sent to Nairobi for more elaborate work which uses a number of computer based statistical packages. The information obtained can then be fed back to the National Co-ordinator and testing laboratory. Either way it is important that results are fed to the epidemiological team in Nairobi for mapping of PARC vaccination achievements on a Regional basis.

8) ESTABLISHMENT OF SERUM BANKS

A serum bank is a planned, catalogued and accessible collection of serum forming a (random) sample which is as representative as possible of a population or populations and which is stored in such a manner that its immunological and biochemical characteristics are preserved.

This activity is ancillary to the sero-monitoring of rinderpest; it is to be recommended but should not be undertaken without careful consideration.

The collection of serum is an expensive exercise and the quantity of serum collected from one animal is normally sufficient for a number of serological tests. There is a lack of good epidemiological information on animal diseases in many of the PARC countries and the sera can be stored and tested against other disease at a later date.

The functions of a serum bank are:

1) to help identify major health problems;
2) to define spatial distribution of diseases.
3) to determine epidemic periodicity;
4) to investigate new diseases;
5) to shed light on aetiology;
6) to monitor vaccination campaigns;
7) to estimate incidence rates of diseases which have low case fatality;
8) to assist in determining losses due to disease.

All these are useful, but the establishment of a bank requires resources of money and manpower. Suitable storage facilities will be needed from the offset and at least one person will have to be appointed to the job of setting up and maintaining the bank. If veterinary authorities wish to set up serum banks, assistance may be possible through EEC, FAO or IAEA.

9) REFERENCES AND FURTHER READING

1) "Veterinary Epidemiology" by Michael Thrusfield, (1986). Butterworths, London.
2) "Patterns of Animal Diseases" by B. Halpin, (1975). Balliere, Tindall, London.
3) "Livestock Disease Surveys — A field manual for Veterinarians". Australian Bureau of Animal Health, Canberra, (1982).
4) "Veterinary Medicine and Human Health" by Calvin W. Schwabe, (1984). Williams and Wilkins, Baltimore, London.
5) "Guide to Statistics of Livestock and Livestock Products". FAO, (1976).

Techniques for Selecting a Simple Random Number Sample

The table consists of sets of two-digit random numbers. It can be used in selecting a random sample from any population of known size.

To select a sample of a required size, start at any point in the table and move systematically down columns and/or along rows until a sample of the required size is obtained. Where a number is duplicated in the group selected it should be ignored and the next random number in the sequence used. Similarly if a number falls outside the range of the population size it should be ignored and the next random number used. This does not affect the randomness of the sample.

For example in selecting a random sample of ten herds from a total population of 40 herds:

a) Number the herds 1 to 40
b) Start at any point in the random number table;
c) Work down the column, writing each number that is before 1 and 40, but rejecting repetition until you have ten numbers. These are then numbers of the ten randomly selected herds.

RANDOM NUMBERS

10	66	53	13	45	41	10	77	97	10	70	12	36	08
24	76	12	17	01	67	54	45	87	74	04	55	13	46
92	99	70	19	11	76	99	07	46	15	94	22	43	56
15	24	91	16	02	78	59	22	12	77	31	77	08	25
02	96	57	75	26	27	00	31	32	49	31	10	94	97
19	10	79	78	35	10	32	23	35	53	32	69	16	44
21	15	44	04	06	35	81	59	07	50	91	92	01	00
21	74	20	78	13	14	80	66	55	71	97	83	98	14
50	00	00	13	73	70	12	12	29	20	01	66	84	45
45	61	13	04	27	96	92	93	06	66	44	03	57	88
86	72	15	57	41	62	13	15	16	39	02	21	67	75
64	21	71	58	86	68	36	32	14	50	10	79	16	94
14	14	27	63	71	03	18	49	48	69	87	61	16	73
72	73	89	89	86	04	81	21	07	60	01	74	74	68
47	94	29	73	90	36	62	86	89	94	43	28	30	13
62	51	73	79	08	78	11	06	44	77	92	76	21	99
07	80	01	36	23	50	33	41	28	69	22	89	50	27
83	26	35	73	57	81	15	55	50	22	97	13	11	74
23	82	82	39	56	47	54	00	42	70	87	50	67	04
48	80	22	91	20	93	20	47	69	19	77	14	52	11
89	12	77	56	13	99	47	30	23	73	90	64	60	34
45	90	47	17	55	50	75	44	07	37	67	86	80	13
72	92	83	11	07	60	99	39	66	48	90	08	08	17
47	52	42	74	93	83	17	39	39	39	40	13	88	18
10	76	55	23	10	54	59	66	69	23	86	98	38	57
53	09	97	90	35	22	23	53	07	75	91	48	74	34
96	90	27	80	69	36	07	11	78	36	09	50	13	74
24	47	09	53	16	47	16	88	30	14	47	33	62	16

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