RIVM report 284500019/2001/2002

Report on the sixth workshop organised by CRL-*Salmonella*
Bilthoven, 11 and 12 June 2001
M. Raes and A.M Henken (editors)

This investigation has been performed by order and for the account of the European Commission, Legislation Veterinaire et Zootechnique, within the framework of project 284500, by the Community Reference Laboratory for *Salmonella*.

RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71
European Commission, Legislation Veterinaire et Zootechnique, Rue de la Loi 86, B-1049 Bruxelles, Belgique, telephone: 32-2-2959 928; telefax: 32-2-2953 144

Contents

Samenvatting

Op 11 en 12 juni 2001 is door het Communautair Referentie Laboratorium voor *Salmonella* (CRL-*Salmonella*) een workshop georganiseerd in Bilthoven, Nederland. Alle Nationale Referentie Laboratoria voor *Salmonella* (NRLs-*Salmonella*) van de EU lidstaten, met uitzondering van die van Ierland, waren vertegenwoordigd. In totaal waren er 37 deelnemers.

Het programma van de workshop bestond uit verschillende delen. Het eerste deel bestond uit de bespreking van de nieuwe draft van de zoönose richtlijn. Daarna vond een evaluatie plaats van het bacteriologische ringonderzoek en de bacteriologische detectie in de verschillende lidstaten. Verder werd gesproken over de opzet en resultaten van typeringsringonderzoeken. De achtergrond en betekenis van kwantitatieve methoden werd als speciaal onderwerp door diverse sprekers toegelicht.

Summary

At 11 en 12 June 2001 a workshop was organised by the Community Reference Laboratory for *Salmonella* (CRL-*Salmonella*) in Bilthoven, The Netherlands. All National Reference Laboratories for *Salmonella* (NRLs *Salmonella*) of the EU Member States, with the exception of the Irish NRL-*Salmonella*, were represented. In total there were 37 participants.

The workshop programme allowed discussion on different subjects, starting with the new draft zoonoses directive. Subsequently an evaluation was held on the bacteriological collaborative study and activities on bacteriological detection in the Member States. The set-up and results of collaborative typing studies and immunological methods were also discussed. As an additional subject several speakers gave presentations on the background and significance of quantitative methods.

1. Opening and introduction of the participants

Mr. A.M. Henken (Director CRL-*Salmonella*, The Netherlands)

First of all I would like to sincerely welcome you all to this workshop. We are with many people, that is, at least 1 to 3 persons of each of 14 EU Member States[1] (See Appendix 2).
A special word of welcome to Mr. Cavitte and Ms. Mäkelä, who are the representatives of the Commission among us. Also a special word of welcome to Ms. Kaesbohrer from the CRL-Epidemiology of Zoonoses in Berlin.
During these days I will be your chairman as the head of CRL-*Salmonella*. I would appreciate it if we would all agree to use the English language during our sessions.
With these words the workshop is opened!

Aims

What can we expect from this workshop? The functions and duties of the CRL-*Salmonella* according to the zoonoses directive are:

1. Providing national laboratories with details of analytical methods and comparative testing;
2. Co-ordinating the application by national reference laboratories of the methods referred to under the first mentioned point, in particular by organising comparative testing;
3. Co-ordinating research into new analytical methods and informing national laboratories of advances in this field;
4. Conducting initial and further training courses for the benefit of staff from national reference laboratories; and
5. Providing scientific and technical assistance to the Commission of the European Community.

The aims of the workshop (see Appendix 3 for the programme) were defined as to discuss:

- The proposed new zoonoses directive;
- Results of collaborative studies organised by the CRL-*Salmonella* with NRLs-*Salmonella*;
- Organisational aspects of collaborative studies among and within states;
- Research activities within Member States;
- Whether or not there are specific needs among NRLs-*Salmonella*; and
- Activities CRL-*Salmonella* 2002.

Participating are: representatives of the EU Commission, a representative of the CRL-Epidemiology of Zoonoses, representatives of NRLs-*Salmonella*, representatives of CRL-*Salmonella* and invited speakers.

[1] At the workshop 2001 Ireland was not represented.

2. Review of the more general presentations

2.1 Current issues on the New Draft European zoonoses directive

Mr. Jean-Charles Cavitte, European Commission

See Appendix 4 for sheets.

Discussion:

- There has not been made a decision whether or not for each of the nine zoonoses a CRL is appointed. It depends on the need for it.
- The ultimate goal of the established targets in the draft directive is for all countries (laboratories) the same, while the starting level is different between countries.
- Firstly the directive is not meant to give measures in case a country is not able to reach the ultimate goal.

2.2 Evaluation of *Salmonella* methods as being discussed in the Scientific Committee

Ms. Pia Mäkelä, European Commission

See Appendix 5 for sheets.

Discussion:

- Somebody of the CRL-*Salmonella* will participate in the meeting of the working group in Brussels and communicate the results with the NRLs-*Salmonella*.
- Same problems with methods revealed in the field of water-microbiology. Kirsten Mooijman (CRL-*Salmonella*) is involved in the working group water-microbiology.
- Activities (e.g. collaborative studies) in regard of PCR are carried out in the framework of the food-PCR project.

2.3 Report of the CRL-Epidemiology

Ms. Anne Käsbohrer, CRL-Epidemiology (See Appendix 6)

Introduction

The main objectives of Directive 92/117/EEC on zoonoses are the collection of epidemiological data to follow the epidemiological trends of the zoonoses concerned and to propose appropriate measures for their control. According to Article 5 of the Directive (as

amended by Directive 97/22/EC) each Member State has to provide a country report by May 31 of every year.
The Community Reference Laboratory for the Epidemiology of Zoonoses (CRL-E) is assisting the Commission with the technical analysis and with the co-ordination of reporting on the EU level. Some of the problems arising from this task are presented now to be discussed with the representatives of the National Reference laboratories for *Salmonella*.

Results

Feed materials and feedingstuff

Information was requested on feed materials (i.e. raw ingredients) by origin (animal or vegetable origin), data gathered during processing (HACCP) and compound feedingstuffs (mixture of feed materials intended for oral animal feeding). Categorisation for feed materials should be based on that laid down in the Annex to Directive 96/25/EC.
Sampling of animal derived feed materials should be based on Dir 76/371/EEC, for vegetable feedingstuffs a sampling scheme was described in Doc. VI/2025/98 Rev.3. There, it is laid down that several incremental samples are taken from a batch of feed materials and combined to one final sample that is investigated in the laboratory. In the report some countries recorded ‘sample’ and others ‘batch’ as the epidemiological unit to which the data are related. Therefore, data had to be evaluated separately. Up to now, no detailed description of the sampling scheme (including the sample sizes) was provided by all Member States to evaluate whether there are real differences in the sampling schemes applied and the consequences thereof for the data.
Other difficulties in the evaluation of the data arose from the fact that the information was not broken down in the categories as requested. Thus, a detailed evaluation on which feed materials are of highest risk could not be performed.
A considerable number of batches and samples of compound feedingstuff for poultry, cattle and pigs were investigated. Unfortunately results of process control and of final products were not reported separately by most of the Member States.
In some countries data on process control were not provided although it was advised that a monitoring programme is in place. A clear distinction was not always provided whether samples were taken before or after heat treatment (or both).

Poultry breeding flocks

For the presentation and evaluation of the data the reporting countries were grouped as follows:

- Countries running an approved *Salmonella* control programme in poultry breeders;
- Countries running a monitoring or control programme using a scheme based on the sampling procedure in Dir.92/117/EEC;
- Countries running a monitoring or control programme using a scheme different from the sampling procedure in Dir.92/117/EEC;
- Other countries.

Data provided from the countries that are running an approved control programme should be considered comparable. Data could not be presented together from these countries due to differences in the way of data presentation. Ireland had to be excluded due to the fact that data resembled multiple sampling of a limited number of flocks.
Some countries apply additional sampling which might ensure a more rapid detection of a new infection in a flock or an infection at a lower prevalence rate within the flock. This has to be taken into account too.
Presentation of data from countries running a monitoring or control programme using a scheme based on the sampling procedure in Dir.92/117/EEC was more difficult.
Data reported from United Kingdom were based on the obligation of all laboratories to report to the Ministry of Agriculture, Fisheries and Food (MAFF) the isolation of all *Salmonella* in samples from livestock. Thus, the number of flocks under control is not known, but the number of finally confirmed infected flocks.
France reported data as an annual weighted average based on the quarterly prevalence rate. The number of flocks under control and the number of positive flocks per year was not reported.
In Austria, the number of breeding flocks investigated for *Salmonella* was considerably lower compared to 1998. Similarly, in Germany, only a few Federal Länder reported examinations of breeding fowl. Compared to previous years, the coverage of the reported investigations has decreased remarkably. No explanation was given for that.
Spain included for the first time data on the *Salmonella* situation. Unfortunately, no flock based data were presented.
In consequence, although a sampling scheme is fixed data could not be evaluated together.
The categorisation of the production level and production type was also not always followed as requested.
Common to all areas is that more detailed knowledge is necessary on the sensitivity of the method taking into account the specimen tested, the sample size and the sample weight.

Antibiotic resistance monitoring

The national report on zoonoses should have been presented until the end of May. There, data on antibiotic resistance testing using the monitoring frame agreed on during the workshop in 2000 should be included:

- 60 isolates of each of the 5 most important *Salmonella* serotypes (by public health relevance);
- 3 main animal species: cattle, pigs, poultry;
- isolates should be selected in randomised way among isolates at NRLs-*Salmonella*, clustering is to be avoided;
- information about whether isolates derive from active or passive surveillance.

It was agreed to test at least the following antimicrobials:
Tetracycline, Chloramphenicol, Florfenicol, Ampicillin, 3rd generation cephalosporin, e.g. cefotaxim, Ciprofloxacin or Enrofloxacin, Nalidixic acid, Sulfonamide/TMP, TMP (optional), Sulfonamide (optional), Streptomycin, Gentamycin, Neomycin, Kanamycin.

Although agar dilution or broth dilution would be desirable, results from agar diffusion testing should be reported too. Details on the test methods used, and the breakpoints should be given.
Furthermore, the report of the National Reference Laboratory for *Salmonella* should include results of serotyping and phage typing stratified by main categories of animal species, feedingstuffs and food, information on the source of the isolates and the results of comparisons of strains using molecular methods.
Details are described in the manual and tables for report 2000 that has been distributed to all Member States.

Summary

To facilitate up to date evaluation of the zoonoses situation on EU-level the National reports should be provided in time (end of May). There, the Member State should provide the information as requested and agreed upon during the annual meetings. Nevertheless, there is a need for standardisation and harmonisation of methods applied.
The sensitivity and specificity of the method for different specimen and sampling schemes should be investigated.

Discussion:

A discussion about on one hand more harmonisation in programmes between the different countries to prevent difficulties in interpretation of the results of the different countries and on the other hand more flexibility in a country to implement the measures in regard of sampling and surveillance programmes (no new discussion).

3. Review of the presentations on bacteriology

3.1 Surveillance trends in *Salmonella* in 2000

Mr. Rob Davies, NRL-*Salmonella* United Kingdom (See Appendix 7)

The number of *Salmonella* incident reports in 2000 was similar to the previous two years.

The decline in the number of reports of *S.* Enteritidis continued in 2000. Reports of this serotype fell by 44% in 2000 and represented only 1% of *Salmonella* incidents in chickens. There were no incidents of *S.* Enteritidis reported in turkeys during 2000. The number of reports in ducks and geese was similar to 1999.

The increase in reports of *S.* Typhimurium in pigs (74% of all porcine incidents related to *S.* Typhimurium) continued in 2000. The most common phage types involved were DT104, DT208 and U302. Reports of *S.* Typhimurium in pigs increased by 50% in 2000 compared with 1999 and most incidents were associated with clinical disease. This contrasted with a continued reduction in reports of Typhimurium in other species, although the decline in reports from cattle was less than in previous years.

The number of reports of *S.* Dublin in cattle continued to increase, and a total of 651 incidents were reported in the year 2000, an increase of 35% since 1999. The pattern of reports shows a distinct seasonal rise in the Autumn each year, however, the autumn peak in 2000 was particularly marked and it was the largest autumn increase in the last 10 years. *S.* Dublin remained rare in other species.
S. enterica subsp. Diarizonae serovar 61:k:1,5,(7) remained the most common *Salmonella* isolated from sheep and accounted for 60% of reports in 2000.

There were marked increases in reports of *S.* Senftenberg, *S.* Give and *S.* Heidelberg in chickens in 2000. However, trends are difficult to interpret as the level of industry monitoring of broiler flocks has increased in recent years

The most common serotype reported in turkey flocks was *S.* Derby, which for the first year since recording began was more common than *S.* Typhimurium. Reports of *S.* Agona increased in 2000 and this became the second most frequently recorded serotype. This is correlated with an increase in reports of this serotype in feeds.

S. Agona was the most common serotype isolated in 2000 from voluntary monitoring of feed ingredients for *Salmonella*; most isolates were recovered from samples of rapeseed or soya bean (protein residues of the vegetable oil industry). *S.* Agona was also the most common serotype isolated from monitoring of compound cattle, pig and poultry feeds in 2000.

There were 2 isolates of *S.* Enteritidis and 17 isolates of *S.* Typhimurium from feed. Compound poultry feed was the most common feedstuff found to contain *S.* Typhimurium.

Nalidixic acid resistance fell in DT104 from all the livestock species although 63.2% of turkey DT104 isolates remained resistant. Other turkey related serovars such as *S.* Newport, *S.* Fischerkietz and *S.* Senftenberg also showed a high level of nalidixic acid resistance.

In pigs and horses there was an increase in the proportion of *Salmonella* isolates found to be resistant to individual antibiotics. The overall proportion of *S.* Typhimurium isolates resistant to at least 1 antimicrobial increased slightly but there was a slight decrease in resistance of *Salmonella* other than *S.* Typhimurium or *S.* Dublin.

Discussion:
The results shown are the first isolates of a certain company. Data about the origin of the farm are known at the institute, so they are sure that only one isolate of each farm is included in the results.

3.2 *Salmonella* in the wild fauna, fur animals and pets in Denmark

Mr. Jens Christian Jørgensen, NRL-*Salmonella* Denmark (See Appendix 8)

During the period 1995-2000, a total number of 726 wild birds, 1,156 wild mammals, 1,335 fur animals and 542 pet animals (dogs and cats) were examined for *Salmonella* as part of the routine or requested bacteriological examination of samples (i.e. carcasses, organs or faecal samples) received at the laboratory.

Material
Samples from wild animals were primarily sent to the laboratory by hunters and nature wardens, but also by farmers and private persons. In connection with marking of birds and systematic collection of traffic killed animals, a larger number of single species were screened. Samples were distributed equally over the year, except when screening migrating birds in the spring.
Samples from fur and pet animals were sent to the laboratory by veterinarians for diagnostic purposes. Samples from fur animals were primarily sent to the laboratory during the breeding season from acute cases of disease, whereas samples from pet animals were received more equally distributed over the year. They often represented chronic clinical cases with a history of long term antibiotic treatment.

Methods
All samples were examined on blood agar with 5% calf blood, Drigalski agar and SSI Enteric medium (last part of 2000) and/or by the routine *Salmonella* cultivation method used by the laboratory, i.e. pre-enrichment in buffered peptone water and selective enrichment in

Rappaport-Vassiliadis Soya peptone broth (RVS), samples from Gallinaceous birds also in selenite-cystine broth, followed by streaking on Rambach agar, and samples from Gallinaceous birds also on brilliant green agar.

Results

A total of 2.3 % of wild birds were *Salmonella* positive during the period (*Table 1*). Twelve of the 17 *Salmonella* isolates were *S.* Typhimurium. Birds of prey were the group of birds with the highest number of *Salmonella* positive, 5.3 %.

Table 1. Number of wild birds Salmonella positive/number examined

Species	**1995-2000**	**Pct. pos.**
Web-footed birds	7 / 233	*3.0*
Gallinaceous birds	0 / 18	*0*
Birds of prey	3 / 57	5.3
Other birds	7 / 418	*1.7*
Total	17 / 726	*2.3*

In wild animals (*Table 2*), *Salmonella* was found in 22.9 % of the hedgehogs examined, 68.8% of these were *S.* Enteritidis PT11. Beside these only a few foxes and a badger were found *Salmonella* positive.

Table 2. Number of wild mammals Salmonella positive/number examined

Species	**Total**	**Pct. pos.**
Deer	0 / 90	*0*
Hare	0 / 122	*0*
Fox	7 / 412	*1.7*
Badger	1 / 224	*0.4*
Wild mink	0 / 61	*0*
Marten	0 / 37	*0*
Polecat	0 / 49	*0*
Hedgehog	32 / 140	*22.9*
Marine mammals	0 / 21	*0*
Total	40 / 944	*4.2*

Hedgehogs were all sent in from nursing stations, taking in young ones in the fall, keeping them in house during the winter and letting out the survivors in the spring. The *Salmonella* status of genuine wild hedgehogs is at present not known.

Salmonella was found sporadically in mink and foxes (*Table 3*). In 2000, a clinical outbreak of *S.* Dublin was seen during the breeding period. The source of infection seemed to be

feed-stuff. One hundred and ten or 91.7 % of all strains isolated from fur animals during 1995-2000 were isolated in connection to this outbreak of salmonellosis. In one of the affected farms also multiresistant *S.* Typhimurium DT104 was isolated from the brain of a fox.

Table 3. Number of fur animal examined/Salmonella positive

Species	Total	Pct. pos.
Chinchilla	11 / 0	*0*
Rabbit	37 / 0	*0*
Mink	1188 / 109	*9.2*
Fox	99 / 11	*11.1*
Total	1335 / 120	*9.0*

In dog and cat, *Salmonella* was only isolated sporadically, 1.5 % of a total of 542 samples were positive.

Conclusions

The material was too biased to be used conclusively, but there was a clear trend, that *Salmonella*, with a single exception of hedgehogs, was not widely distributed in neither wild animals nor in fur or pet animals.

An ongoing study of *Salmonella* in domestic animals and the wild fauna inside and around twelve farms (Wildlife as a source of *Salmonella* infection in food animal production) may add further to our knowledge.

The value of this kind of data can be improved by registering more details. This, however, was not possible in the database used at present.

Also, it should be remembered that *Salmonella* is not the only pathogen of interest dealing with wild animals as a possible source of disease in domestic animals and man.

Discussion:

- No resistance against the strains are found. The same serotypes as in other animals were found.
- Furthermore seagulls were tested and no *Salmonella* was isolated, so probably in Denmark seagulls are *Salmonella* free. Samples were not tested in combination with waste plants.

  In Finland (Sweden?) seagulls were also tested and 8 – 10 % of the samples were contaminated with *Salmonella.* They were tested in combination with waste plants.

3.3 National slaughter surveys for *Salmonella* in pigs, cattle and sheep

Mr. Rob Davies, NRL-*Salmonella* United Kingdom (See Appendix 9)

To determine the prevalence of *Salmonella* in pigs and cattle at slaughter in Great Britain samples of caecal contents (10 g) and 0.1 m^2 carcass surface swabs were collected from 2,509 pigs between March 1999 and February 2000 in a randomised national study. Caecal carriage of *Salmonella* was identified in 578 (23.0%) pigs (95% confidence interval [CI] 21.4 to 24.7). Surface contamination with *Salmonella* was detected on 135 (5.3%) carcasses (95% CI 4.5 to 6.2). The predominant *Salmonella* serotypes in both type of sample were *S.* Typhimurium (11.1% caeca, 2.1% carcasses) and *S* Derby (6.3% caeca, 1.6% carcasses). Neck muscle from the carcasses was also tested using an indirect ELISA based on lipopolysaccharide somatic antigens from *S.* Typhimurium and *S.* Choleraesuis ('mix' or 'meat juice' ELISA). At the 40% optical density standard cut-off, 15.2% of pigs were positive (95% CI 13.7 to 16.6).

The distribution of *Salmonella* serotypes largely corresponded with results of passive surveillance derived from reports of clinical investigations, although *S.* Derby was more common in the abattoir samples and less common in clinical samples, with the reverse being the case for *S.* Typhimurium. The predominant phage types of *S.* Typhimurium found were DT104 (21.9% of *S.* Typhimurium isolated from caeca), DT193 (18.7%) untypable (17.6%), DT208 (13.3%) and U302 (13.3%). On later checking with an experimental phage the untypables contained a number of U310 strains. This is a newly designated phage type which has also been involved in human cases associated with processed pig meat products in Great Britain.

In the cattle and sheep surveys, 891 and 973 samples of rectal contents were collected, respectively, between January 1999 and February 2000. *Salmonella* was found in 1g samples of rectal contents from two cattle (0.2% [95% CI 0.0 to 0.5]) and one sheep (0.1% [95% CI 0.0 to 0.3]). The serotypes found were all *S.* Typhimurium and were DT193 and DT12 in cattle and DT41 in sheep.

Discussion:

- The programme presented was not a part of the EU programme SALINPORK. In great details the results of both programmes differ significantly.
- It is of interest to see if there are relations between the decline of certain phagetypes and the development of others. There are a lot of theories about this topic.

3.4 Results of the fifth bacteriological collaborative study

Mr. Maurice Raes, CRL-*Salmonella* (See Appendix 10)

See Appendix 10 for sheets.

Discussion:

- It is unknown whether there is a relation between bad results, pre-heating the BPW and incubating the pre-enrichment medium longer than the prescribed time.
- Detailed information about the PCR used by the different countries is not yet available at the CRL, but it is possible to still get this information.
- PCR: more than 5% BPW in a PCR reaction is an inhibitor. Therefore an internal control had to be included. Feces is a bad matrix, so there is a need for improvement. A possibility is involvement of the CRL in the food-PCR project.
- There are differences between different sets of media and therefore it is recommended that the CRL send all media needed for a collaborative study to the participating laboratories. However the overall purpose is to decrease the variation and to increase the average over the years. So we go on with the procedure used in the last studies for about 4 years and then we will make a statistical evaluation.

Remarks for future studies:

- The use of feces obtained from other animals, e.g. pigs.
- Total number of capsules is enough, especially for laboratories that would like to test their own method beside the prescribed method.
- Be careful with comparing the results obtained with MK. Never put them in one column, because there are many different formulas.

3.5 Reference Materials

Ms. Kirsten Mooijman, CRL-*Salmonella*

Discussion:

It is difficult to buy reference materials. PHLS is maybe a possibility. In France the rms are not for sale. The institute in Brussels do not produce rms themselves and these materials cannot be used for collaborative studies.

3.6 Revision of ISO6579

Ms. Kirsten Mooijman, CRL-*Salmonella* (See Appendix 11)

Discussion:

- Maybe it is better to have two ISO methods; one for food and one for S. Typhi / S. Paratyphi. It is uncertain if it will make it so far.
- The incubation temperature of MK (37 °C or 42 °C) depends on the matrix. This will be discussed at the next meeting of the revision of the ISO 6579 in Bern.

3.7 Discussion on bacteriological collaborative study VI

- Changes in the ISO 6579 can be included, otherwise we will go on on the same way.
- Again, PCR is included.
- Because Maurice Raes and Nelly Voogt are leaving the CRL, the next collaborative study will possibly not be carried out in autumn, but early next year.

4. Review of the presentations on typing

4.1 Preliminary results sixth typing study

Mr. André Henken, CRL-*Salmonella*. (CRL-*Salmonella*)

See Appendix 12 for sheets.

Discussion:
No questions

4.2 Resistance monitoring of *Salmonella* (human and non-human isolates) in Austria

Mr. Christian Berghold, NRL-*Salmonella* Austria (See Appendix 13)

In 1992 the Austrian Reference Laboratory received the highest number of human isolates, over 13,400 strains. Since then the numbers of human isolates have gradually declined, with less then 7,500 isolates in the year 2000. The predominant serotype in Austria remains *S.* Enteritidis with over 85% of all human isolates. In our country, we believe that poultry and eggs are still the principal cause of infections.
Phage type 4 (PT) is the most important phage type in *S.* Enteritidis, with more than sixty percent of all human *S.* Enteritidis-isolates, second is PT 8, third PT 21. The distribution of phagetypes of *S.* Enteritidis from non-human origin is similar.
The overall resistance-rates of *Salmonella* in Austria remain low. But there is much concern about ciprofloxacin resistance in campylobacter. Up to fifty percent of human campylobacter isolates are now resistant against ciprofloxacin in Austria. In *Salmonella* there had been a rising tendency in the resistance against nalidixic acid over the last years, but at a much lower level (as has been shown before, the resistance to nalidixic-acid represents a borderline resistance to ciprofloxacin. These nalidixic-acid resistant strains are normally provided with a point-mutation in one of the gyrase-genes, conferring them higher MICs to all chinolons in comparison to wildtype-strains). Five strains showed high-level resistance to ciprofloxacin, most of them secondary strains or non-human-strains. Three strains were resistant to cefotaxim in 2000.
It is remarkable, that the rates of resistance against nalidixic-acid differ very much between serotypes and even phagetypes. Some serotypes, as *S.* Hadar show high rates of resistance to nalidixic acid. About four to five percent of all human *S.* Enteritidis isolates are resistant to nalidixic acid. Some phagetypes of *S.* Enteritidis, as PT 21, are quite often resistant against nalidixic acid. PT 4 has an average level of resistance against chinolons, and PT 8 is still fully sensitive to all antibiotics.
Isolates from children, who normally are not treated with chinolons, show more or less the same degree of resistance to nalidixic acid as the older age-groups. Nevertheless the older

age-groups tend to have minimally higher rates, indicating the possibility of a human factor in the development of resistance against chinolons (nalidixic-acid).
Multiresistant *S.* Typhimurium DT 104 has been of much concern in many countries in the last decade. In former years in Austria less than 1 percent of all human isolates belonged to this multiresistant DT 104 clone. Since the beginning of this year we observe a marked increase of resistant *S.* Typhimurium DT 104. Comparing the first 5 months of 2001 to the first five months of 2000 there has been a nearly threefold increase. In the last months nearly 5 % of all human isolates belonged to this resistant clone.
Only very few non-human isolates of multiresistant *S.* Typhimurium DT 104 were sent to the Reference Laboratory in the last one and a half years. There have been no isolates from chicken and pork, only two isolates from cattle, but 8 isolates from turkey. These 8 isolates were sent in the year 2000. This year we have not received any isolates from turkey at the Austrian Reference Laboratory. The reason for this sudden increase of multiresistant *S.* Typhimurium DT 104 remains therefore unclear.

Discussion:
It is not clear what has caused the increase in Malidixic resistant *Salmonella* in the late eighties, however, this was seen all over Europe.

4.3 Studies performed on *Salmonella* isolates at LNIV in 2000

Ms. Maria do Rosário, NRL-*Salmonella* Portugal

See Appendix 14 for sheets.

Discussion:
The samples originating from Azores Island originated from an outbreak.
The rather high rate of resistance is quite normal for nowadays, it is seen all over Europe.

4.4 Discussion on the typing study 7

It was decided to standardise the antibiotic resistance pattern typing a bit more. This can be done by prescribing the antimicrobials used and let the laboratory use their own method.
The strains to be tested should be strains that are detected more often in humans. In addition to these, strains that look like them should be included.

4.5 Principle and design for a DNA chip for *Salmonella*

Mr. Reiner Helmuth, NRL-*Salmonella* Germany (See Appendix 15)

The techniques of classical DNA/DNA-Hybridisation and polymerase chain reaction (PCR) are principally based on the homology of double stranded DNA molecules. Both methods are however limited in their capacity to analyse many targets at the same time. The DNA Chip technology in contrast is a straight forward development of this principle, which allows the simultaneous detection of hundreds to thousands of target sequences. Furthermore, not only the presence but also the expression of a particular gene can be analysed. DNA arrays are used for this purpose and represent an orderly arrangement of single stranded DNA molecules. They offer a medium for matching known and unknown DNA samples based on base-pairing rules and its large throughput by automatisation provides a way to identify numerous unknown biological traits.
Macro-arrays contain sample spot sizes of about 300 microns or larger. The sample spot sizes in micro-arrays are typically less than 200 microns in diameter and these arrays usually contain thousands of spots. Micro-arrays require specialised robotics for their production and imaging equipment. DNA micro-array, or DNA chips are fabricated by so called spotters which apply DNA probes mainly on glass.
Micro-arrays can contain several types of DNA molecules as probes such as oligonucleotides, PCR products or plasmids. They are either synthesised in situ (on-chip) or by conventional synthesis followed by on-chip immobilisation.
A typical analysis of a sample involves DNA extraction, labelling of the complementary target DNA, hybridisation on the chip, detection, and automatical analysis of the results.
The NRL-*Salmonella* of Germany has designed a prototype DNA chip which will detect important characters of *Salmonella* isolates. Among those are DNA elements responsible for O and H antigens, phagetypes, virulence markers, drug resistance, and subtyping.
The application of this technology will make a deeper, faster and more efficient characterisation of isolates received at the NRL possible. Automatic data collection and analysis is anticipated.

Discussion:
The targets for phage typing are determined by using RAPD PCR on different phage types of Salmonella. The different bonds can be sequenced, and primers can be made for the specific piece of DNA. Same for the targets for O antigens, only a limited number of genes code for the O antigens, all of these genes have been sequenced.
The sensitivity and specificity for the DNA chip were already tested by validating PCR.
The development of DNA chips is mostly performed by research institutes. However, it is important to follow the development of this technique.

4.6 General aspects of antimicrobial resistance typing

Mr. Reiner Helmuth, NRL-*Salmonella* Germany (See Appendix 16)

In the last four decades the development of antimicrobial resistance has led to an intensified discussion about the rational use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture. Consequently numerous governmental and non governmental organisations have discussed the issue in detail with experts and interested stakeholders. On the European level several WHO conferences, the EU Conference on the Microbial Threat 1998, its follow up conference in Visby 2001, and the report of the Scientific Steering Committee on Antimicrobial Resistance presented in Brussels in 1999 are good examples. One common result of these and other conferences was the conclusion that a scientific risk assessment and further action must be based on reliable data on the prevalence of resistance against antimicrobial agents.
As determined by questionnaires of the CRL for *Salmonella*, comparable, standardised and harmonised data are not available on an European level.
The same experience was made within the concerted action ARBAO (**A**ntibiotic **R**esistance in **B**acteria of **A**nimal **O**rigin, FAIR PL 97 3654) in a survey on current monitoring programmes among the EU member and associated states. Both initiatives revealed that for *Salmonella* the testing methods, antibiotics and sampling strategies varied tremendously. Nevertheless, the results so far obtained have given conclusive, persuading and scientifically sound evidence in many areas of resistance development, spread and mechanisms.
Among the experts of the ARBAO group and NRLs for *Salmonella*, it was therefore concluded that there is a need for a harmonised and standardised European method.
However, such an approach is not easily realised due to lacking financial resources and historical differences in the health services and NRLs of the individual member countries. As a result, the minimal requirements for surveillance of antimicrobial resistance for *Salmonellae* were defined by the ARBAO working group.
They include:

- The definition of a minimal set of antimicrobial agents which should be tested.
- The collection of quantitative data obtained by agar diffusion or dilution tests.
- The development of a list of data which should be collected.

During the presentation, details on the points listed above were given.

Discussion:
It is hard to find 900 isolates as mentioned in the presentation. If it is not possible to find this much isolates, it simply can not be performed, this is not a problem, however, the number of 900 was statistically calculated as a minimum needed.
The methods for antimicrobial resistance typing need to be standardised. For this it is necessary to name someone who will choose the specific method.

4.7 Molecular characterisation of genetic elements involved in antimicrobial resistance in *Salmonella*

Ms. Beatriz Guerra Roman, NRL-*Salmonella* Germany (See Appendix 17)

During the last decades the world-wide use of antimicrobials in human and veterinary medicine and as growth promoting agents has created an enormous selective pressure for the selection of antimicrobial resistances among bacterial pathogens. Since 1990 there has been an dramatic increase in the occurrence of multi-drug resistant (MDR) strains of zoonotic agents causing human infections in many countries all over the world. In numerous investigations on MDR *Salmonellae*, the prevalence of multiple resistant isolates, of the same serotype, resistance profile, phage type and molecular markers, have repeatedly been described. However, in the last years a few MDR clones have received special scientific and public attention, because of their epidemiological importance and well-documented molecular characteristics.

Studies about the molecular epidemiology of some resistant clones point to the fact that different resistance genes and mechanisms are responsible for the resistance phenotypes. In fact, different antimicrobials act on different cell targets, and bacteria become resistant when they are able to interfere with the action of substances with antimicrobial activity. Consequently, bacteria have developed different mechanisms for resistances: i. e. changes in the permeability of the cell, active transport of the antimicrobial to the outside of the cell, inactivation of the antimicrobial molecule, modification of the cell targets, etc. In some cases, resistance to one antimicrobial can result from the combination of more than one resistance mechanism. The characterisation of the genes implicated in the resistance of one strain is a first step in the survey of MDR-strains.

In addition the transfer of antibiotic resistance genes between different species of bacteria can be facilitated by mobile DNA elements, such as plasmids, transposons, and integrons which often contain one or more genes or gene cassettes that encode antibiotic resistance. The characterisation of the vehicles implicated in the spread of resistance among strains is another very important fact which must be taken into account in the survey of MDR-strains.

Presently, PCR-detection of different antimicrobial genes, integrons and transposons using different sets of primers is the first step in the molecular characterisation. In combination with the hybridisation of the whole DNA of the strains with the resistance PCR products the location of the different resistance structures can be achieved. Both techniques are very valuable tools for the characterisation of MDR-strains.

On the other hand, the genetic characterisation of the resistant strains using different typing methods such as PFGE, ribotyping, RAPD-typing, etc., is also necessary. All the information cited above will allow the allocation of resistant strains to clones or lineages, and to study their propagation, spread and evolution.

One example in which studies at these three levels have been carried out successfully, is the identification of the molecular mechanisms underlying the penta-drug-resistance

(PDR)-in the *S.* Typhimurium DT104 clone. In fact, it has been found that their resistance is based on the presence of a resistance-island which includes a resistance gene coding for streptomycin-resistance (*aadA2*) located in a class 1 integron, followed by genes responsible for chloramphenicol and tetracycline resistance (*floR* and *tet(G)*), and a gene responsible for the ampicillin-resistance (*pse1*) located in another class 1 integron. Both integrons also carried genes encoding sulfadiazine-resistance. All those resistances are chromosomally located, and the strains present a very homogeneous typing pattern.

Discussion:
Some genes, i.e. the Efflux genes, can be activated by other factors than antimicrobials only. This complicates the molecular typing.

5. Review of the presentations on quantitative methods

5.1 Numeration / quantification of *Salmonella*

Ms. Florence Humbert, NRL-*Salmonella* France (See Appendix 18)

The problem of quantification of *Salmonella* is not a recent one and is quite recurrent in the scientific literature related to *Salmonella.* First, one must consider if it is a natural or an artificial contamination. In the simplest case, when artificial contamination is to be followed in a food matrix or in inoculated animals, an antibiotic marked strain of *Salmonella* is often used because, in that particular case, the detection limit may be lowered to 10-20 *Salmonella*/g of sample by direct plating or pouring of a minimal dilution of the sample onto/in the selective medium supplemented with the corresponding antibiotic. But there are some limitations to artificial contamination. The major one is that the level of artificial contamination used in these experiments are much higher than those naturally encountered.

There are 3 main strategies for numeration or quantification of *Salmonella*.
The first one is direct counting using the immuno-fluorescence technology, the two other ones are culture techniques : direct isolation and the so called Most Probable Number (MPN) method.

Immuno-fluorescence is a well known technique often used by people working on Viable but Not Cultivable (VNC) states of bacteria in environmental studies. It is also referenced for *Salmonella* in the Bacteriological Analytical Manual of the FDA and in the Official Methods of Analysis of the Association Of Analytical Chemists in the US. But, in practice, immuno-fluorescence is limited by technical problems (filtration of the sample, qualities of the antibody, choice and linkage of the fluorochrome…). The major problem is the non viability or culturability of some of the cells counted. To overcome this, immuno-fluorescence may be applied after a short period of incubation in order to detect micro-colonies instead of cells.

Direct isolation is feasible only if the 3 following conditions are fulfilled:
- if the number of *Salmonella* exceeds 100-1000 per g or ml;
- if the ratio of *Salmonella* cell number to that of competitive flora is not too low;
- if each *Salmonella* cell can develop into a colony on a solid selective agar.

In practice, without any further strategy to concentrate bacteria, sensitivity of direct plating is limited to the numeration of solid samples containing more than 1000 *Salmonella*/g or liquid ones containing more than 100 *Salmonella*/g.
If media proposed for selective isolation following pre-enrichment and enrichment steps are used for direct isolation, results may not be reliable. So, direct isolation needs specially

formulated and balanced media (selective enough to impair growth of competitive micro-organisms and sensitive enough to allow growth of injured *Salmonella*). Using Dulcitol Bile Novobiocine (DBN) some results have been obtained with poultry products.
Direct plating is most often used after different strategies to concentrate the bacterial flora of the sample in a lower volume or space (filtration, centrifugation or immuno-concentration) even if these strategies are of limited efficacy.
An other limitation of direct plating is the difficulty to recover stressed or injured *Salmonella*.
The overlay procedure (derived from coliform counting technique) using XLD, HE or SS agar to overlay the non selective TSA previously allowed to stand at room temperature for 4 hours, was tested to count stressed *Salmonella*.

The MPN technique is the method of choice to quantify *Salmonella*. The theory of MPN assumes 2 hypotheses (that the organisms are randomly distributed in the sample and that each solution of the sample when incubated in the appropriate medium is certain to exhibit growth whenever it contains one or more organisms), both of which are not usually fulfilled. But these underline the need, when MPN analysis is performed :

- to perfectly homogenise the sample,
- to use the most sensitive detection technique.

The MPN is based on the analysis of replicate volumes of a serially diluted sample. So, each sample may be analysed 9 times (3 repetitions/3 dilutions) or 12 times (3 repetitions/4 dilutions) or even more. Then the number of positive samples at each dilution is recorded to give the 'characteristic number' (for ex. 321, 555…). By reference to MPN tables or files, using statistical assumptions, each characteristic number is related to a MPN of bacteria in the amount of sample included in one tube of the first dilution. This MPN must be given with its confidence limits as these are usually very large (for ex. 321 gives 15 *Salmonella*/g, [3-38] confidence limits at 95% probability). In its traditional scheme, MPN method is a time consuming and labour intensive technique to obtain approximate results.
Some authors have tried to shorten the last step of detection of *Salmonella* by using 'rapid' methods like 1-2 test, DNA probe, ELISA, VIDAS or a luminescent phage assay. But the only improvement of this last step of the MPN method does not offer great advantage.
The miniaturisation of the whole procedure is more convenient because it offers the possibility to work under the microplate format. Using centrifugation of the suspension in BPW of the sample to be analysed, the whole bacterial flora of this sample (including *Salmonella*) is concentrated in a pellet which is weighted and re-suspended in the required volume of BPW in order to be divided in the number of repetitions chosen.
Therefore pre-enrichment, enrichment and post enrichment (if necessary) are realised in 3 successive plates containing micro-tubes of 2 ml volume each, and detection which can be based on the 'enrichment serology' technique or any other detection method suitable with the microplate format is realised in a microplate of 96 wells. In order to avoid cross-contamination between individual repetitions or dilutions, it is recommended to work only in every second row or line of the microplate. One plate is therefore used for 6 dilutions and 4 repetitions or conversely. Using this miniaturised MPN method, the analysis of 26 neck skin

samples from carcasses of two abattoirs gives a MPN of Salmonella of 12 *Salmonella*/g of neck skin with confidence limits between 0.9 and 5560.
Using traditional MPN method (3repetitions/3dilutions) applied on the whole liquid from carcass rinse, some authors also give numbers of *Salmonella* per carcass. These numbers are from <1 to 3200 *Salmonella* per carcass.
From a study on environmental contamination by *Salmonella* of all kind of human and animal wastes (manure and slurry from farms, water and sewage from sludge treatment plants) in a defined region of Brittany in France, the most contaminated waste is sludge (60% of samples contain more than 1 *Salmonella*/g or ml) and the least contaminated one is water from sludge treatment plants. For animal wastes, waste from poultry and pig origin are less contaminated than those from cattle.

Some references concerning quantification or numeration of *Salmonella* are shown in chapter 7, page 32-33.

Discussion:
No questions

5.2 Research on MPN performed at RIVM

Mr. Frans van Leusden, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

See Appendix 19 for sheets.

Discussion:
The samples have the same origin and therefore you expect the same number of *Salmonella* on them. The difference in MPN results between the frozen and the fresh samples can be explained by the way samples are frozen. The fresh samples are cooled by air, while the frozen samples are treated with liquid.

5.3 Theoretical background of MPN tables

Mr. Eric G. Evers, National Institute for Public Health and the Environment, Bilthoven, The Netherlands (See Appendix 20)

MPN stands for Most Probable Number, a term used for a specific experimental set up. For MPN, usually three dilutions of a sample are incubated, in 3, 5, or 10 replicates.
Absence/presence is then observed on selective plates. The concentration of the micro-organism in the sample is estimated from these absence/presence measurements using e.g. MPN tables. Cases for which MPN is preferred to direct plating are characterised by a

concentration that is too low for direct plating and/or by a surplus of competitive flora in direct plating.

For MPN calculations, three assumptions must be made:
1) The variation in number of micro-organisms between samples can be described by a Poisson distribution;
2) The micro-organism has a high growth rate during incubation;
3) The micro-organism, if present on the plate, is detected with high probability.

One statistical technique is necessary:
4) Maximum likelihood estimation.

sub 1)
The Poisson distribution can be applied if the micro-organism is randomly distributed in the medium. It describes the 'natural' variation of numbers in multiple sampling that is a direct consequence of this random distribution. This variation is termed variability. It is important to realise that this variation is not caused by experimental error. Experimental error would only increase the variation.

sub 2) and 3)
MPN formulas assume that one single micro-organism in the sample is detected. This assumption is probably not realistic, but it can be approximated if growth rate and detection probability are high. If this is not the case, MPN table values are an underestimation. High growth rate is important as the experimental procedure usually involves taking a relatively small volume from a larger volume after incubation. Starting with one micro-organism, growth has to be sufficiently high during incubation to have at least one micro-organism in the small volume that is taken from the large volume.

sub 4)
The maximum likelihood technique consists of two steps:
a) Assume a probability distribution for the variation in numbers between the samples;
b) Given the measurements, what is the most probable value for the parameter(s) of this distribution.

Parameter values can be estimated for measurements such as direct plating, absence/presence, or MPN. For simple cases, the estimation of the parameter value(s) by intuition agrees with estimation by maximum likelihood equations that can be derived mathematically. For more complicated cases, such as MPN tests, intuition is not sufficient and then only maximum likelihood equations provide estimated values. However, the principle of maximum likelihood does not change, whether cases are simple or complicated.

In using MPN tables, an important point is to be aware of the dimension of the MPN values given in these tables. Usually this is ‘number per highest amount tested’. MPN tables can also be used for other amounts than tested, if the proper dimension is used. If e.g. a tenfold higher

amount of sample is used than indicated in the MPN table, the estimated concentration is a factor 10 lower than the value in the MPN table.

A disadvantage of MPN tests is that the resulting MPN value has relatively large confidence intervals. This is probably caused by the fact that concentrations are estimated using only absence/presence information. In addition, one must realise that the MPN confidence interval is only an estimation of the uncertainty of lambda, the mean of the Poisson distribution. The actual variation in numbers between samples is larger than that, due to the variability of the corresponding Poisson distributions. The uncertainty can be reduced by additional measurements, whereas the variability cannot be reduced.

Discussion:
No questions

5.4 Quantitative estimation of *Salmonella* at VLA

Mr. Rob Davies, NRL-*Salmonella* United Kingdom (See Appendix 21)

Enumeration of *Salmonella* is desirable for risk assessment purposes. Unfortunately it is never possible to achieve a true value for the number of organisms present in a sample because of constraints of sampling, homogenisation and culture sensitivity. It is therefore more useful to carry out a larger number of semi-quantitative estimates than a small number of pseudo-exact counts, where microcolonies attached to small particles of the sample rather than individual cells are likely to be the units measured.

The larger number of estimations can then be used to provide a distribution of values which will give a greater degree of realism to the risk models.

The *Salmonella* culture method used at VLA involves pre-enrichment in Buffered Peptone Water (BPW) for 18 hours, selective enrichment in DIASALM agar for a minimum of 24 hours and plenty of Rambach Agar. The use of the Diasalm/Rambach combination reduces the number of confirmatory tests needed as a widely spread DIASALM at 24 hours incubation combined with profuse typical colonies on Rambach agar needs no further confirmation.

For quantifying *Salmonella* in feed samples we use a 3 tube MPN technique (3x10g, 3x1g, 3x0.1g), concentrating on avoiding settlement whilst dispensing the portions of the sample. Further decimal dilutions are added to test if *Salmonella* numbers are expected to be higher than 1,100 per 100g. The use of DIASALM permits direct slide agglutination testing of fluid from the plate thus saving time and media costs. A few representative subsamples and any suspect cultures in DIASALM are fully plated and confirmed.

In the field a decimal dilution approach is used, although serial dilutions may be also used to more accurately define the range of *Salmonella* numbers if these are expected to be low, e.g. on surfaces after disinfection. Samples of materials and surface swabs are collected directly into BPW and dispersed as much as possible by vigorous shaking then stirring with a pipette. Dilutions are normally made on site unless the premises is close to the laboratory when ice-cold BPW is used for the samples which are then rapidly returned to the laboratory. Normally only the initial sample and sometimes the 1st dilution are cultured fully, the other dilutions being incubated then stored at 4°C until sample results are available when full cultures of all dilutions from positive samples can be completed. Using this method 2 people can complete sampling and dilution of 100-200 samples in the field without difficulty.

Salmonella in tissue samples is estimated by direct plating of swabs from tissue on brilliant green or McConky agar or by dilution/enrichment on macerated tissues in the same way as for field samples. When these tests are carried out in parallel it is clear that the plate count lacks sensitivity and only levels of 10^4 *Salmonella*/g or more will be consistently detected by direct plating on selective media. Highly selective media such as XLT-4, which is often recommended for direct counts, is even more inhibitory.
In the future it is possible that quantitative PCR, and immunofluorescent flow cytometry may replace these traditional methods but currently the sensitivity and throughput capacity of such tests is too low to be practicable for routine use.

Discussion:
The poly H antiserum is used on the DIASALM plate. For this, a bit of agar is taken from the plate. After this the hole will be filled with fluid from the agar. This fluid is taken from the plate and agglutination is performed on an objective.

5.5 Quantification of DT104 in slurry from infected pig herds

Dorte Lau Baggesen, Danish Veterinary Laboratory, **Jakob Bagger**, **Vibeke Møgelmose**, **Bent Nielsen**, **Birgitta Svensmark**, Danish Bacon and Meat Counsil, **John Elmerdahl Olsen**, Royal Veterinary and Agricultural University (See Appendix 22)

Slurry from Danish swine herds infected with multiresistant *Salmonella* Typhimurium DT104 (DT104) must be deposited with a hose applicator and ploughed in immediately after spreading on fields according to rules laid down by the Danish Veterinary and Food Administration. To investigate if these special precautions are relevant in order to prevent or reduce the spread of DT104 to wildlife and livestock, a quantitative examination (MPN) of DT104 in slurry was carried out. In addition it was studied if parameters of infection at herd level e.g. serology and bacteriological examination of faecal samples can be used as predictors of the contamination level in the slurry.

Slurry-samples were collected from 18 DT104 infected swineherds during February and Marts 2001. In each herd, nine samples were collected from the slurry tank, three samples from the top, middle and bottom layers respectively.
Information on bacteriological findings in the herd and storage time of the slurry was recorded. For the 14 finisher herds, the prevalence of seropositive meat juice samples (OD%>20) in the last month of filling the slurry tank was obtained from the Danish Zoonosis Register. Culturing, sero- and phagetyping were done by standard procedures. The three samples from each layer were assessed together by using a three tube MPN-method.
Before the results of the microbiological examination were known, a group, consisting of five of the authors, had agreed on a classification of all 18 herds in to high risk, medium risk and low risk herds based on knowledge of the infection on the individual farm e.g. bacteriological examination of faecal samples, serology, duration of infection and information on last input of contaminated slurry to the slurry tank.

DT104 was isolated in slurry from 16 of the 18 herds. The most probable number estimates were ranging from less than 0.02 to 23 cfu per gr. slurry.
All 18 slurry tanks were divided in to three levels based on the most probable number of cfu per.gr slurry: high level (>1cfu/gr.), middle level (0.1-1 cfu/gr), low level (<0.1 cfu/gr.). Table 4 shows the correlation between these three microbiological levels and the risk classification done by the expert group.

Table 4. Correlation between microbiological examination of slurry and information on infection at herd level

	Classification of herds by a group of experts		
Bact. exam	High risk	Medium risk	Low risk
High level > 1 cfu/gr.	4 herds	1 herd	
Middle Level 0.1-1 cfu/gr	2 herds	2 herds	1 herd
Low Level < 0.1 cfu/gr	2 herds*	2 herds	4 herds

* In two herds, pig slurry were diluted with non DT104 cattle slurry in the tank

All herds in the study having less than 1 cfu/gr. slurry were granted exemptions from the special slurry handling on different conditions. A normal use of slurry in Danish agriculture is approximately 40 ton slurry per 1 hectare (10.000 m^2). Giving a contamination level of 1 cfu per gram, the spread of DT104 will thus be 4.000 cfu per m^2 landfill. It has not been possible to evaluate if this constitutes a risk of spreading the infection to livestock and wild life.

Discussion:
Increase or decrease on a farm of the level of contamination can be influenced by the season, or by import or export from a farm.

5.6 Enumeration of *Salmonella* from raw poultry meat using MPN technique

Ms. Tuula Johansson, NRL-*Salmonella* Finland

In Finland raw broiler meat at retail level has been analysed for *Salmonella* every second year during 1979-1987 (Hirn et al., 1992) and yearly since 1989. Samples have been deep-frozen carcasses during 1979-1991 (Hirn et al., 1992) and since then fresh broiler cuts (breasts and legs), because deep-frozen products are lees commonly marketed in Finland. MPN-technique for enumeration has been used since 1989.

Sample preparation

Deep-frozen carcasses, weight about 1 kg, were packaged in sterile plastic bags, thawed in refrigerator and thereafter shaken vigorously in 225 ml buffered peptone water (BPW) for 3 minutes.

Fresh broiler cuts, 500 g, have been shaken vigorously in 225 ml BPW. Rinsing liquid has been used for investigation.

Investigations

The method of the Nordic Committee on Food Analysis, NMKL No. 71 has been applied for MPN-analysis. The method has been modified by using Önöz agar as solid selective medium and Modified Semisolid Rappaport-Vassiliadis medium (MSRV; in 1991-1992) for selective enrichment. The rinsing liquid has been divided into the tubes: 4 x 25 ml, 4 x 5 ml filled to 10 ml with BPW and 4 x 1 ml filled to 10 ml with BPW. High sample quantities are chosen for analysis, because cell densities are suspected to be low. Dilutions of 1:5 instead of 1:10 are chosen, because variation in the cell densities between samples is suspected to be rather low (Niemelä, 1983). The tubes have been incubated at 37 °C for 20 h. Thereafter 100 µl has been transferred to secondary enrichment broth RVS or 3 x 33 µl to MSRV, both incubated at 41.5 °C for 18-24 h before inoculation on the selective plates.

Calculation of MPN-indices

Thomas' approximate MPN formula (Niemelä, 1983) has been used for calculation of MPN-indices, because no tabulated indices are available:

MPN (organisms per ml) = P / ÖNT

P = total number of positive tubes

T = total volume of original sample cultured

N = total volume of original sample in negative tubes

Limit of determination was 2 MPN/kg, when 1 kg was rinsed in 225 ml BPW, and 4 MPN/kg, in case of rinsing 500 g in 225 ml BPW.

Choice of dilutions for calculations

Series of dilutions containing both positive and negative tubes are chosen for calculations. The approximate value differs most from the theory, when the proportion of positive tubes is great. Three dilutions are usually included in calculation. However, two dilutions are preferred, if all the tubes of the least diluted series are positive (Niemelä, 1983).

Results

Numbers of Salmonella have been low, usually 2-50 MPN/kg (Table 5).

Table 5. Salmonella in broiler meat in Finland during 1989-2000

	Year	No of samples	% positive	MPN/kg
Frozen	1989	195	5.1	2-7
	1990	90	11.1	2-54
	1991	105[1)]	10.5	2-25
Fresh	1992	95	9.5	4-33[2)]
	1993	100	4.0	NE
	1994	109	9.2	NE
	1995	101	1.0	NE
	1996	100	3.0	NE
	1998	114	0.9	NE
	1999	158	0.0	-
	2000	161	0.0	-

NE = not enumerated, 1) 25 fresh, 2) > 387 in one sample

References

Hirn, J., Nurmi, E., Johansson, T. and Nuotio, L. (1992) Long-term experience with competitive exclusion and salmonellas in Finland. Int. J. Food Microb., 15, 281-285.

Niemelä, S. (1983) Statistical evaluation of results from quantitative microbiological examinations. Nordic Committee on Food Analysis, Ord & Form, Uppsala.

Discussion:
No questions

5.7 Discussion on quantitative methods

Most of the time MPN is performed, the results are used for risk assessment. It is necessary to know what you want with the data from quantitative methods. It is possible to do a lot of work and get a lot of data, however, you can also perform a bit less work and obtain data which is good enough to use.

If calculating with a certain interval of MPN, both the range and the distribution of observations within the range are important.

The media used for quantitative methods make a big difference between the results obtained. The precise influence of different media is hard to measure. It can be done with artificially contaminated samples. However, these are much easier to investigate than naturally contaminated samples. Also the level of contamination as the distribution within naturally contaminated samples are unknown and are the cause of different results between media.

It was concluded that more data is necessary to evaluate quantitative methods. This is however restricted to the time and money which is available within laboratories.

5.8 Reliable ELISAs showing between-lines differences in hens orally inoculated with *Salmonella* Enteritidis

Ms. Karine Proux, NRL-*Salmonella* France

See Appendix 23 for sheets.

Discussion:
More studies have been performed to test the resistance in the hens to other serotypes.

5.9 CRL-*Salmonella* internet-site

Mr. Maurice Raes, CRL-*Salmonella*

Some suggestions for further development of the internet site:

- adding links;
- partly accessible (e.g. the addresses of NRLs) to others than the NRLs. This has to be discussed with the EU;
- list of persons of the NRLs with their experiences/specialisation;
- information about the application of the directive in the different Member States.

6. Closing remarks

General discussion

The work plan for the year 2002 has been discussed.
Next year, the workshop will be held in Ploufragan, France, at Tuesday 28 May 2002, i.e. one day before the World *Salmonella* Symposium.
The next (= 6th) bacteriological collaborative study will have the same set-up as the 4th and 5th study to allow comparison of study results over time. Some media and reagents may be additionally prescribed depending on the outcome of the discussion elsewhere about the new ISO.
The next (=6th) typing collaborative study will have the same set-up as the last study (sero typing, phage typing and antibiotic resistance typing). The following suggestions have been made:

- choose serotypes that are the most important with respect to public health and choose further also sero types that can easily be confounded with those most important ones;
- phage typing will be organised again in co-operation with the PHLS Colindale;
- the antibiotics against which resistance should be determined will be prescribed and restricted to the ones as presented during the meeting by Reiner Helmuth from NRL-*Salmonella* Germany;
- the method of antibiotic resistance testing will not be prescribed, but results should be reported in a quantitative way (e.g. report of diameter in case of disk diffusion in stead of report in terms of 'resistant' (R), 'sensitive' (S) and 'intermediate' (I).

No special comments were given on the other activities (newsletter, web site, research, ad hoc activities) of the CRL-*Salmonella* meaning that they should be continued without further changes.

Evaluation of the workshop

The objectives set for the workshop were realised, although this year no reports were given by NRLs-*Salmonella* on national ring trials which among other has to do with the present situation that reference materials other than *Salmonella* Panama are not (commercially) available. The plan of the CRL-*Salmonella* for the near future is clear, i.e. the goals in terms of activities are set. Whether these will be performed according to the normal annual time table is not yet clear as this will depend on the recruitment of new personnel as Ms. Nelly Voogt and Mr. Maurice Raes will leave RIVM this summer. The CRL-*Salmonella* is complimented for their work and strongly advised to continue with their work.

Discussion:
Concerning the collaborative studies organised within Member States, it was asked if there are many laboratories in the Member States that work with the samples as described in the directive. In Denmark just a few laboratories do, however, the number of laboratories increases. In The Netherlands, about 25 laboratories participate in the studies organised by NRL-*Salmonella* The Netherlands. These laboratories are obliged to participate by the productboard for cattle, meat and eggs in order to get permission to investigate samples from the directive. In Great Britain no such samples are investigated by laboratories. Mixed cultures are tested for *Salmonella,* these samples are however much easier to find positive compared to the samples from the collaborative studies.

Closing remarks

The participants from the Member States were thanked for their active participation in the workshop programme. Every year participants step forward willingly to contribute and thus making the workshop a success. This is much appreciated. The EU Commission is acknowledged for their support also in financial terms to make this workshop possible. The participants consider the workshop a necessary element in the annual programme. The CRL-*Salmonella* team is acknowledged for their work of the previous year. The team that organised the workshop is thanked for the efficient organisation of the logistics and the workshop programme. And, lastly, Ms. Nelly Voogt, who has been involved in the CRL-*Salmonella* work from the start in 1994, and Mr. Maurice Raes, who joined the team in 1998, were thanked for their contributions and wished success as they both will leave the RIVM.

References

References from Florence Humbert concerning quantification or numeration of *Salmonella.*

- BANFFER J.R.J., VAN ZWOL-SAARLOOS J.A. and BROERE L.J., 1993. Evaluation of a commercial latex agglutination test for rapid detection of *Salmonella* in fecal samples. Eur. J. Clin. Microbiol. Infect. Dis., Vol. 12, 633-636.
- BERRY E. D. and SIRAGUSA G. R., 1997. Hydroxyapatite adherence as a means to concentrate bacteria. Applied and environmental Microbiology, 4069-4074.
- BLAIS B. W. and YAMAZAKI H., 1991. Application of polymyxin-coated polyester cloth to the semi-quantitation of *Salmonella*. International Journal of Food Microbiology, 14, 43-50.
- de MAN J. C., 1974. The probability of most probable numbers, Anal. Chem., 67-78.
- de MAN J. C., 1983. MPN tables, corrected. European Journal Applied Microbiology and Biotechnology, 17, 301-305.
- FLEET G.H., KARALIS T., HAWA A. and LUKONDEH T., 1991. A rapid method for enumerating *Salmonella* in milk powders. Letters in Applied Microbiology, 13, 255-259.
- HAAS C. N. and HELLER B., 1988. Test of the validity for the Poisson assumption for analysis of most-probable-number results. Applied and Environmental Microbiology, 2996-3002.
- HAMMACK T. S., ANDREWS W. H., AMAGUANA R. M., JUNE G. A. and SHERROD P. S., 1994. Effectiveness of a one-day procedure for recovery of *Salmonella* from milk powders. Journal of AOAC International, Vol. 77, 6, 1681-1684.
- HARRIGAN W.F. and McCANCE M., 1966. Laboratory Method in Microbiology. Probability tables for the estimation of bacterial numbers by the dilution tube technique. Academic Press, 319-324.
- HAWA S.G., MORISSON G.J. and FLEET G.H., 1984. Method to rapidly enumerate Salmonella on poultry carcasses. Jornal of Food Protection, 47, 932-936.
- HO B.S.W. and TAM T. Y., 2000. Rapid enumeration of *Salmonella* in environmental waters and wastewater. Wat. Res., Vol. 34, 8, 2397-2399.
- HOSZOWSKI A., FRASER A.D.E., BROOKS B.W and RICHE E.M., 1994. Rapid detection and enumeration of *Salmonella* in chicken carcass rinses using filtration, enrichment and colony blot immunoassay. International Journal of Food Microbiology, 28, 341-350.
- HUGUES B. and PLANTAT J.L., 1982. Calcul du nombre le plus probable et de son intervalle de confiance dans le cas où le nombre d'inocumums par dilution est important. Chemosphere, Vol. 11, N°11, 1135-1140.
- HUMBERT F., LAHELLEC C., 1987. Essais de dénombrement des *Salmonella* présentes sur les carcasses de volailles. Science des Aliments, 7, VII, 253-259.
- HUMBERT F., SALVAT G., LALANDE F. and COLIN P., 1997. Miniaturised Most Probable Number and enrichment serology technique for the enumeration of Salmonella spp. on poultry carcasses. Journal of Food Protection, 60, 11, 1306-1311.
- HUSSONG. D., ENKIRI N. K. and BURGE W. D., 1984. Modified agar medium for detecting environmental Salmonellae by the most-probable-number method. Applied and Environmental Microbiology, 1026-1030.
- IZAT A.L., KOPEK J.M. and McGINNIS J.D., 1990. Incidence, number and serotypes of *Salmonella* on frozen broiler chickens at retail. Poultry Science, 15, 1438-1440.
- JETTON J.P., BILGILI S.F., CONNER D.E., KOTROLA J.S. and REIBER M.A., 1992. Recovery of *Salmonella* from chilled broiler carcasses as affected by rinse media and enumeration method. Journal of Food Protection, Vol. 55, 5, 329-332.

- LILLARD H.S., 1988. Incidence and recovery of *Salmonella* and other bacteria from commercially processed poultry carcasses at selected pre-and post-evisceration steps. Journal of Food Protection, Vol. 52, 2, 88-91.
- McLELLAND R.G. and PINDER A.C., 1994. Detection of low levels of specific Salmonella species by fluorescent antibodies and flow cytometry. Journal of Applied Bacteriology, 77, 440-447.
- MAHON J. and LAX A.J., 1993. A quantitative polymerase chain reaction method for the detection in avian faeces of Salmonellas carrying the *spvR* gene. Epidemiol. Infect., 111, 455-464.
- PROST H. and HUGUES B., 1981. Dénombrement des microorganismes par la technique du nombre le probable (indice NPP) : Emploi statistique de cet indice. Ann. Fais. Exp. Chim., 75, n°807, 185-207.
- PUMFREY L. and NELSON C. E., 1991. Use of a most probable number method modified with a deoxyribonucleic acid probe to monitor control by food preservatives of natural *Salmonella* contamination in animal meat meals. Poultry Science 70, 780-784.
- RIGBY C.E., 1982. Most probable number cultures for assessing *Salmonella* contamination of eviscerated broiler carcasses. Canadian Journal comp. Medecine, 46, 279-282.
- RODRIGUES U.M. and KROLL R.G., 1990. Rapid detection of Salmonellas in raw meats using a fluorescent antibody-microcolony technique. Journal of Applied Bacteriology, 68, 213-223.
- SLAVIK M. F., KIM J. W. and WALKER J. T., 1995. Reduction of *Salmonella* and *Campylobacter* on chicken carcasses by changing scalding temperature. Journal of Food Protection, 58, 689-691.
- STRANTZ A. A. and ZOTTOLA E. A., 1989. A modified plating technique for the recovery and enumeration of stressed *Salmonella* typhimurium Hf. Journal of Food Protection, Vol. 52, 10, 712-714.
- TOKUMARU M., KONUMA H., UMESAKO M., KONNO S. and SHINAGAWA K., 1990. Rates of detection of *Salmonella* and *Campylobacter* in meats in response to the size and the infection level of each species. International Journal of Food Microbiology, 13, 41-46.
- THOMASON B. M., 1981. Current status of immunofluorescent methodology for *Salmonell*a. Journal of Food Protection, 44, 381-384.
- TURPIN P. E., MAYCROFT K. A., BEDFORD J., ROWLANDS C. L. and WELLINGTON E. M. H., 1992. A rapid luminescent-phage based MPN method for the enumeration of *Salmonella* typhimurium in environmental samples. Letters in Applied Microbiology, 16, 24-27.
- TURPIN P. E., MAYCROFT K. A., ROWLANDS C. L. and WELLINGTON E. M. H., 1993. An ion-exchange based extraction method for the detection of Salmonellas in oil. Journal of Applied Bacteriology, 74, 181-190.
- WHITTEMORE A.D., 1993. A modified most probable technique to enumerate total aerobes, *Enterobacteriaceae* and *Salmonella* on poultry carcasses after the whole carcass rinse procedure. Poultry Science, 72, 2353-2357.
- YAMAGUCHI W., KOPEK M. J. and WALDROUP A. L., 1994. Comparison of a membrane filtration procedure and a MPN procedure for enumerating *Salmonella* in whole carcass rinse samples. Publication February 16, 287-297.
- YU L.S.L. and FUNG D.Y.C., 1993. Five-tube most-probable-number method using the Fung-Yu tube for enumeration of *Listeria moncytogenes* in restructured meat products during refrigerated storage. International Journal of Food Microbiology, 18, 97-106.

Appendix 1 Mailing list

01	European Commission, Director of Directorate D	P. Testori-Coggi
02	European Commission, head of Unit D.2	E. Poudelet
03	European Commission	J.C. Cavitte
04	European Commission	P. Mäkelä
05	President of the Council of Health, the Netherlands	prof. dr. J. J. Sixma
06	Veterinary Public Health Inspector	drs. H. Verburg
07-43	Participants of the workshop	
44	Dutch National Library for Publications and Bibliography	
45	Board of Directors RIVM	H.A.P.M. Pont
46	Head of Microbiological Laboratory for Health Protection and Director CRL-*Salmonella*	dr. ir. A.M. Henken
47	Authors	
48	SBD/Information and Public Relations	
49	Registration agency for Scientific Reports	
50	Library RIVM	
51-65	Sales department of RIVM Reports	
66-70	Spare copies	

Appendix 2 Participants

National Reference Laboratories for *Salmonella*

Austria
Christian Berghold
Lassnig Heimo
Belgium
Ingrid Wybo
Denmark
Dorte Lau Baggesen
Jens Chr. Jorgensen
Gitte Sørensen
Finland
Tuula Johansson
Sinikka Pelkonen
France
Florence Humbert
Karine Proux
Germany
Reiner Helmuth
Beatriz Guerra Roman
Greece
Maria Passiotou-Gavala
Italy
Stefano Marangon
Giovanni Pezzoti
Luxembourg
Joseph Schon
The Netherlands
Nelly Voogt
Portugal
Alice Amado
Maria Do Rosario Vieira
Spain
Consuelo Rubio Montejano
Cristina de Frutos Escobar
Sweden
Ingrid Hansson
Anna Aspan
United Kingdom
Robert Davies
Stanley McDowell

CRL-*Salmonella*
André Henken
Maurice Raes
Wim Wannet
Kirsten Mooijman

Invited speakers/guests
Eric Evers
Wilfrid van Pelt
Frans van Leusden
Jan Nieuwenhuijs
Henny Maas

CRL-Epidemiology of Zoonoses
Annemarie Käsbohrer

Commission
Jean-Charles Cavitte
Pia Mäkelä

Appendix 3 Programme of the workshop

Sunday 10 June

20.30 - 21.30 Social get together, bar hotel Mitland

Monday 11 June

8.30 - 8.40 Opening and introduction of participants (André Henken)
8.40 - 9.30 Current issues on the New Draft European zoonoses directive (Jean-Charles Cavitte)
9.30 - 9.45 Evaluation of *Salmonella* methods as being discussed in the Scientific Committee (Pia Makela)
9.45 - 10.05 Report of the CRL-Epidemiology of Zoonoses (Annemarie Kaesbohrer)
10.05 - 10.35 Coffee/tea
10.35 - 10.55 Surveillance trends in *Salmonella* in 2000 (Rob Davies)
10.55 - 11.15 *Salmonella* in the wild fauna, fur animals and pets in Denmark (Jens Chr. Jørgensen)
11.15 - 11.35 National slaughter surveys for *Salmonella* in pigs, cattle and sheep (Rob Davies)
11.35 - 11.55 Results fifth bacteriological study (Maurice Raes)
11.55 - 12.05 Revision of ISO 6579 (Kirsten Mooijman)
12.05 - 12.15 Reference Materials (Kirsten Mooijman)
12.15 - 12.30 Discussion on bacteriological collaborative study VI (and the use of Tetrathionate) (André Henken)
12.30 - 14.00 Lunch
14.00 - 14.20 Preliminary results sixth typing study (Wim Wannet)
14.20 - 14.30 Resistance monitoring of *Salmonella* (human and non-human isolates) in Austria (Christian Berghold)
14.30 - 14.50 Studies performed on *Salmonella* isolates at LNIV in 2000 (Maria do Rosário)
14.50 - 15.05 discussion on 7th typing study
15.05 - 15.30 Coffee/tea
15.30 - 16.15 Principle and design for a DNA chip for *Salmonella* (Reiner Helmuth)
16.15 - 16.35 General aspects of antimicrobial resistance typing (Reiner Helmuth)
16.35 - 17.00 Molecular characterisation of genetic elements involved in antimicrobial resistance in *Salmonella* (B. Guerra Roman)

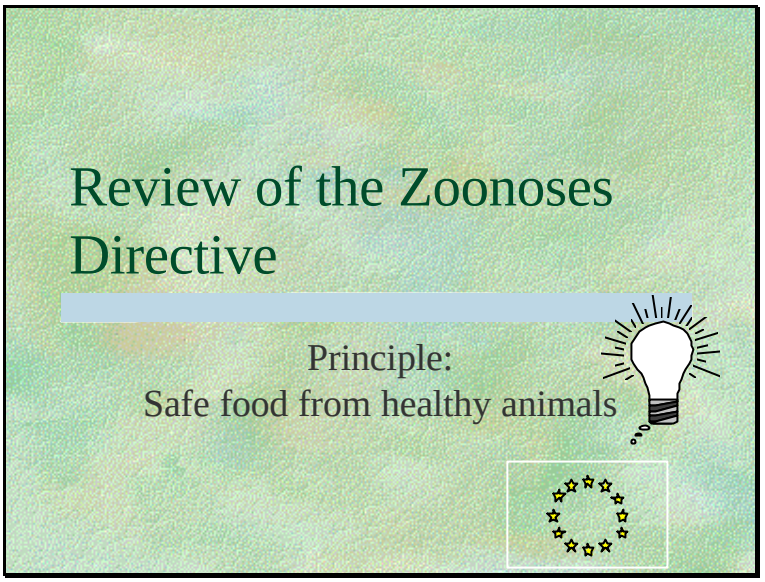
18.00 Evening programme, departure to Utrecht

Tuesday 12 June

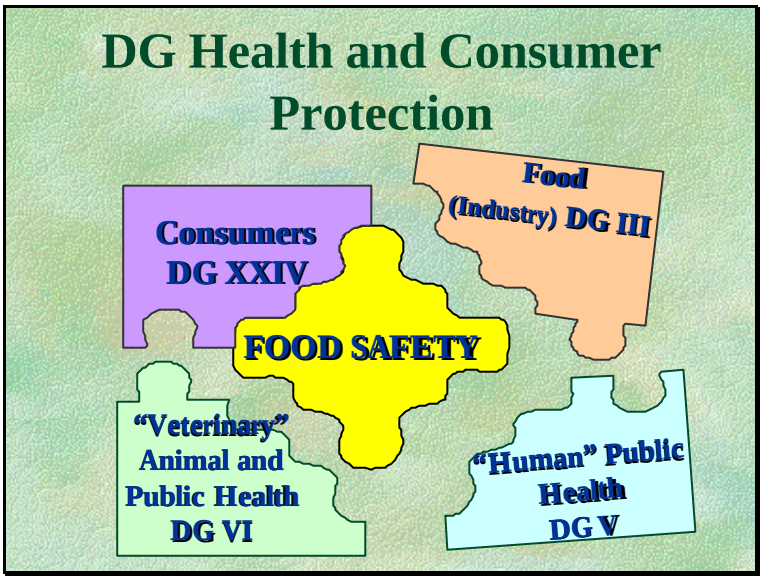
8.30 - 9.00	Numeration / quantification of *Salmonella* (Florence Humbert)
9.00 - 9.20	Research on MPN performed at RIVM (Frans van Leusden)
9.20 - 10.00	Theoretical background of MPN tables (Eric Evers)
10.00 - 10.10	Quantitative estimation of *Salmonella* at VLA (Rob Davies)
10.10 - 10.40	coffee / tea
10.40 - 11.00	Quantification of DT104 in slurry from infected pig herds (Dorte Lau Baggesen)
11.00 - 11.15	Enumeration of *Salmonella* from raw poultry meat using MPN technique (Tuula Johansson)
11.15 - 11.35	discussion on quantitative methods
11.35 - 11.55	Reliable ELISAs showing between-lines differences in hens orally inoculated with *Salmonella* Enteritidis (Karine Proux)
11.55 - 12.05	preview CRL-*Salmonella* internet site
12.05 - 12.30	Closing remarks
12.30-14.00	lunch
±14.00	departure to airport

Appendix 4 Sheets of presentation 2.1

Slide 1



Slide 2



Slide 3

DG Mission statement

The mission of DG SANCO is to "ensure a high level of protection of consumers' health, safety and economic interests as well as of public health at the level of the European Union".

Slide 4

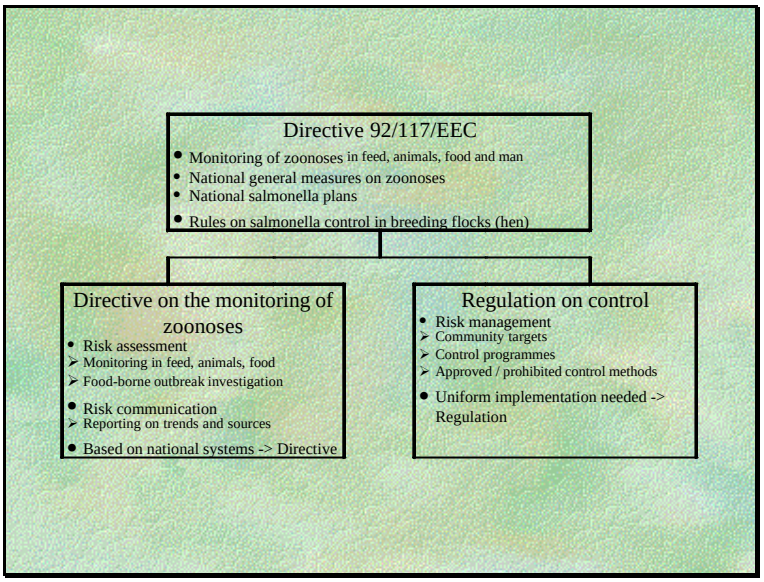
The proposals on zoonoses are at the final stage of the procedure before adoption by the Commission.

(Inter-service consultation has been finalised)

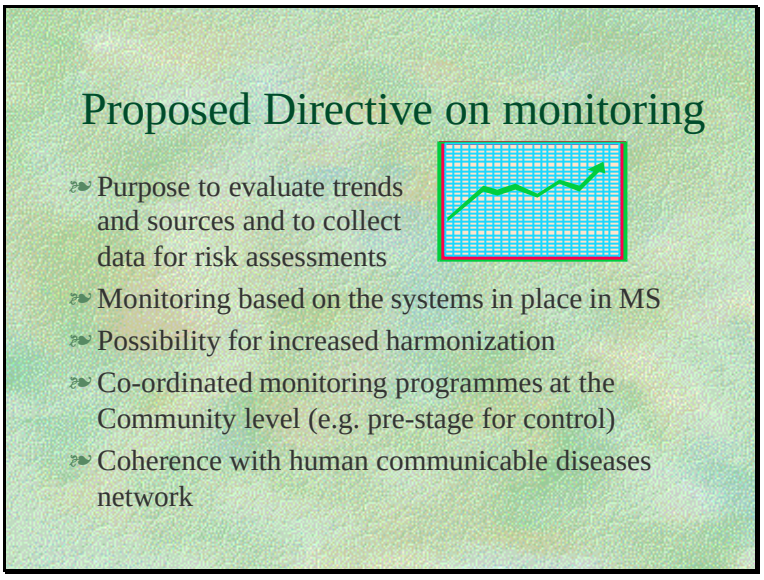
Slide 5

The proposals have been significantly amended since last year, but the structure and most elements remain

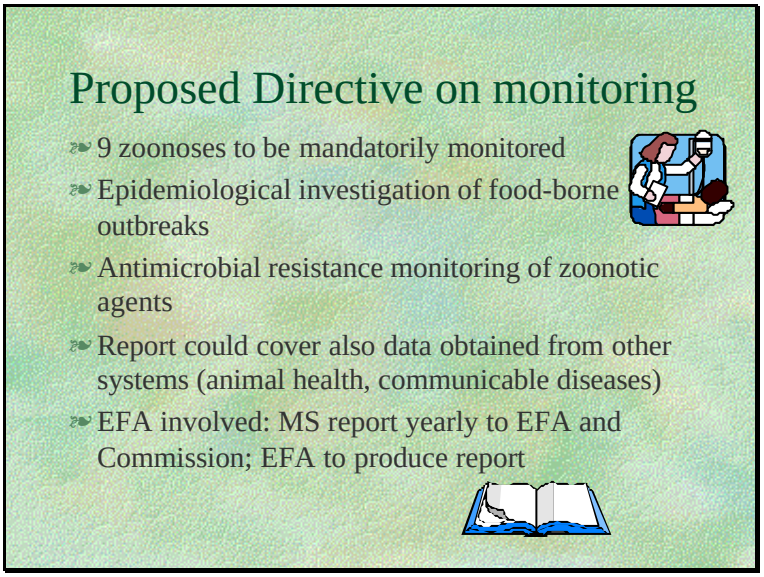
Slide 6



Slide 7



Slide 8



Slide 9

Proposed Directive on monitoring

- 9 zoonoses to be mandatorily monitored
  - **•Brucellosis ***
  - **•Campylobacteriosis**
  - **•Cryptosporidiosis**
  - **•Echinococcosis**
  - **•Listeriosis**
  - **•Salmonellosis ***
  - **•Trichinellosis ***
  - **•Tuberculosis due to *Mycobacterium bovis* ***
  - **•Verotoxigenic *E. coli***
  - *•* covered by 92/117*

Slide 10

Proposed Directive on monitoring

- All financial provisions to be laid down in Decision 90/424/EEC on the financing of veterinary measures. The chapter on zoonoses in that decision will be amended
  - Financing would be maintained for same measures as in Directive 92/117/EEC (global review of financial support for disease control intended by the Commission)
  - co-ordinated monitoring programmes to be financed
  - CRLs

Slide 11

Directive on monitoring

- Co-ordinated programmes (Commission)
  - increased harmonisation
  - limited duration
  - specific needs:
    - emerging pathogens
    - restricted interest (ST DT104)
    - exposure assessment
    - pre-testing surveillance requirements

Slide 12

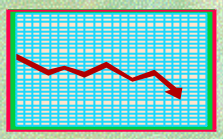
Proposed Regulation on control of salmonella and other foodborne zoonotic agents

- Creates a framework for zoonoses control
- Measures will be defined more closely by Commission Decisions
- Linked to Directive on monitoring, hygiene and animal health legislation

Slide 13

Proposed Regulation on control of specified zoonoses

- Pathogen reduction targets decided by Comitology procedure
  - target is 'XX prevalence OR XX % reduction in prevalence by year YY'
  - targets to be adopted step-by-step
  - Scientific Committee opinion needed



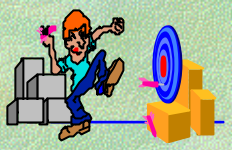
Slide 14

Proposed Regulation on control of specified zoonoses

- Progressive enlargement:
  - Currently: SE and ST in poultry breeders
  - Salmonella with public health significance:
    - breeding and commercial flocks (SE and ST in layers); turkey and breeding herds of pigs
  - possibility to include other zoonoses or other stages of food-chain

Slide 15

Proposed Regulation for control of specified zoonoses



- When targets established
  - MS prepare a control programme
  - methods for controlling decided by MS;
  - Comm may forbid / approve certain methods
  - MS programmes approved by Comm
  - Food businesses may create own programmes -> MS approves

Slide 16

Proposed Regulation for control

Proposal in force	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
***S.* serotypes** with P.H. importance; **breeding flocks Gallus gallus**		**Target established**	**National control programmes Certification**			
***S.* Enteritidis and *S.* Typhimurium : laying hens**			Target est.			
***S.* serotypes** with P.H. importance; **broilers**				Target est.		
***S.* serotypes** with P.H. importance; **turkeys**					Target est.	
***S.* serotypes** with P.H. importance; **breeding herds of pigs**					Target est.	

Slide 17

Proposed Regulation for control of specified zoonoses

- Rules on trade in live animals (incl. poultry for slaughter) and hatching eggs
  - link to Directives 90/539 (poultry) and 64/432 (pigs)
  - after target established -> certificate for dispatches with test results (certificates to be amended in due course)
  - MS may require same testing results requirements as it applies itself (Comm. Dec., transitional period)

Slide 18

Proposed Regulation for control

- Predefined specific measures
  - Fowl breeding flocks infected with SE/ST: slaughter/heat treatment/destruction
  - Table eggs: to come from flocks tested negative (transitional period up to 1.1.2008)
  - Poultry meat: criterion of absence in 25g or industrial heat treament or other treatment able to eliminate salm (transition period up to 1.1.2009)

Slide 19

Proposed Regulation for control

- Rules on imports : equivalent conditions
  - control programmes;
  - certification to be laid down or amended in due course

Slide 20

Transition

- Directive 92/117/EEC to be repealed
- However, measures foreseen for the control of salmonella (incl. approved national plans) shall be in force until respective programmes have been approved according to proposed Regulation

Slide 21

Conclusions:

- Directive 92/117 is being revised, as well as other legislation on hygiene: the whole chain will be covered
- Revision of Dir 92/117: controls to be defined on risk based approach; step-by-step pathogen reduction targets (but also predefined measures)
- The financial impact of measures must not be underestimated
- Risk communication to consumers should be developed

Slide 22

White Paper on Food Safety

- the responsibility for food safety rests primarily with food businesses, including feed manufacturers and farmers;
- risk based systems (HACCP);
- creating conditions for traceability;
- being based on risk analysis, including the possibilities to take into account precautionary principle and other legitimate factors, where appropriate.

Slide 23

Other proposals/ pre-harvest food safety

- **Review of specific legislation for animal feed**
- **Intended modernisation of meat inspection, both ante and post mortem:**
  - make it more risk based,
  - introduce a stable to table approach (information accompanying animals to slaughter)
  - introduce microbiological testing

Slide 24

Proposal on EFA (missions)

- Setting up an integrated, coherent system of scientific and technical support for the EC legislation and policies; provision of independent information; risk communication
- all fields having a direct or indirect impact on the safety of food; animal health and welfare, plant health; nutrition; any matter relating to GMOs;
- operation of RASFF

Slide 25

Proposal on EFA (tasks)

- Provide Community Inst and MS with scientific opinions in cases provided for in EC legis and on any question within its missions
- promote and coordinate harmonisation of RA methodologies
- provide scientific and technical support to Comm (establishment/evaluation of technical criteria, guidelines, guides)
- commission scientific studies

Slide 26

Proposal on EFA (tasks)

- search, collate, analyse and summarise scientif and techn data
  - food consumption and exposure to food related risks; incidence and prevalence of biological risks; contaminants in food/feed
  - close cooperation with all organisations operating in field of data collection including T/C, intern bodies
- to undertake action to identify and characterise emerging risks
- to establish a network or organisationsoperating in the fields within its mission and be responsible for their operation

Slide 27

Proposal on EFA (tasks)

- operation of RASFF
- assistance to Comm in the crisis management
- assistance upon request by Comm to improve cooperation between Community and T/C, international organisations
- assistance upon request by Comm concerning communication on nutritional issues
- to ensure that public and interested parties receive rapid, reliable, objective and comprehensible info in the fields within its missions

Slide 28

Proposal on EFA (staff/budget)

- Staff: 255 by year n+3; +/- 340 by year n+5
- Budget: from 9 million euros in year n, to 44 (year n+3) and 67 (year n+5)

Slide 29

Laboratories and EC legislation on official control of food: QA systems

- Directive 89/397/EEC on the official control of foodstuffs (*without prejudice to provisions in more specific Community rules*)
- Directive 93/99/EEC: additional measures concerning the official control of foodstuffs

Slide 30

National Laboratories and EC legislation on official control of food:

- Directive 93/99/EEC, Art 3: Labs used for off control of foodstuffs shall comply with EN 45001 (*recently changed into EN/ISO 17025*); subject to assessment
- applicable from November 1998

Slide 31

National Laboratories and EC legislation on official control of food:

- Uncertainty because of the old separation between veterinary (*which refers to "approved" laboratories*) and non veterinary "food" legislation
- LS opinion: applicable to both, *without prejudice*...

Slide 32

Community Reference Laboratories in the area of food control

- To provide NRLs with details of analytical methods and comparative testing and co-ordinate application by NRLs by ring trials;
- To co-ordinate research on new methods;
- To conduct initial and further training for NRLs...

Slide 33

Community Reference Laboratories in the area of food control

- 5 CRLs for microbiologcal risks (Paris;Berlin; Bilthoven; Vigo; Weymouth); 4 CRLs for residues (Bilthoven; Fougères; Berlin; Roma)
- According to LS, CRLs are covered also by provisions in Directive 93/99/EEC (*accreditation*), *without prejudice*...

Slide 34

Community Reference Laboratories in the area of food control

- 2 CRLs Vigo and Weymouth appointed by Council Decisions in 1993 and 1999 and CRLs for Residues: as part of their duties:
  - helping NRLs to implement an appropriate system of QA based on the principles of GLP and EN 45000

Slide 35

Zoonoses proposals

- Responsibilities and tasks of CRLs and possibly NRLs to be defined
- Labs for control to be approved, to apply QA systems conforming to EN 17025 (from 1.1.2005), using methods and protocols recommended by international bodies as reference methods or validated alternative methods or…)

Slide 36

Forthcoming revision of legislation on official feed/food control

- Draft Regulation (prelim. working doc)
- Sampling and analysis
  - Methods to be validated in accordance with internationally accepted methods
  - Approved laboratories applying EN/ISO 17025

(ISO Guide 43-1 on the organisation of ring trials?)

Slide 37

Quality Standards for Laboratories involved in official control of food in the Community

- There are and/or there will be soon requirements for QA standards in Laboratories in the EC: GLP and EN/ISO 17025

Slide 38

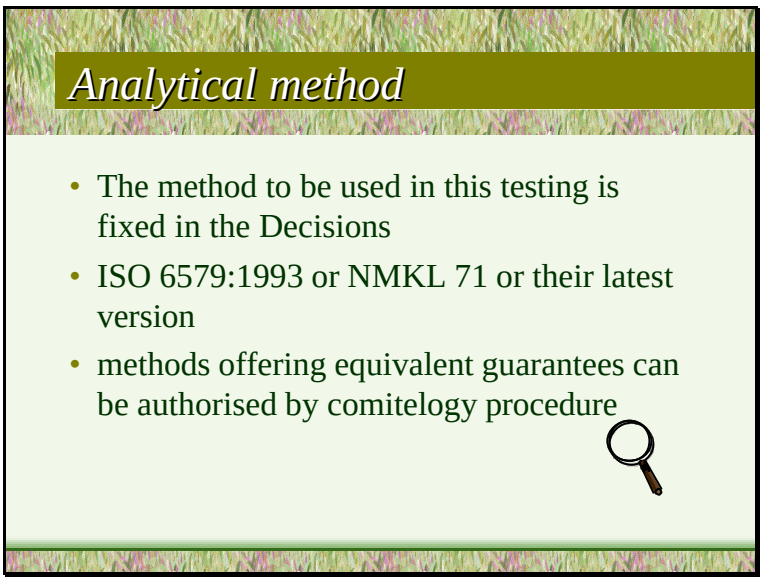
National Laboratories and EC legislation on official control of food:

- Also Directive 96/23/EC for residues and subsequent Decision 98/179/EC:
  - Laboratories approved for off residue control
  - participation to internationally recognised external quality control assessment and accreditation scheme (accreditation must be obtained before 1.1.2002)
  - participation in proficiency testing schemes recognised or organised by NRLs or CRLs

Appendix 5. Sheets of presentation 2.2

Slide 1

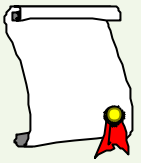
Slide 2



Slide 3

Alternative methods

- Applications for approval:
- EN 12824:1997
- DIN 10135:1999-11 (PCR method)
- EiaFoss Salmonella
- Transia Plate Salmonella Elisa kit



Slide 4

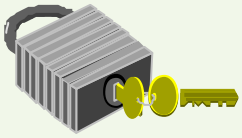
Question to Scientific Committee

- Asked to evaluate the equivalency of the methods:
  the Committee refused
- Instead: general question
  advice on criteria to be applied in the evaluation of new salmonella methods

Slide 5

Possible options ??

- MICROVAL
- ISO and CEN standard on validation of alternative methods (still draft)



Appendix 6. Sheets of presentation 2.3

Slide 1

Report on trend and sources of zoonotic agents in the EU 1999 Results and problems

Annemarie Käsbohrer
Community Reference Laboratory for the Epidemiology of Zoonoses
Berlin, BgVV

Slide 2

Feed

- Objectives:
  - For identification of risk material
  - Useful information for other MS
- Data requested:
  - Feed materials separated by animal and vegetable origin
  - Process control samples
  - Compound feedingstuffs

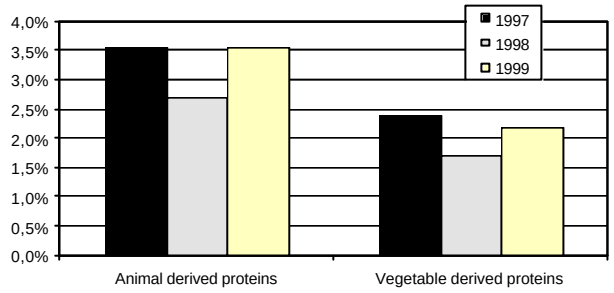
Slide 3

Feed

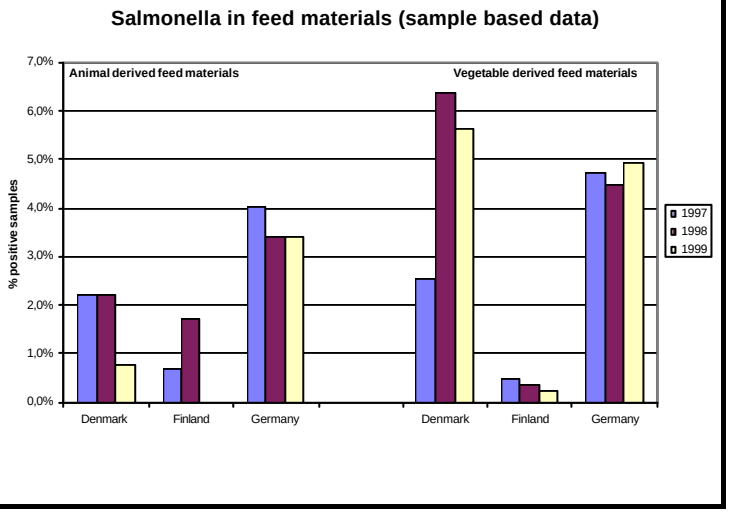
- Sources of information
  - Official sampling using a statistically based sampling plan, random sampling
  - Self control system (based on legal requirements)
  - Findings are reportable
  - Voluntary programmes
  - Laboratory reports

Slide 4

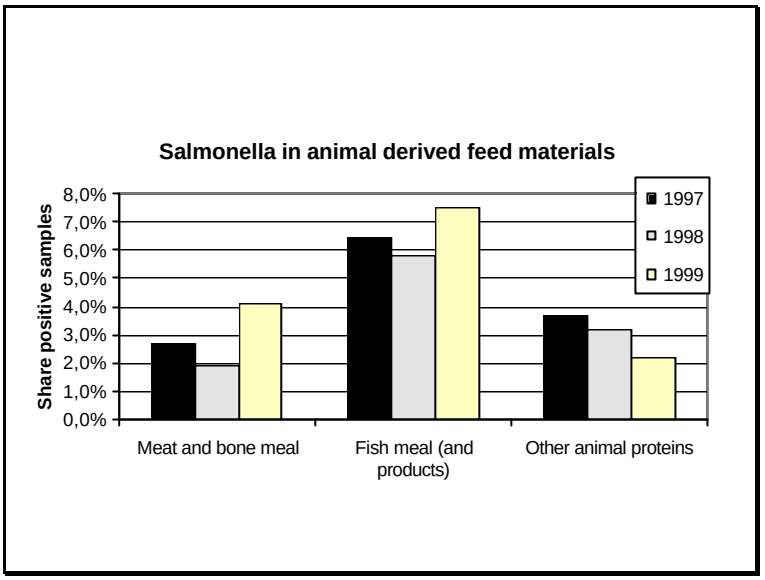
Salmonella in feed materials



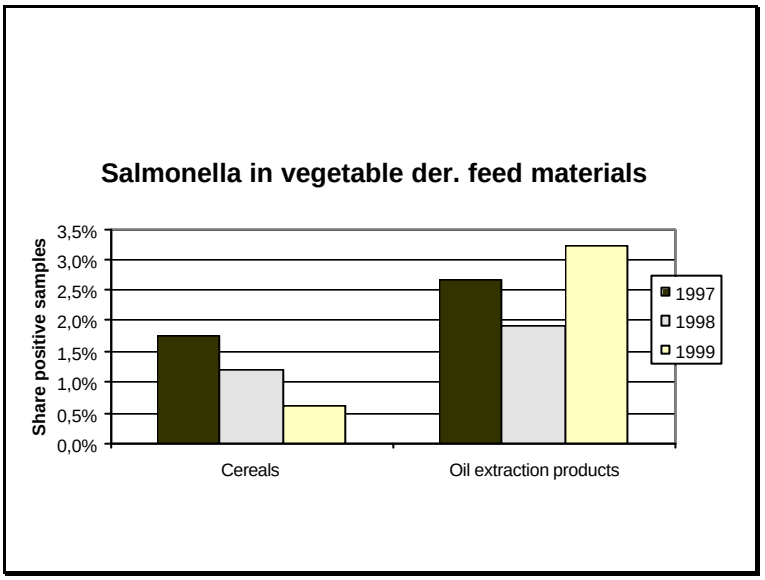
Slide 5



Slide 6



Slide 7



Slide 8

Process control samples

		n	% positive
Austria		?	?
Denmark		3306	6,3%
Finland		?	?
Sweden	Before heat treatment	2851	0,84%
	After heat treatment	4276	0,07 %
Norway		8750	0,07%

Slide 9

Problems with the current data Feed

- Epidemiological unit: batch or sample ?
  - Pooled samples
  - Separate samples from one batch
  - Sample size: 1500g, 1000g, 500g, 100g, 25g
- Feed materials investigated ?
- Level of sampling in the chain
- Sensitivity of the method ?

Slide 10

Poultry

- Objectives
  - estimation of exposure to human
  - estimation of prevalence
    - rough estimate of prevalence
    - determination of the baseline (for control measures)
    - to set targets for each MS
    - adaption of the sampling size
  - measurement of the impact of control activities

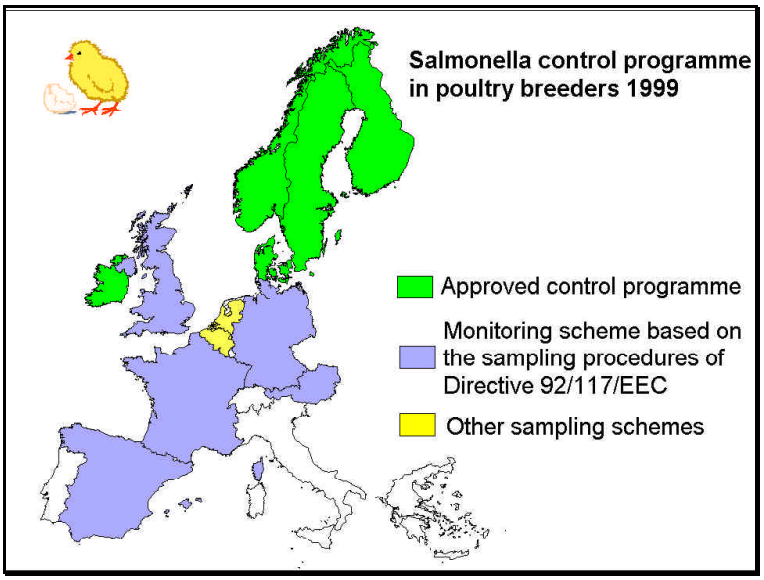
Slide 11

Breeding flocks

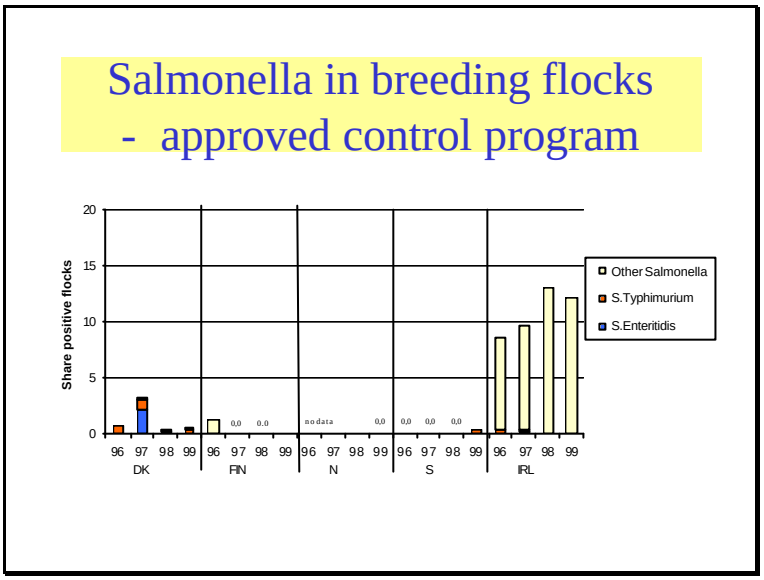
Grouping of countries

- Countries running an approved Salmonella control programme in poultry breeders
- Countries running a monitoring or control programme using a scheme based on the sampling procedure in Dir.92/117/EEC
- Countries running a monitoring or control programme using a scheme different from the sampling procedure in Dir.92/117/EEC
- Other countries

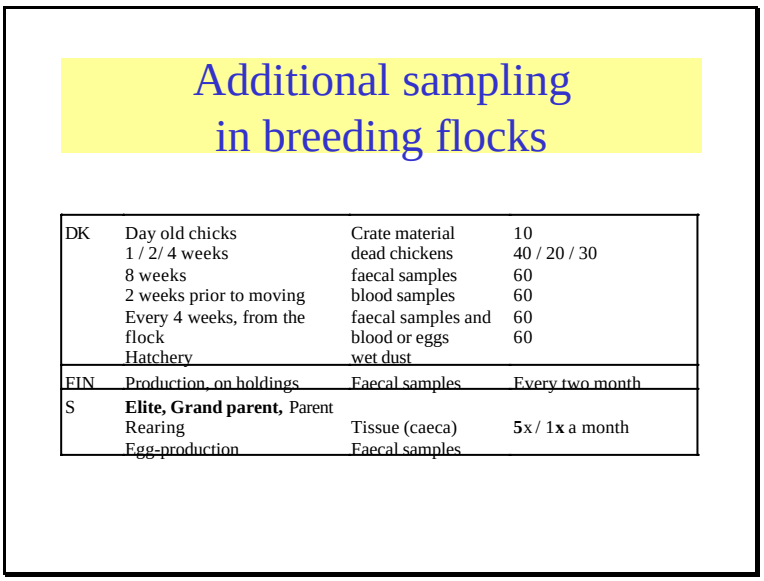
Slide 12



Slide 13

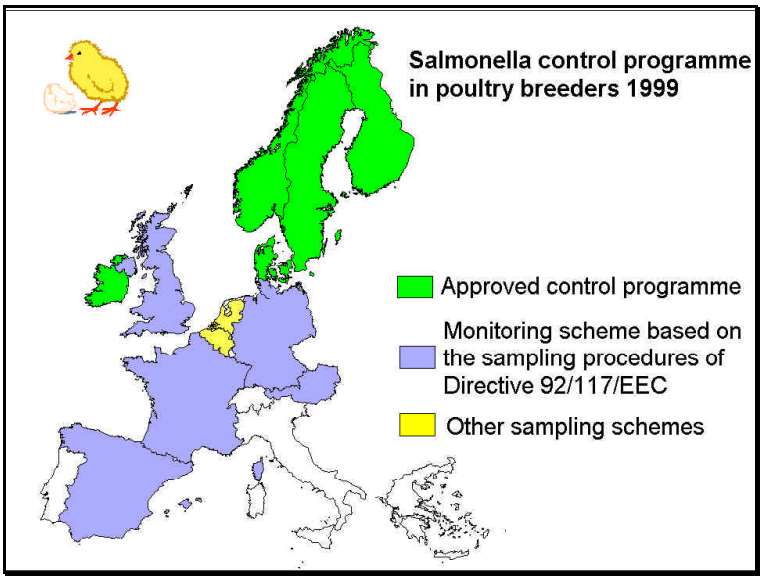


Slide 14

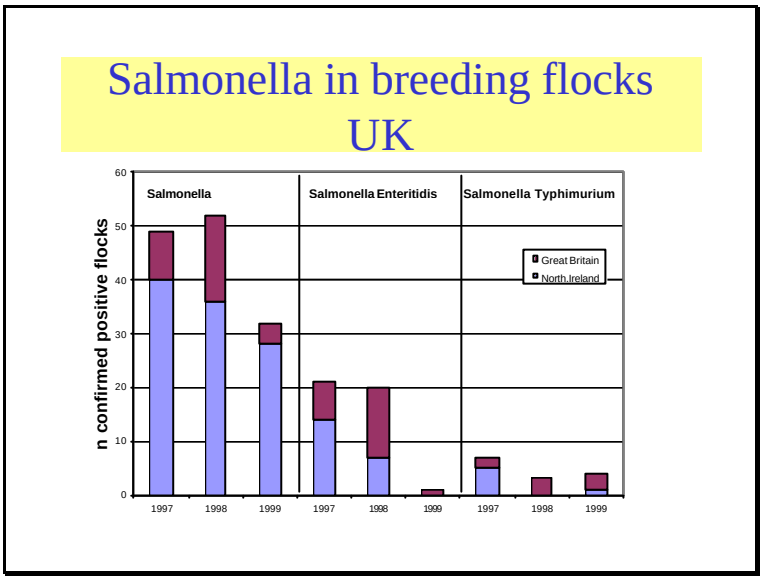


DK	Day old chicks	Crate material	10
	1 / 2/ 4 weeks	dead chickens	40 / 20 / 30
	8 weeks	faecal samples	60
	2 weeks prior to moving	blood samples	60
	Every 4 weeks, from the flock	faecal samples and blood or eggs	60
	Hatchery	wet dust	
FIN	Production, on holdings	Faecal samples	Every two month
S	**Elite, Grand parent,** Parent		
	Rearing	Tissue (caeca)	**5**x / 1**x** a month
	Egg-production	Faecal samples	

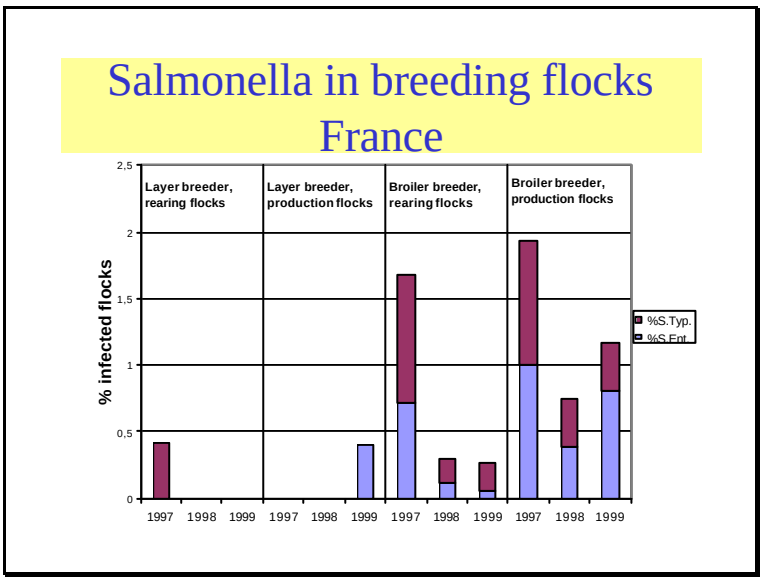
Slide 15



Slide 16

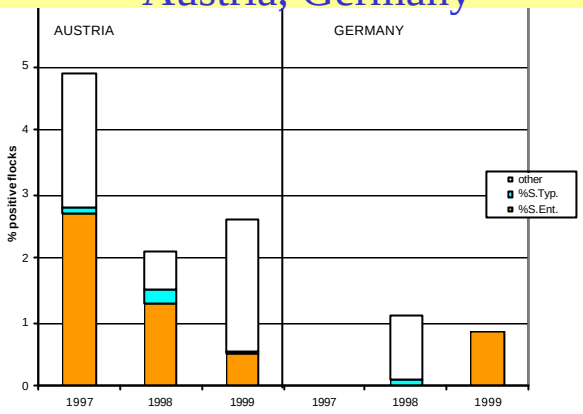


Slide 17



Slide 18

Salmonella in breeding flocks Austria, Germany



Slide 19

Problems with the current data Breeding flocks

- **Flocks investigated**:
  - no. of examinations vs flocks under control?
  - All flocks - rearing period - production period
- **Production level:**
  - all breeders vs parents and grandparents
- **Production type:**
  - all breeders vs egg and meat production line
- **Sensitivity of the method**

Slide 20

Food

- Objectives
  - estimation of exposure to human close to consumer
  - Quantitative data needed for risk assessment

Slide 21

Food: poultry meat

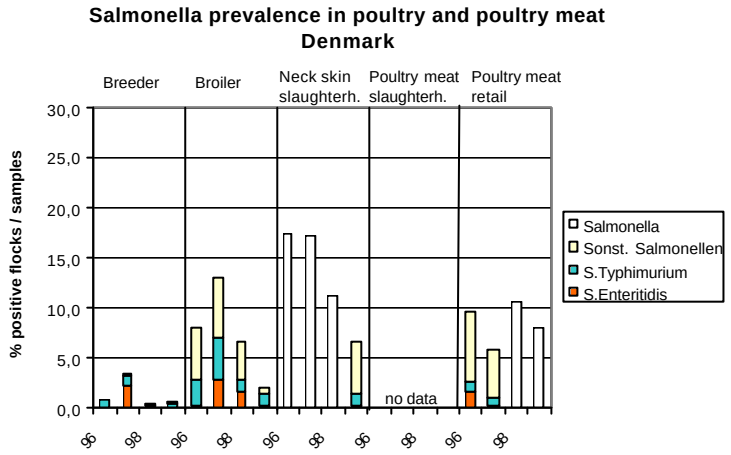
Sources of information

- Official sampling
- Self control system
- Screening programmes
- Voluntary programmes

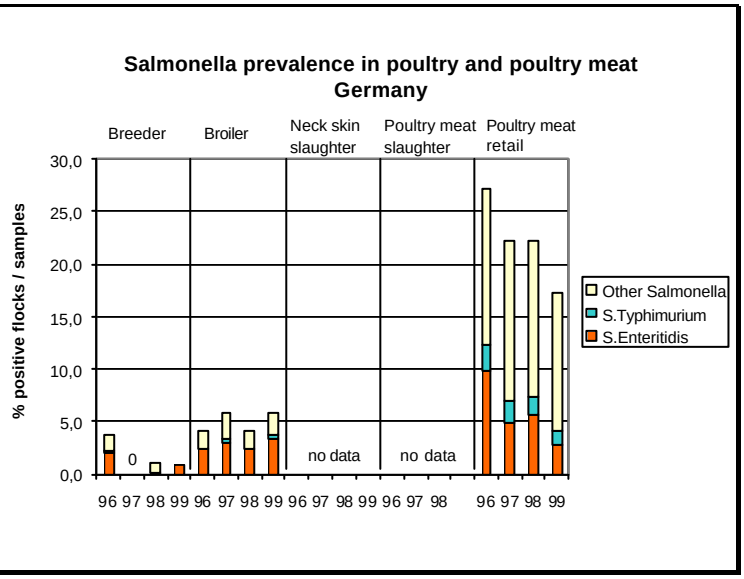
} at slaughterhouses, at cutting plants, at retail level

- Findings are reportable
- Laboratory reports

Slide 22



Slide 23



Slide 24

Antibiotic resistance testing

- **Monitoring frame**
  - 60 isolates of the 5 most important Salmonella serotypes
  - 3 main animal species:cattle, pigs, poultry
  - isolates should be selected in **randomized way** among isolates at NRLs, clustering **is to be avoided**
  - information about whether isolates derive from **active** or **passive** surveillance

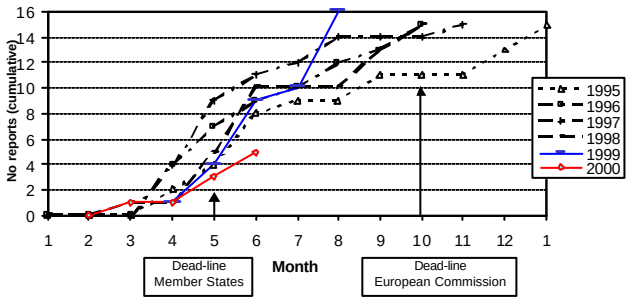
Slide 25

Reports by the Reference Laboratories

– Results of serotyping stratified by main categories of animal species, feedingstuffs and food
– Information on the source of the isolates
– Results of phagetyping
– Results of comparisons of strains using molecular methods

Slide 26

Timeliness of the reports



Slide 27

Summary

- We need a report in time
- MS should provide the information as requested
- There is a need for standardisation and harmonisation of methods
- Sensitivity of the method for different specimen and sampling schemes

Slide 28

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate D - Food Safety: production and distribution chain
D2 - Biological risks

SANCO/1069/2001

TRENDS AND SOURCES OF ZOONOTIC AGENTS

IN ANIMALS, FEEDINGSTUFFS, FOOD AND MAN

IN THE EUROPEAN UNION AND NORWAY

IN 1999

SUMMARY

Based on individual reports submitted by the Member States
pursuant to Article 5 of Council Directive 92/117/EEC

http://europa.eu.int/comm/dgs/health_consumer/library/pub/pub09_en.pdf

Slide 29

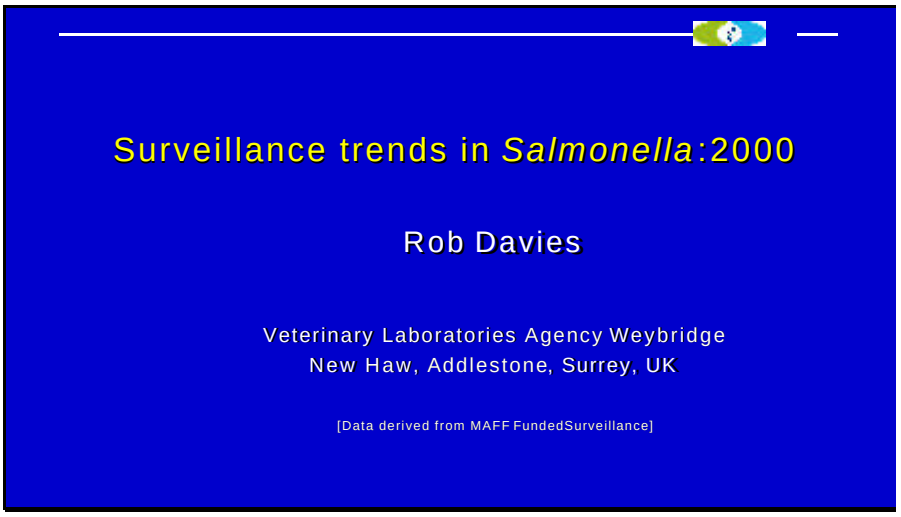
Acknowledgement

We would like to thank all persons/institutions

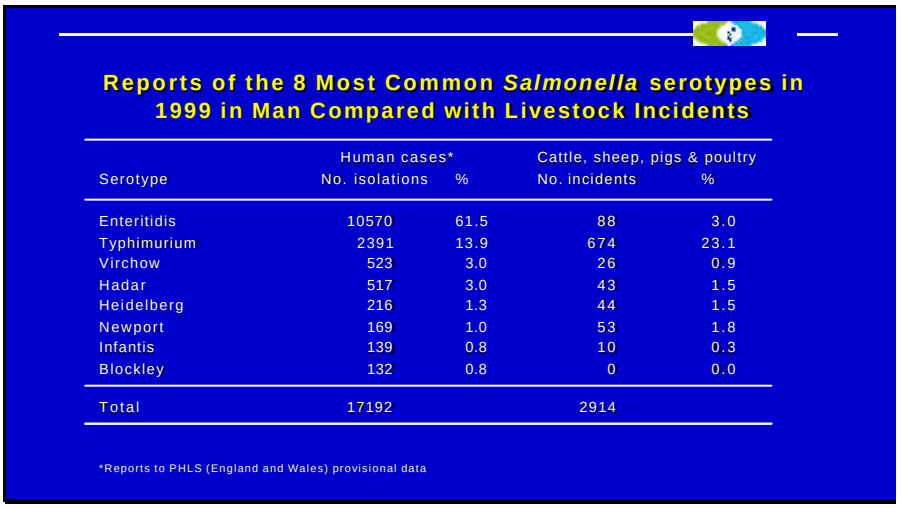
contributing to the National zoonoses reports
and
assisting us in preparing a summary report

Appendix 7. Sheets of presentation 3.1

Slide 1



Slide 2

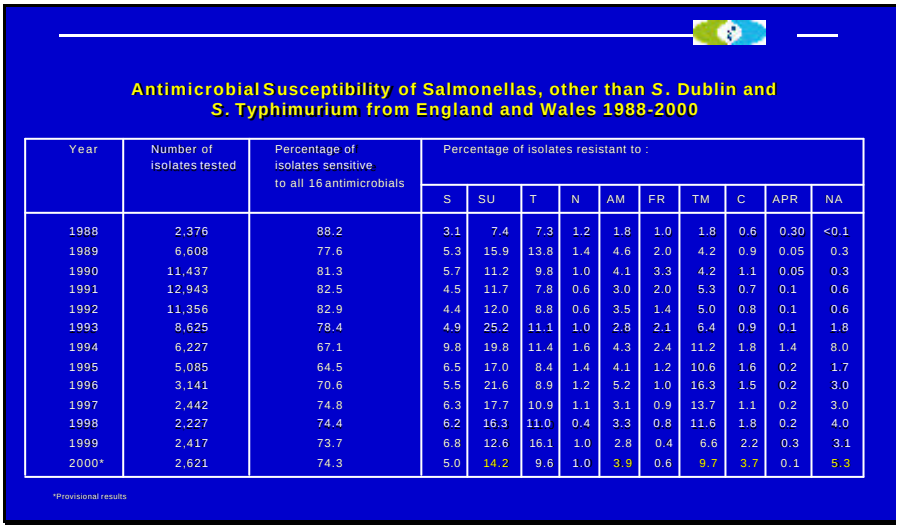


Reports of the 8 Most Common *Salmonella* serotypes in 1999 in Man Compared with Livestock Incidents

Serotype	Human cases* No. isolations	%	Cattle, sheep, pigs & poultry No. incidents	%
Enteritidis	10570	61.5	88	3.0
Typhimurium	2391	13.9	674	23.1
Virchow	523	3.0	26	0.9
Hadar	517	3.0	43	1.5
Heidelberg	216	1.3	44	1.5
Newport	169	1.0	53	1.8
Infantis	139	0.8	10	0.3
Blockley	132	0.8	0	0.0
Total	17192		2914	

*Reports to PHLS (England and Wales) provisional data

Slide 3

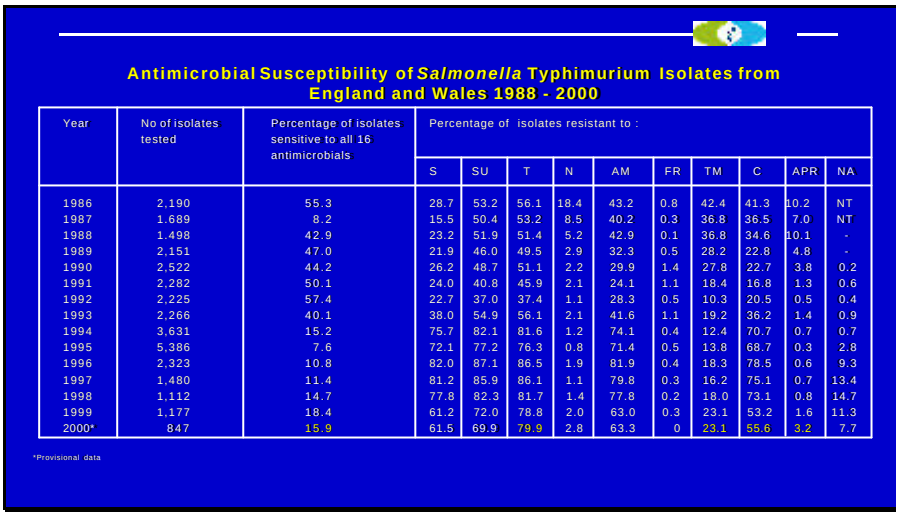


Antimicrobial Susceptibility of Salmonellas, other than S. Dublin and S. Typhimurium from England and Wales 1988-2000

Year	Number of isolates tested	Percentage of isolates sensitive to all 16 antimicrobials	Percentage of isolates resistant to :									
			S	SU	T	N	AM	FR	TM	C	APR	NA
1988	2,376	88.2	3.1	7.4	7.3	1.2	1.8	1.0	1.8	0.6	0.30	<0.1
1989	6,608	77.6	5.3	15.9	13.8	1.4	4.6	2.0	4.2	0.9	0.05	0.3
1990	11,437	81.3	5.7	11.2	9.8	1.0	4.1	3.3	4.2	1.1	0.05	0.3
1991	12,943	82.5	4.5	11.7	7.8	0.6	3.0	2.0	5.3	0.7	0.1	0.6
1992	11,356	82.9	4.4	12.0	8.8	0.6	3.5	1.4	5.0	0.8	0.1	0.6
1993	8,625	78.4	4.9	25.2	11.1	1.0	2.8	2.1	6.4	0.9	0.1	1.8
1994	6,227	67.1	9.8	19.8	11.4	1.6	4.3	2.4	11.2	1.8	1.4	8.0
1995	5,085	64.5	6.5	17.0	8.4	1.4	4.1	1.2	10.6	1.6	0.2	1.7
1996	3,141	70.6	5.5	21.6	8.9	1.2	5.2	1.0	16.3	1.5	0.2	3.0
1997	2,442	74.8	6.3	17.7	10.9	1.1	3.1	0.9	13.7	1.1	0.2	3.0
1998	2,227	74.4	6.2	16.3	11.0	0.4	3.3	0.8	11.6	1.8	0.2	4.0
1999	2,417	73.7	6.8	12.6	16.1	1.0	2.8	0.4	6.6	2.2	0.3	3.1
2000*	2,621	74.3	5.0	14.2	9.6	1.0	3.9	0.6	9.7	3.7	0.1	5.3

*Provisional results

Slide 4

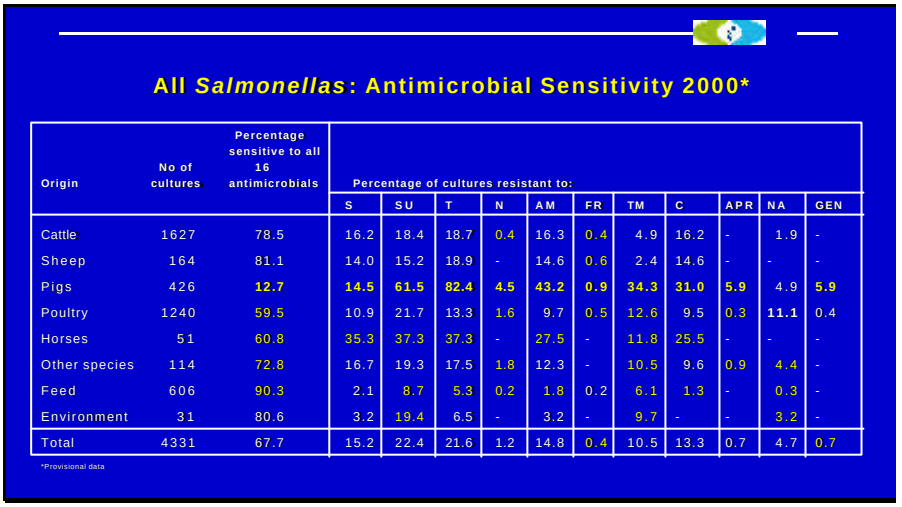


Antimicrobial Susceptibility of *Salmonella* Typhimurium Isolates from England and Wales 1988 - 2000

Year	No of isolates tested	Percentage of isolates sensitive to all 16 antimicrobials	Percentage of isolates resistant to :									
			S	SU	T	N	AM	FR	TM	C	APR	NA
1986	2,190	55.3	28.7	53.2	56.1	18.4	43.2	0.8	42.4	41.3	10.2	NT
1987	1,689	8.2	15.5	50.4	53.2	8.5	40.2	0.3	36.8	36.5	7.0	NT
1988	1,498	42.9	23.2	51.9	51.4	5.2	42.9	0.1	36.8	34.6	10.1	-
1989	2,151	47.0	21.9	46.0	49.5	2.9	32.3	0.5	28.2	22.8	4.8	-
1990	2,522	44.2	26.2	48.7	51.1	2.2	29.9	1.4	27.8	22.7	3.8	0.2
1991	2,282	50.1	24.0	40.8	45.9	2.1	24.1	1.1	18.4	16.8	1.3	0.6
1992	2,225	57.4	22.7	37.0	37.4	1.1	28.3	0.5	10.3	20.5	0.5	0.4
1993	2,266	40.1	38.0	54.9	56.1	2.1	41.6	1.1	19.2	36.2	1.4	0.9
1994	3,631	15.2	75.7	82.1	81.6	1.2	74.1	0.4	12.4	70.7	0.7	0.7
1995	5,386	7.6	72.1	77.2	76.3	0.8	71.4	0.5	13.8	68.7	0.3	2.8
1996	2,323	10.8	82.0	87.1	86.5	1.9	81.9	0.4	18.3	78.5	0.6	9.3
1997	1,480	11.4	81.2	85.9	86.1	1.1	79.8	0.3	16.2	75.1	0.7	13.4
1998	1,112	14.7	77.8	82.3	81.7	1.4	77.8	0.2	18.0	73.1	0.8	14.7
1999	1,177	18.4	61.2	72.0	78.8	2.0	63.0	0.3	23.1	53.2	1.6	11.3
2000*	847	15.9	61.5	69.9	79.9	2.8	63.3	0	23.1	55.6	3.2	7.7

*Provisional data

Slide 5

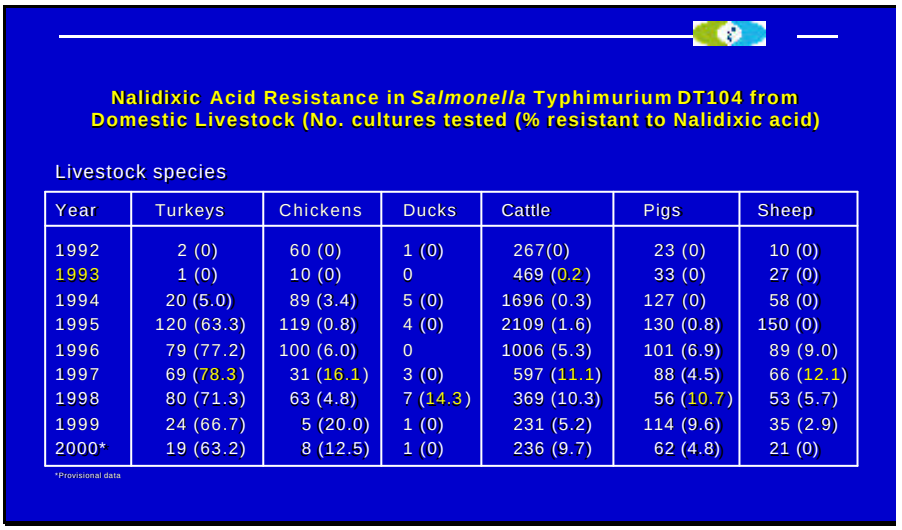


All *Salmonellas*: Antimicrobial Sensitivity 2000*

Origin	No of cultures	Percentage sensitive to all 16 antimicrobials	Percentage of cultures resistant to:										
			S	SU	T	N	AM	FR	TM	C	APR	NA	GEN
Cattle	1627	78.5	16.2	18.4	18.7	0.4	16.3	0.4	4.9	16.2	-	1.9	-
Sheep	164	81.1	14.0	15.2	18.9	-	14.6	0.6	2.4	14.6	-	-	-
Pigs	426	**12.7**	**14.5**	**61.5**	**82.4**	**4.5**	**43.2**	**0.9**	**34.3**	**31.0**	**5.9**	4.9	**5.9**
Poultry	1240	59.5	10.9	21.7	13.3	1.6	9.7	0.5	12.6	9.5	0.3	**11.1**	0.4
Horses	51	60.8	35.3	37.3	37.3	-	27.5	-	11.8	25.5	-	-	-
Other species	114	72.8	16.7	19.3	17.5	1.8	12.3	-	10.5	9.6	0.9	4.4	-
Feed	606	90.3	2.1	8.7	5.3	0.2	1.8	0.2	6.1	1.3	-	0.3	-
Environment	31	80.6	3.2	19.4	6.5	-	3.2	-	9.7	-	-	3.2	-
Total	4331	67.7	15.2	22.4	21.6	1.2	14.8	0.4	10.5	13.3	0.7	4.7	0.7

*Provisional data

Slide 6



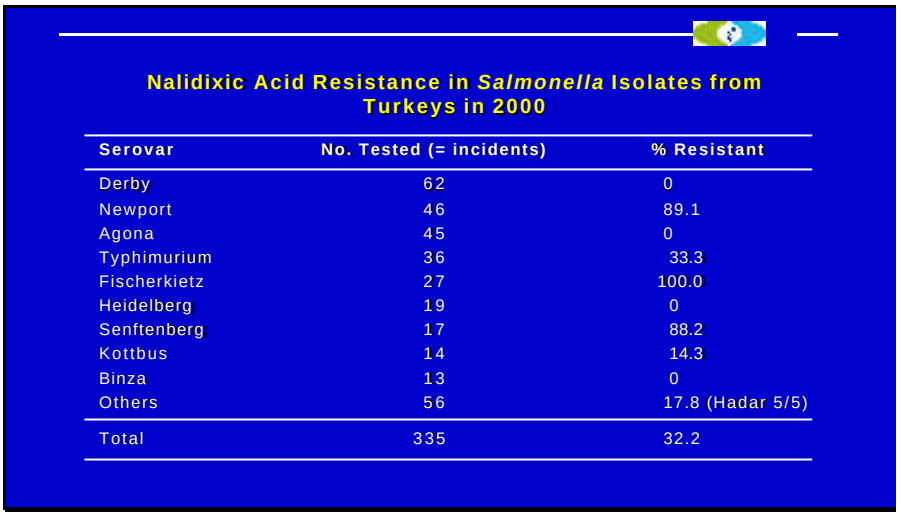
Nalidixic Acid Resistance in *Salmonella* Typhimurium DT104 from Domestic Livestock (No. cultures tested (% resistant to Nalidixic acid)

Livestock species

Year	Turkeys	Chickens	Ducks	Cattle	Pigs	Sheep
1992	2 (0)	60 (0)	1 (0)	267(0)	23 (0)	10 (0)
1993	1 (0)	10 (0)	0	469 (0.2)	33 (0)	27 (0)
1994	20 (5.0)	89 (3.4)	5 (0)	1696 (0.3)	127 (0)	58 (0)
1995	120 (63.3)	119 (0.8)	4 (0)	2109 (1.6)	130 (0.8)	150 (0)
1996	79 (77.2)	100 (6.0)	0	1006 (5.3)	101 (6.9)	89 (9.0)
1997	69 (78.3)	31 (16.1)	3 (0)	597 (11.1)	88 (4.5)	66 (12.1)
1998	80 (71.3)	63 (4.8)	7 (14.3)	369 (10.3)	56 (10.7)	53 (5.7)
1999	24 (66.7)	5 (20.0)	1 (0)	231 (5.2)	114 (9.6)	35 (2.9)
2000*	19 (63.2)	8 (12.5)	1 (0)	236 (9.7)	62 (4.8)	21 (0)

*Provisional data

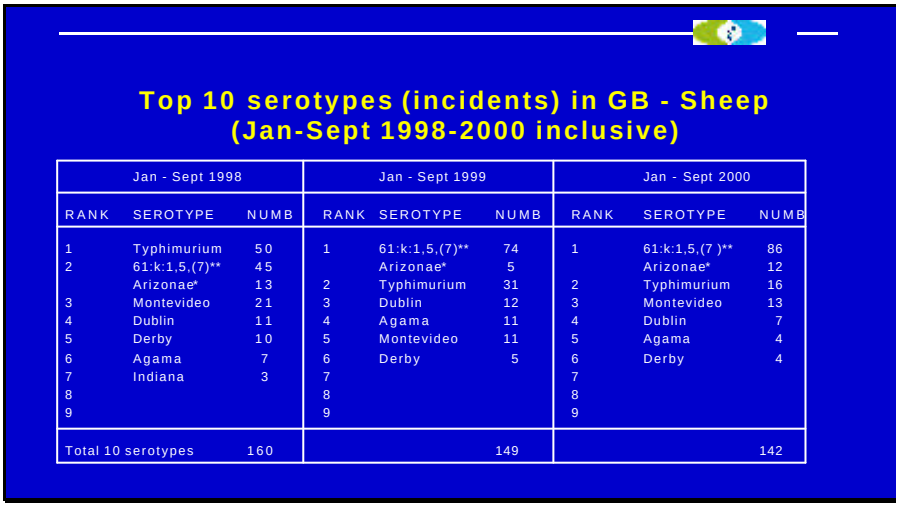
Slide 7



Nalidixic Acid Resistance in *Salmonella* Isolates from Turkeys in 2000

Serovar	No. Tested (= incidents)	% Resistant
Derby	62	0
Newport	46	89.1
Agona	45	0
Typhimurium	36	33.3
Fischerkietz	27	100.0
Heidelberg	19	0
Senftenberg	17	88.2
Kottbus	14	14.3
Binza	13	0
Others	56	17.8 (Hadar 5/5)
Total	335	32.2

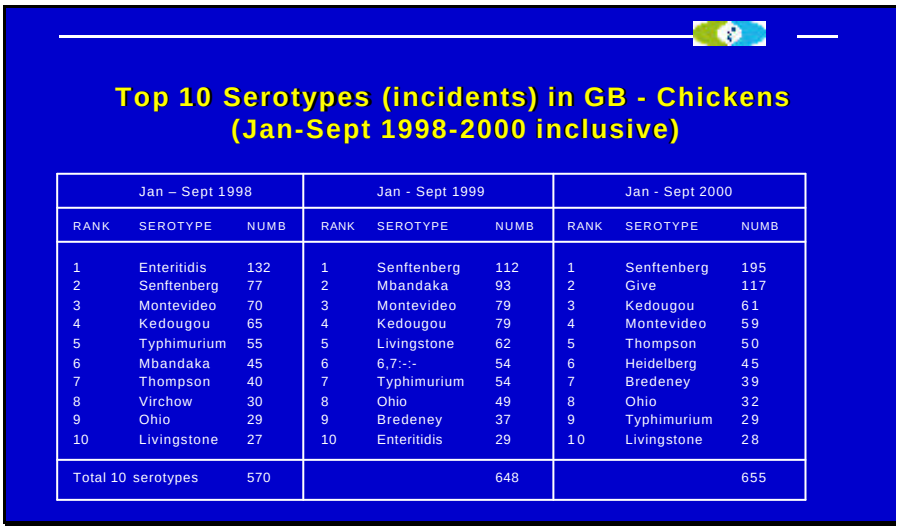
Slide 8



Top 10 serotypes (incidents) in GB - Sheep (Jan-Sept 1998-2000 inclusive)

Jan - Sept 1998			Jan - Sept 1999			Jan - Sept 2000		
RANK	SEROTYPE	NUMB	RANK	SEROTYPE	NUMB	RANK	SEROTYPE	NUMB
1	Typhimurium	50	1	61:k:1,5,(7)**	74	1	61:k:1,5,(7)**	86
2	61:k:1,5,(7)**	45		Arizonae*	5		Arizonae*	12
	Arizonae*	13	2	Typhimurium	31	2	Typhimurium	16
3	Montevideo	21	3	Dublin	12	3	Montevideo	13
4	Dublin	11	4	Agama	11	4	Dublin	7
5	Derby	10	5	Montevideo	11	5	Agama	4
6	Agama	7	6	Derby	5	6	Derby	4
7	Indiana	3	7			7		
8			8			8		
9			9			9		
Total 10 serotypes		160			149			142

Slide 9

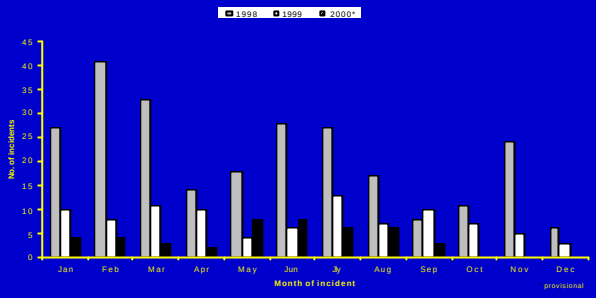


Top 10 Serotypes (incidents) in GB - Chickens (Jan-Sept 1998-2000 inclusive)

Jan – Sept 1998			Jan - Sept 1999			Jan - Sept 2000		
RANK	SEROTYPE	NUMB	RANK	SEROTYPE	NUMB	RANK	SEROTYPE	NUMB
1	Enteritidis	132	1	Senftenberg	112	1	Senftenberg	195
2	Senftenberg	77	2	Mbandaka	93	2	Give	117
3	Montevideo	70	3	Montevideo	79	3	Kedougou	61
4	Kedougou	65	4	Kedougou	79	4	Montevideo	59
5	Typhimurium	55	5	Livingstone	62	5	Thompson	50
6	Mbandaka	45	6	6,7:-:-	54	6	Heidelberg	45
7	Thompson	40	7	Typhimurium	54	7	Bredeney	39
8	Virchow	30	8	Ohio	49	8	Ohio	32
9	Ohio	29	9	Bredeney	37	9	Typhimurium	29
10	Livingstone	27	10	Enteritidis	29	10	Livingstone	28
Total 10 serotypes		570			648			655

Slide 10

All incidents of *S.* Enteritidis in all species in GB 1998 - 2000



Slide 11

Isolations of *S.*Enteritidis & *S.*Typhimurium from all Feedingstuffs and Feed Ingredients being Monitored Under MAFF Codes of Practice (January to September 2000)

Type of material	1993		1994		1995		1996		1997		1998		1999		2000*	
	Se	St	Se	St	Se	St	Se	St	Se	St	Se	St	Se	St	Se	St
Finished feeds	5	9	4	25	2	20	0	18	2	7	0	8	7	0	0	8
Animal protein	0	1	0	4	0	1	0	10	0	2	0	0	0	1	0	2
Vegetable material	7	15	1	6	4	10	5	6	0	9	0	9	1	9	1	3
Minerals	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous	2	1	0	4	1	5	1	2	1	6	2	3	1	1	1	3
TOTALS	14	26	5	39	7	36	6	36	3	24	2	20	2	18	2	16

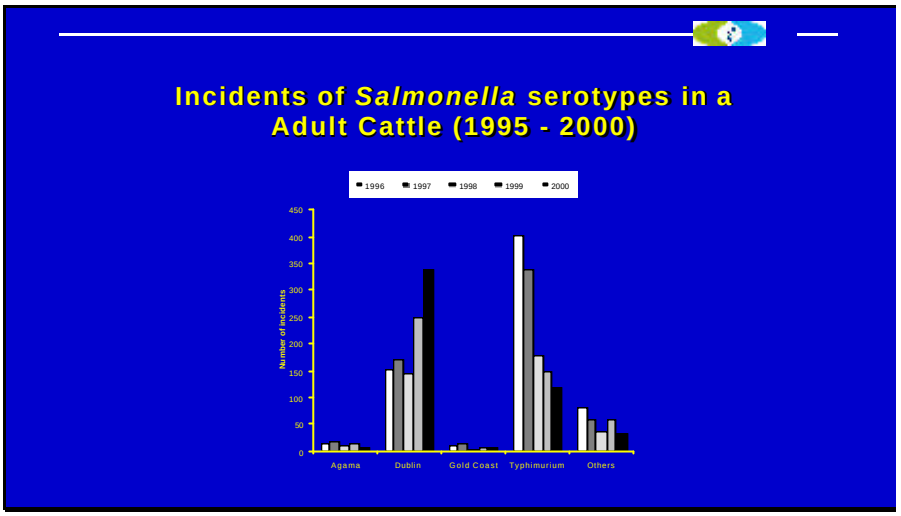
*Figures for 2000 cover the period January to September 2000

Slide 12

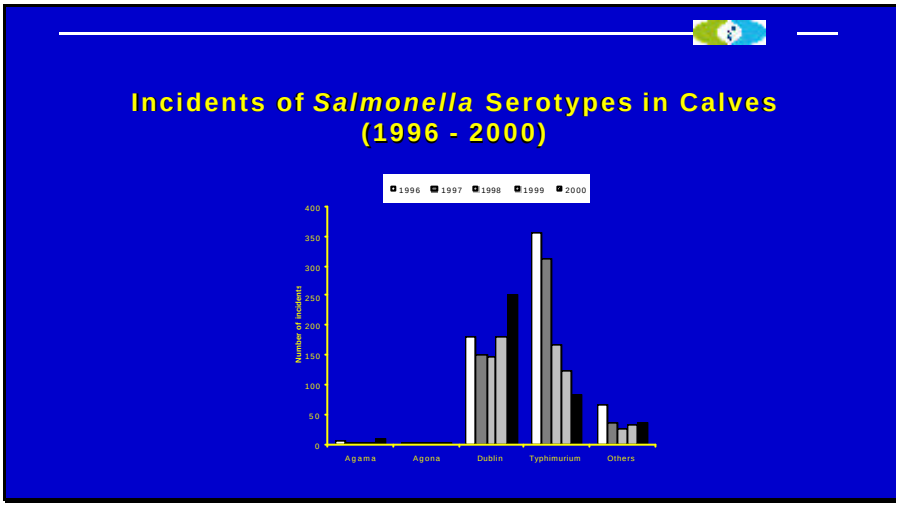
Isolations of *S.* Enteritidis and *S.* Typhimurium from products monitored under the MAFF Codes of Practice, 2000

Salmonella serotype	Feedingstuff	Number
Enteritidis	barley	1
	other feedingstuff	1
Typhimurium	cattle compound feed	2
	poultry compound feed	4
	unspecified compound feed	4
	fishmeal	2
	soya	3
	wheat	1
	other feedingstuff	1
	feedmill environment	2

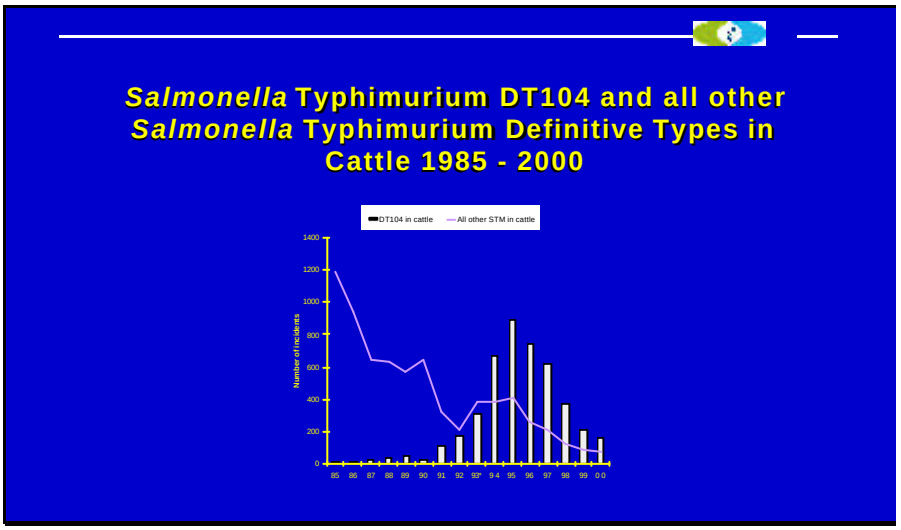
Slide 13



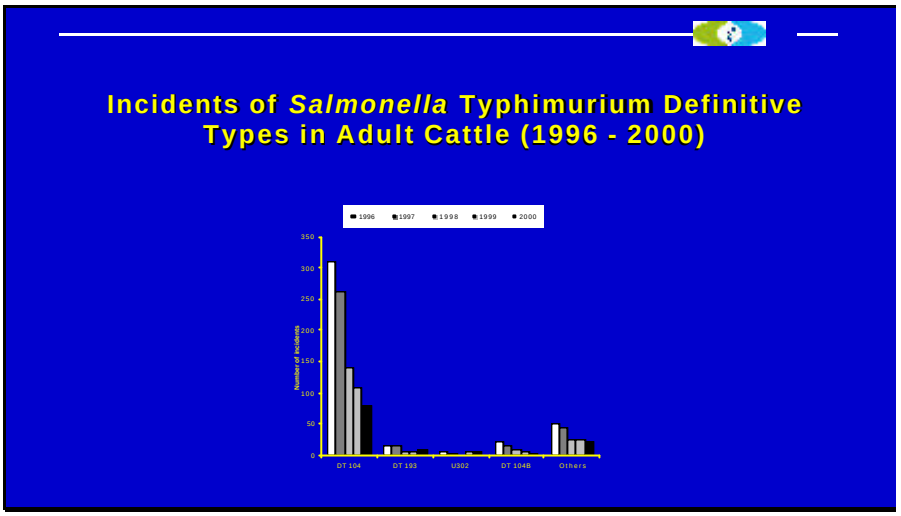
Slide 14



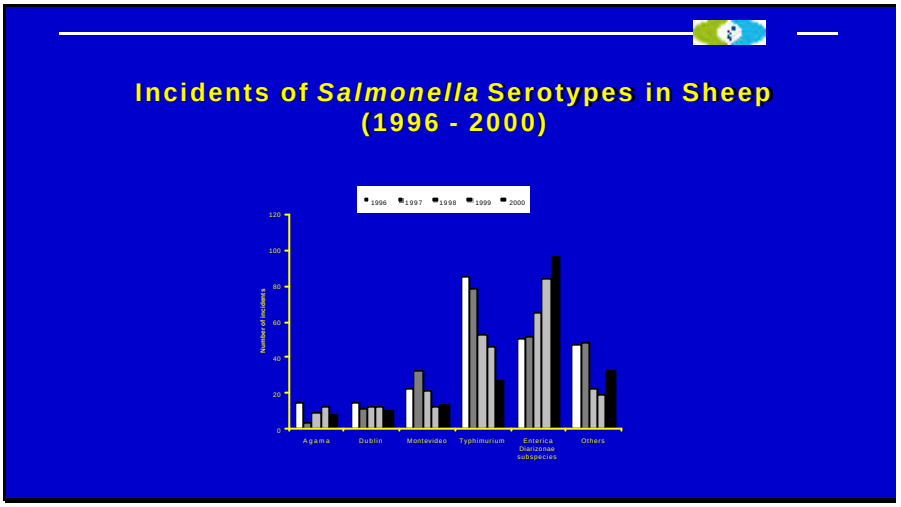
Slide 15



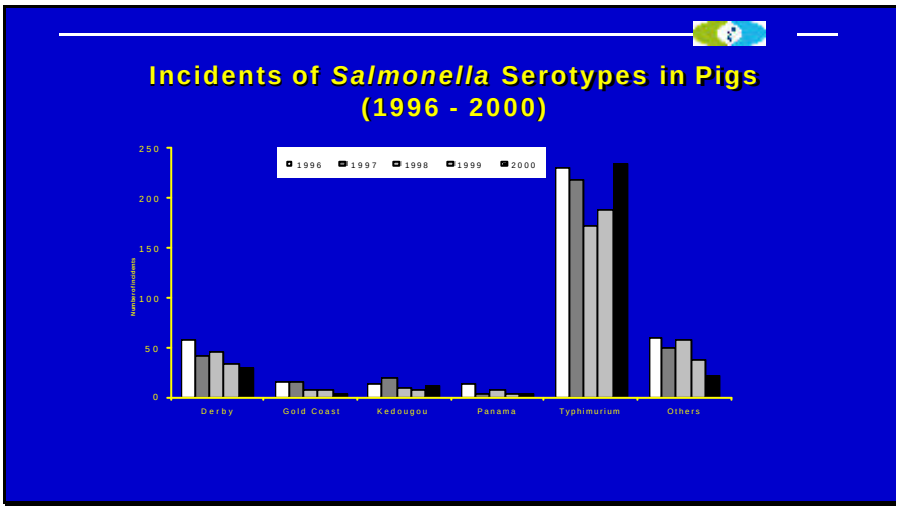
Slide 16



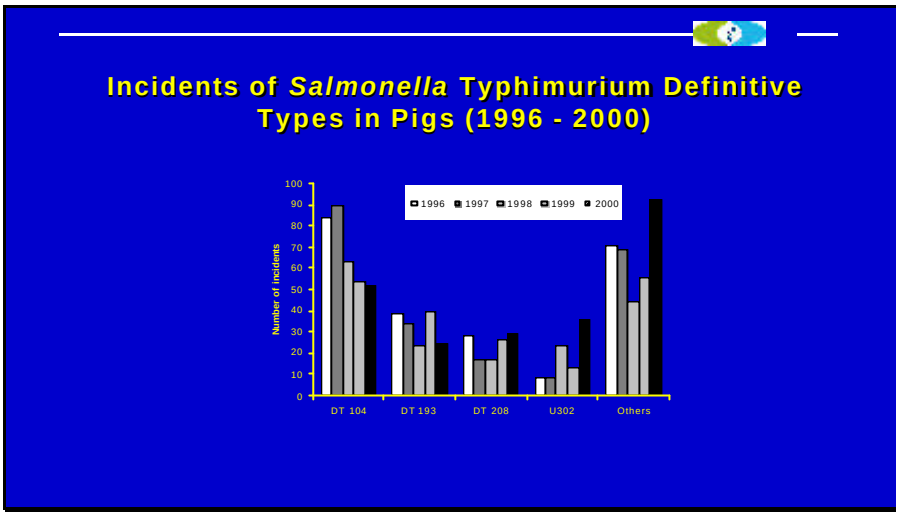
Slide 17



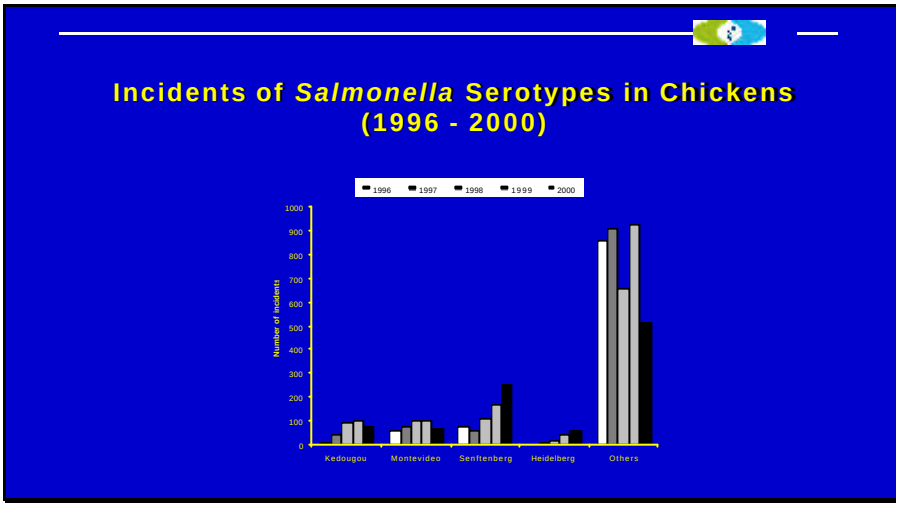
Slide 18



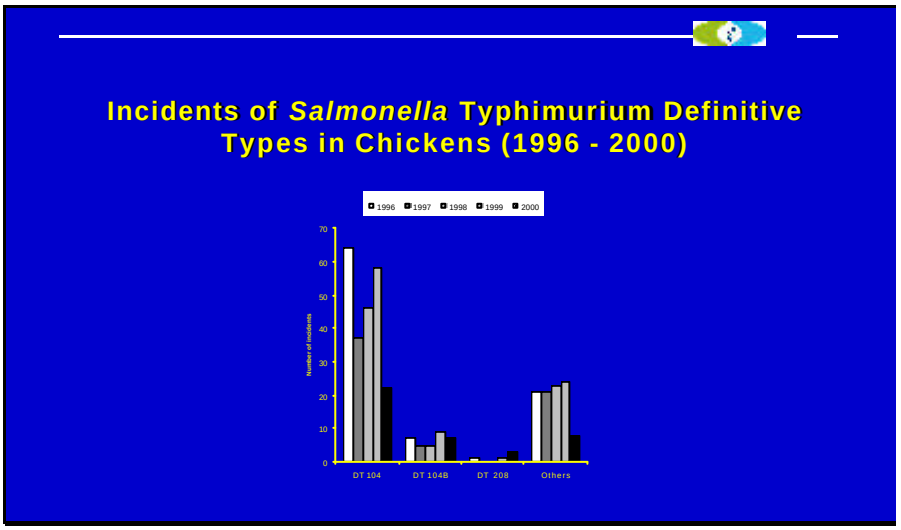
Slide 19



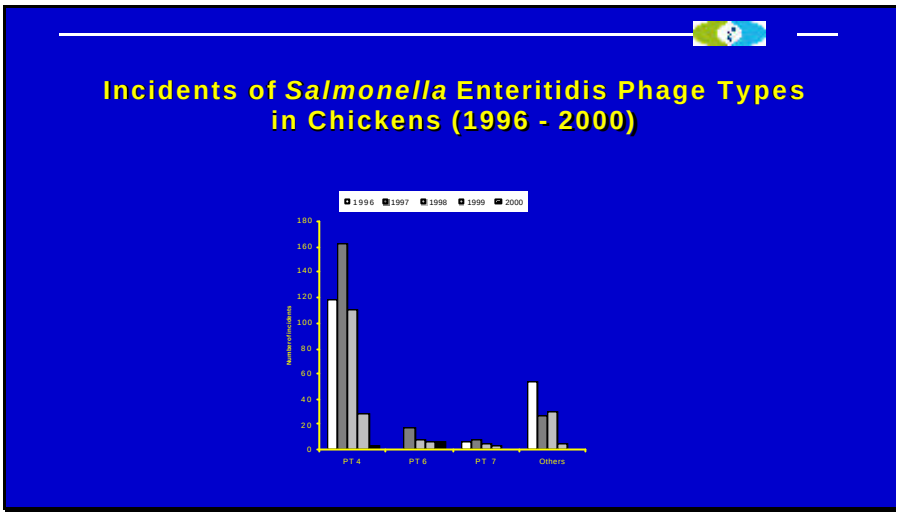
Slide 20



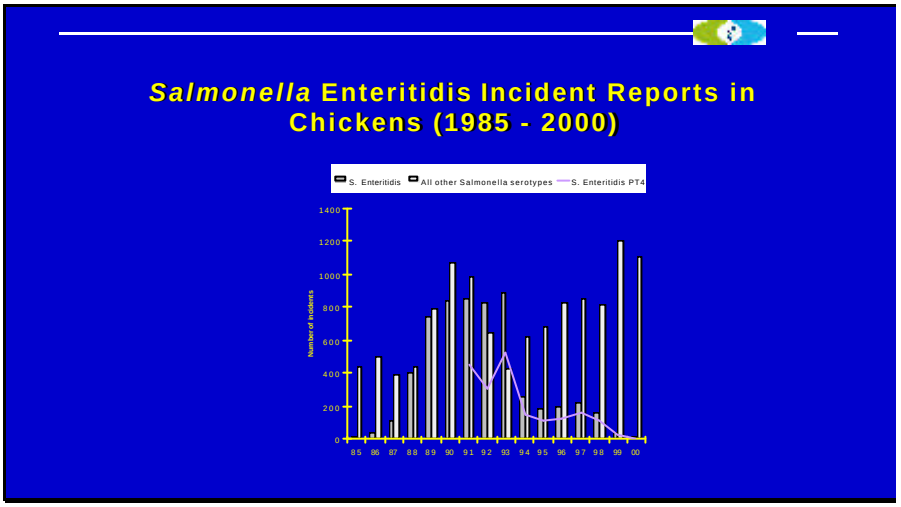
Slide 21



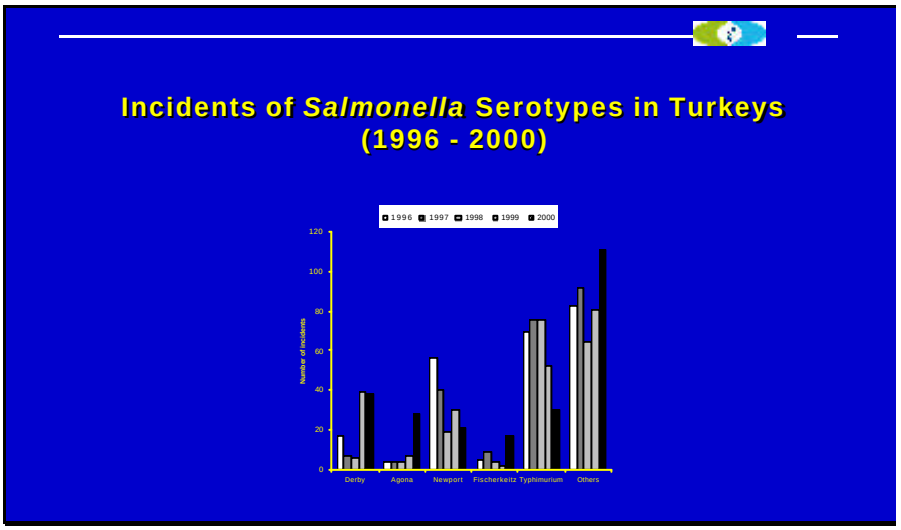
Slide 22



Slide 23

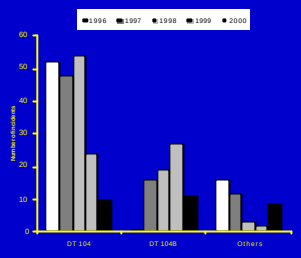


Slide 24



Slide 25

Incidents of *Salmonella* Typhimurium Definitive Types in Turkeys (1996 - 2000)



Slide 26

Incidents of *Salmonella* in Livestock 2000 (1999)

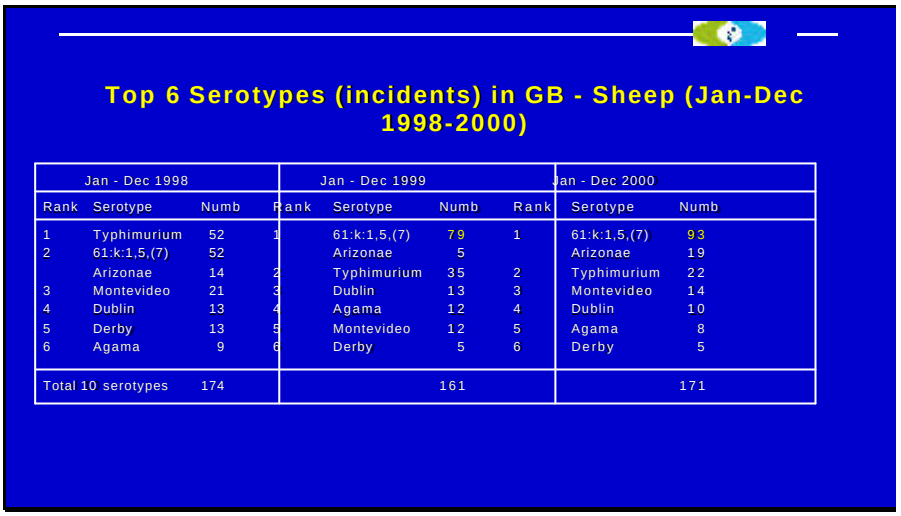
	*S.*Enteritidis	*S.*Typhimurium	Other Serovars
Broiler breeders	1 (14)	3 (11)	64 (77)
Layer breeders	2 (5)	1 (0)	13 (29)
Layers	6 (7)	0 (3)	5 (5)
Unknown fowl types	0 (0)	0 (0)	0 (4)
Broilers	2 (11)	31 (65)	978 (1120)
Turkeys	0 (4)	23 (47)	217 (149)
Ducks	13 (21)	9 (6)	11 (21)
Geese	5 (0)	2 (1)	3 (2)
Other birds	4 (7)	14 (15)	51 (85)
Total Poultry	32 (69)	83 (148)	1342 (1492)
Cattle	9 (6)	230 (264)	721 (562)
Sheep	0 (2)	27 (35)	161 (138)
Pigs	0 (1)	235 (159)	68 (84)
Goats	0 (0)	1 (2)	2 (3)
Horses	4 (6)	31 (18)	15 (10)
Other	7 (9)	28 (40)	70 (62)
Total	53 (94)	671 (711)	2381 (2308)

Slide 27

Top 10 Serotypes (incidents) in GB - Cattle (Jan-Dec 1998-2000)

Jan - Dec 1998			Jan - Dec 1999			Jan - Dec 2000		
Rank	Serotype	Numb	Rank	Serotype	Numb	Rank	Serotype	Numb
1	Typhimurium	431	1	Dublin	473	1	Dublin	632
2	Dublin	391	2	Typhimurium	265	2	Typhimurium	211
3	Enteritidis	20	3	Agama	20	3	Agama	19
4	Agama	15	4	Enteritidis	8	4	Gold Coast	11
5	Gold Coast	6	5	Newport	7	5	Enteritidis	9
6	Newport	6	6	Gold Coast	7	6	Newport	8
7	Anatum	6	7	Brandenburg	6	7	Agona	5
8	Mbandaka	5	8	Mbandaka	6	8	Montevideo	4
9	Thompson	4	9	Agona	6	9	Give	4
10	O-rough	4	10	Anatum	6	10	9,12:-:-	4
Total 10 serotypes		888			804			907

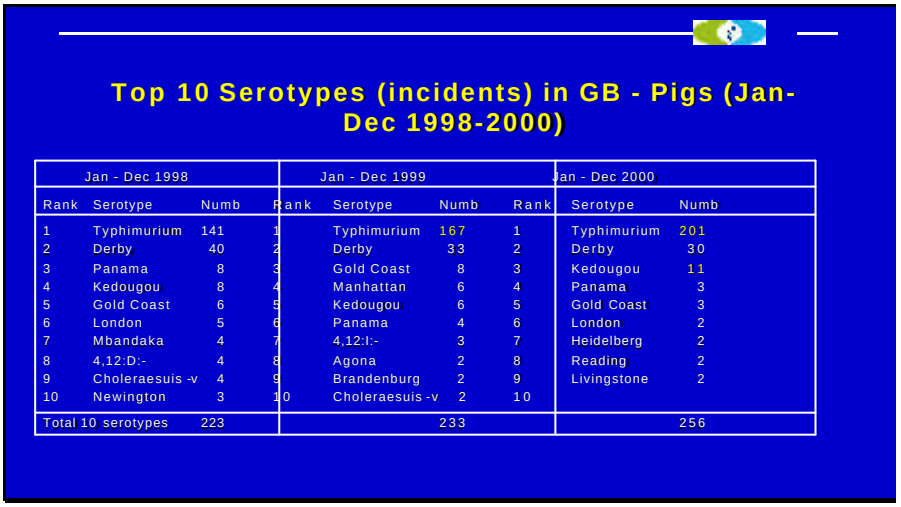
Slide 28



Top 6 Serotypes (incidents) in GB - Sheep (Jan-Dec 1998-2000)

Jan - Dec 1998			Jan - Dec 1999			Jan - Dec 2000		
Rank	Serotype	Numb	Rank	Serotype	Numb	Rank	Serotype	Numb
1	Typhimurium	52	1	61:k:1,5,(7)	79	1	61:k:1,5,(7)	93
2	61:k:1,5,(7)	52		Arizonae	5		Arizonae	19
	Arizonae	14	2	Typhimurium	35	2	Typhimurium	22
3	Montevideo	21	3	Dublin	13	3	Montevideo	14
4	Dublin	13	4	Agama	12	4	Dublin	10
5	Derby	13	5	Montevideo	12	5	Agama	8
6	Agama	9	6	Derby	5	6	Derby	5
Total 10 serotypes		174			161			171

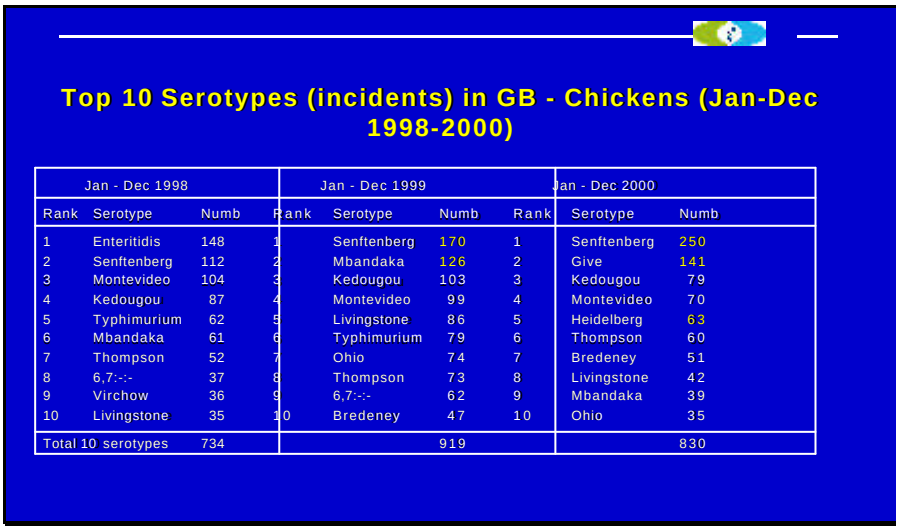
Slide 29



Top 10 Serotypes (incidents) in GB - Pigs (Jan-Dec 1998-2000)

Jan - Dec 1998			Jan - Dec 1999			Jan - Dec 2000		
Rank	Serotype	Numb	Rank	Serotype	Numb	Rank	Serotype	Numb
1	Typhimurium	141	1	Typhimurium	167	1	Typhimurium	201
2	Derby	40	2	Derby	33	2	Derby	30
3	Panama	8	3	Gold Coast	8	3	Kedougou	11
4	Kedougou	8	4	Manhattan	6	4	Panama	3
5	Gold Coast	6	5	Kedougou	6	5	Gold Coast	3
6	London	5	6	Panama	4	6	London	2
7	Mbandaka	4	7	4,12:l:-	3	7	Heidelberg	2
8	4,12:D:-	4	8	Agona	2	8	Reading	2
9	Choleraesuis -v	4	9	Brandenburg	2	9	Livingstone	2
10	Newington	3	10	Choleraesuis -v	2	10		
Total 10 serotypes		223			233			256

Slide 30



Top 10 Serotypes (incidents) in GB - Chickens (Jan-Dec 1998-2000)

Jan - Dec 1998			Jan - Dec 1999			Jan - Dec 2000		
Rank	Serotype	Numb	Rank	Serotype	Numb	Rank	Serotype	Numb
1	Enteritidis	148	1	Senftenberg	170	1	Senftenberg	250
2	Senftenberg	112	2	Mbandaka	126	2	Give	141
3	Montevideo	104	3	Kedougou	103	3	Kedougou	79
4	Kedougou	87	4	Montevideo	99	4	Montevideo	70
5	Typhimurium	62	5	Livingstone	86	5	Heidelberg	63
6	Mbandaka	61	6	Typhimurium	79	6	Thompson	60
7	Thompson	52	7	Ohio	74	7	Bredeney	51
8	6,7:-:-	37	8	Thompson	73	8	Livingstone	42
9	Virchow	36	9	6,7:-:-	62	9	Mbandaka	39
10	Livingstone	35	10	Bredeney	47	10	Ohio	35
Total 10 serotypes		734			919			830

Slide 31

Top 10 Serotypes (incidents) in GB - Turkeys (Jan-Dec 1998-2000)

Jan - Dec 1998			Jan - Dec 1999			Jan - Dec 2000	
Rank	Serotype	Numb	Rank	Serotype	Numb	Rank	Serotype	Numb
1	Typhimurium	67	1	Typhimurium	46	1	Derby	38
2	Newport	19	2	Derby	39	2	Agona	28
3	Senftenberg	16	3	Newport	30	3	Typhimurium	23
4	Indiana	15	4	Senftenberg	17	4	Newport	21
5	Enteritidis	10	5	Indiana	12	5	Fischerkeitz	17
6	Derby	6	6	Binza	8	6	Binza	15
7	Binza	5	7	Agona	7	7	Montevideo	14
8	Agona	4	8	Kottbus	6	8	Senftenberg	11
9	Fischerkeitz	4	9	Heidelberg	5	9	Kottbus	10
10	Saint Paul	3	10	Enteritidis	4	10	Indiana	9
Total 10 serotypes		149			174			186

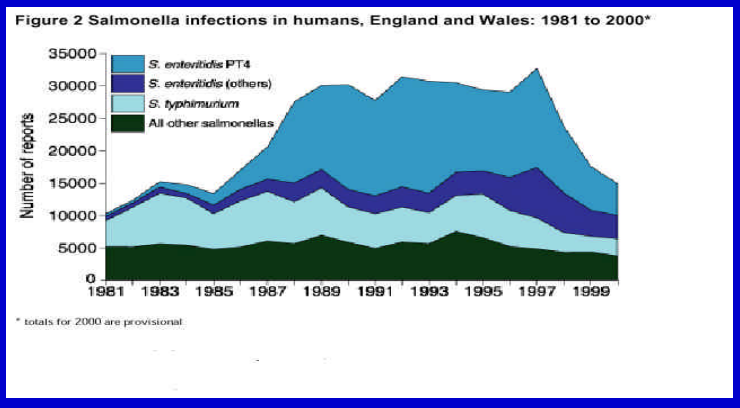
Slide 32

Other Developments in *Salmonella* Surveillance/Control

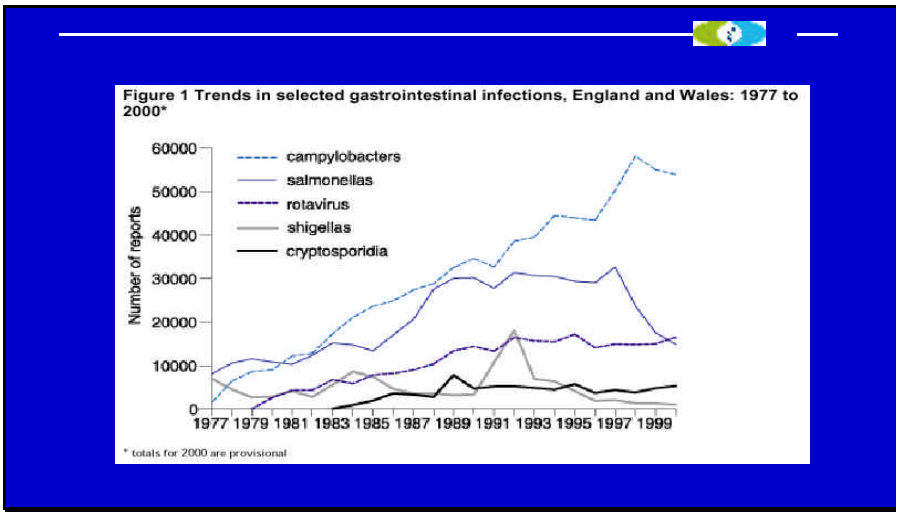
- 2000 killed combined SE/STM vaccine licenced
  live SE vaccine licenced
- 2000 Completion of pig, cattle and sheep slaughter surveys
  Pig Code of Practice
  Retailer/Industry Monitoring schemes
- 2000 FSA targets : 50% reduction in poultry meat contamination in 5 years
  20% reduction in human food poisoning cases in 5 years
- ? EU monitoring and prevalence targets

[RUMA, BVA, Species Groups etc. codes for antimicrobial use in livestock]

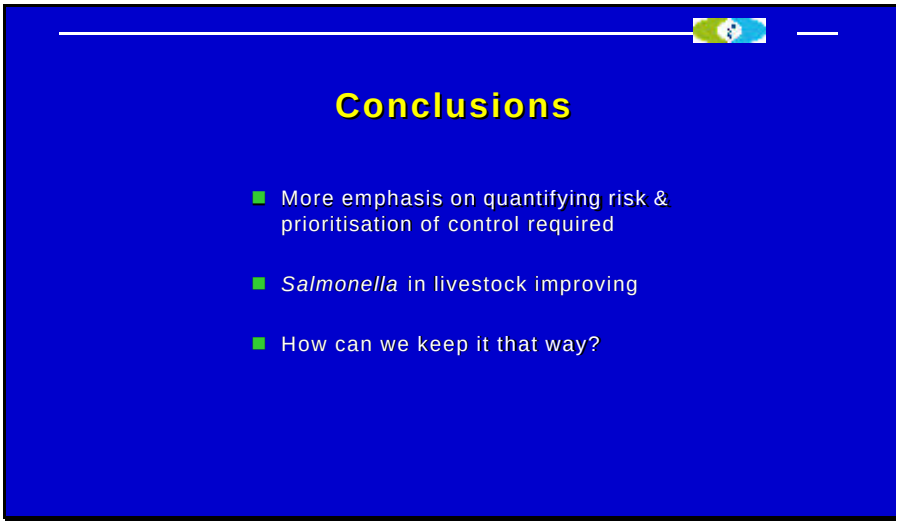
Slide 33



Slide 34

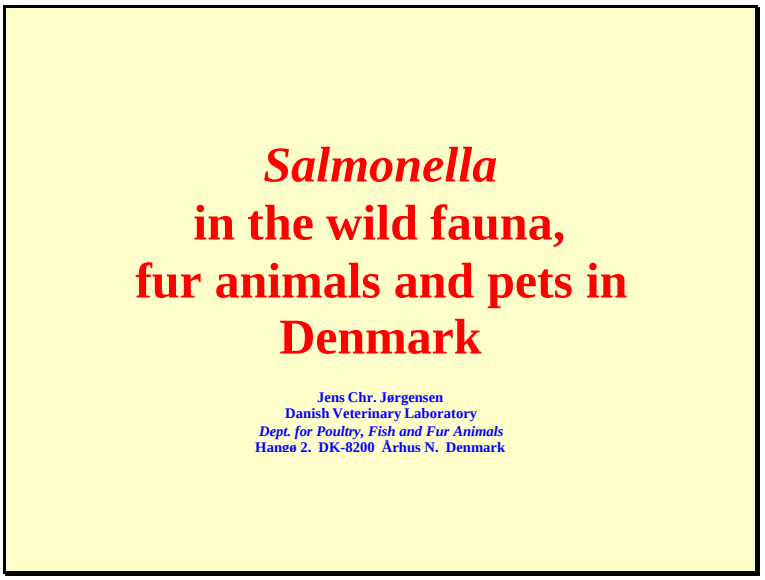


Slide 35

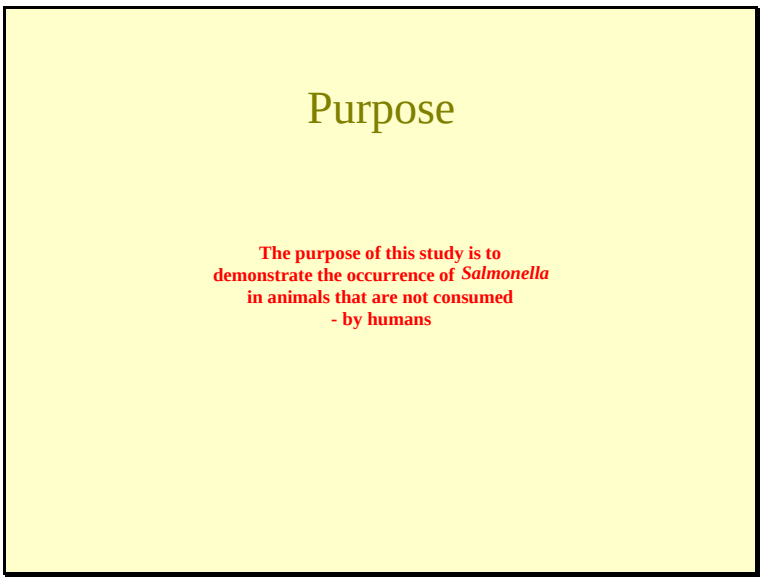


Appendix 8. Sheets of presentation 3.2

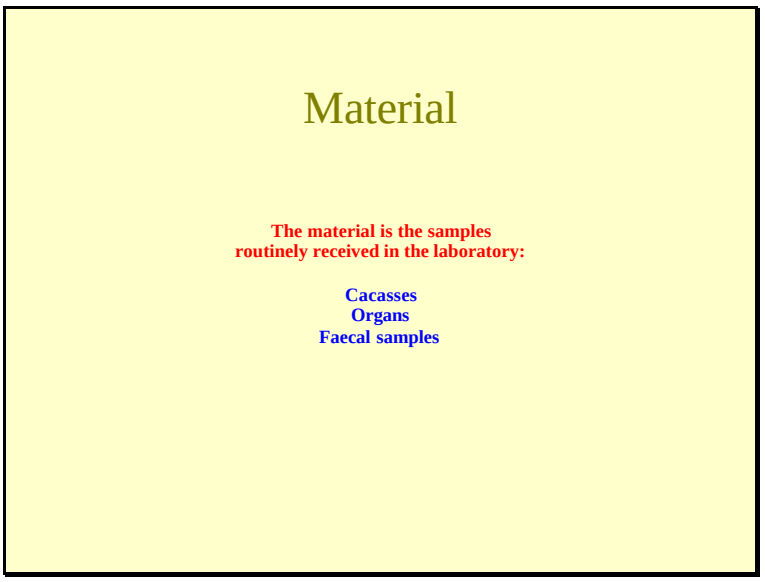
Slide 1



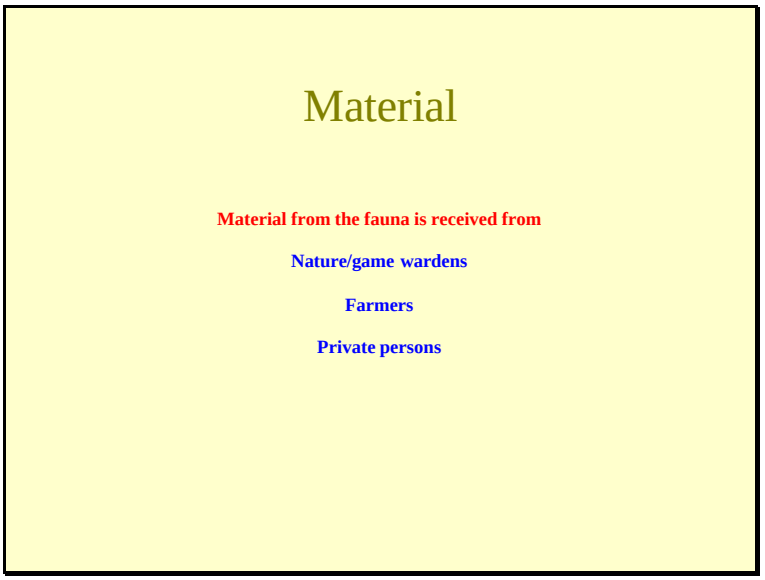
Slide 2



Slide 3

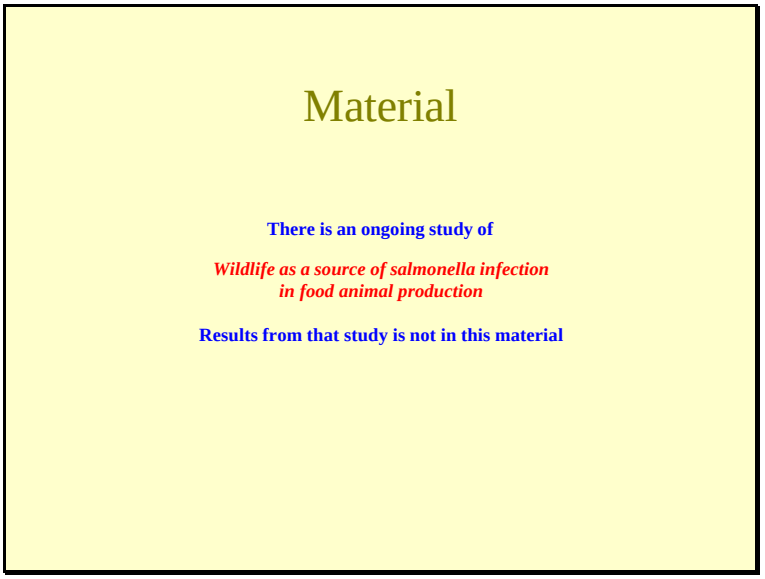


Slide 4

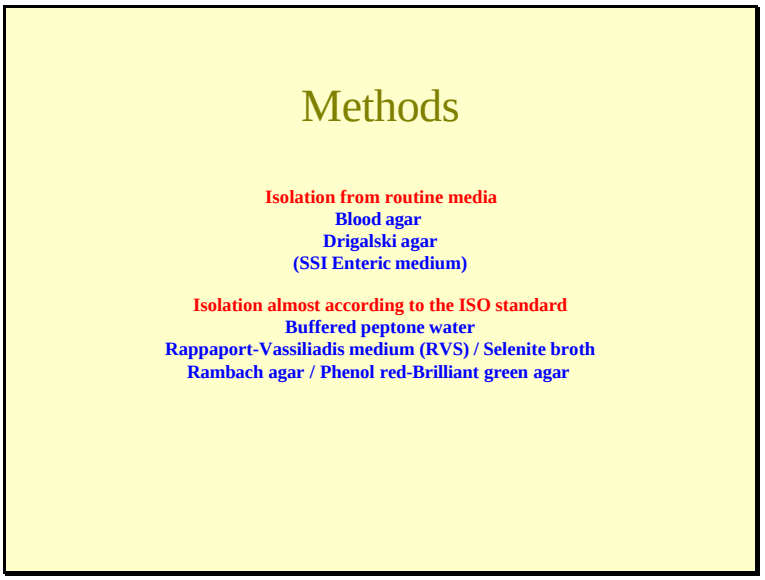


Slide 5

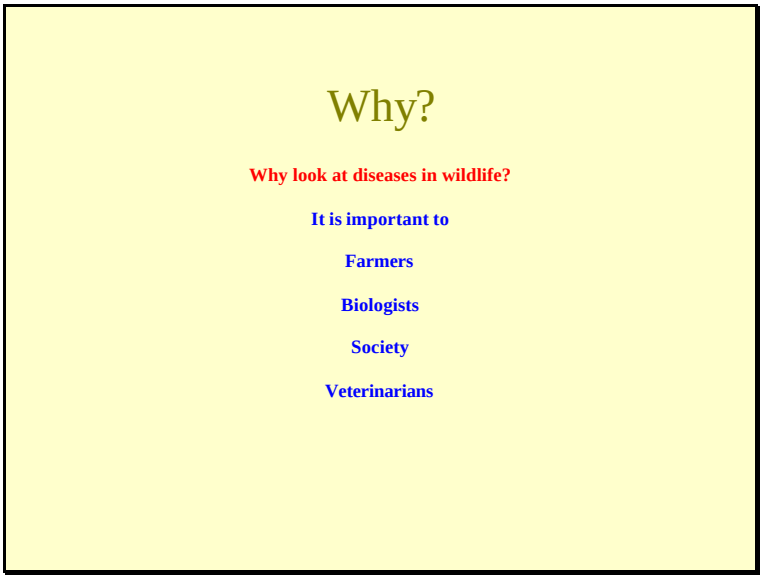
Slide 6



Slide 7



Slide 8



Slide 9

Slide 10

Wild birds

Number of wild birds examined/*Salmonella* positive

Species	1995	1996	1997	1998	1999	2000	Total	Pct. pos.
Web-footed birds	10 / 0	9 / 1	9 / 0	6 / 0	179 / 5	20 / 1	233 / 7	*3.0*
Gallinaceous birds	0 / 0	0 / 0	0 / 0	3 / 0	6 / 0	9 / 0	18 / 0	*0*
Birds of prey	3 / 1	3 / 1	18 / 0	6 / 0	6 / 0	21 / 1	57 / 3	5.3
Other birds	65 / 3	84 / 1	13 / 0	30 / 1	3 / 0	223 / 2	418 / 7	*1.7*
Total	78 / 4	96 / 3	40 / 0	45 / 1	194 / 5	273 / 4	726 / 17	*2.3*

Slide 11

Wild birds

***Salmonella* serotypes in wild birds**

Serotype	Web-footed birds	Birds of prey	Other birds	Total
***S.*Typhimurium**	5	2	5	12
***S.*4,12:b:-**	0	0	1	1
***S.*Not typeable**	1	0	0	1
***S.*Unknown**	1	1	1	3
Total	7	3	7	17

Slide 12

Wild mammals (game)

Number of game examined/*Salmonella* postive

Species	1995	1996	1997	1998	1999	2000	Total
Deer	7/0	4/0	12/0	12/0	26/0	29/0	90/0
Hare	3/0	17/0	46/0	11/0	13/0	32/0	122/0
Total	10/0	21/0	58/0	33/0	39/0	51/0	212/0

Slide 13

Wild mammals

Number of wild mammals examined/*Salmonella* positive

Species	1995	1996	1997	1998	1999	2000	Total	Pct. pos.
Fox	19 / 1	10 / 0	26 / 0	117 / 2	185 / 3	55 / 1	412 / 7	*1.7*
Badger	169 / 0				42 / 1	13 / 0	224 / 1	*0.4*
Wild mink	0 / 0	0 / 0	0 / 0	44 / 0	11 / 0	6 / 0	61 / 0	*0*
Marten	0 / 0	0 / 0	0 / 0	0 / 0	15 / 0	22 / 0	37 / 0	*0*
Polecat	0 / 0	0 / 0	0 / 0	0 / 0	3 / 0	46 / 0	49 / 0	*0*
Hedgehog	123 / 29				11 / 2	6 / 1	140 / 32	*22.9*
Marine mammals	0 / 0	0 / 0	0 / 0	0 / 0	4 / 0	17 / 0	21 / 0	*0*
Total	508 / 32				271 / 6	165 / 2	944 / 40	*4.2*

Slide 14

Wild mammals

***Salmonella* serotypes in wild mammals**

Serotype	Fox	Badger	Hedgehog	Total
***S.*Typhimurium**	3	0	0	3
***S.*Enteritidis**	1	0	22	22
***S.*Agona**	1	0	0	1
***S.*Hadar**	1	0	0	1
***S.*Thompson**	0	1	0	1
***S.*Dublin**	0	0	1	1
***S.*Unknown**	1	0	9	10
Total	7	1	32	40

Slide 15

Fur animals

Number of fur animals examined/*Salmonella* positive

Species	1995	1996	1997	1998	1999	2000	Total	Pct. pos.
Chinchilla	1 / 0	6 / 0	0 / 0	0 / 0	0 / 0	4 / 0	11 / 0	*0*
Rabbit	9 / 0	9 / 0	3 / 0	2 / 0	5 / 0	9 / 0	37 / 0	*0*
Mink	243 / 0	98 / 2	11 / 0	17 / 5	26 / 1	793 / 101	1188 / 109	*9.2*
Fox	18 / 0	11 / 1	3 / 0	24 / 0	1 / 0	42 / 10	99 / 11	*11.1*
Total	271 / 0	124 / 3	17 / 0	43 / 5	32 / 1	848 / 111	1335 / 120	*9.0*

Slide 16

Fur animals

Salmonella **serotypes in fur animals**

Serotype	Mink	Fox	Total
S.Agona	3	0	3
S.Enteritidis	4	0	4
S.Hadar	1	0	1
S.Dublin	101	6	107
S.Typhimurium	0	2	2
S.Derby	0	2	2
S.Unknown	0	1	1
Total	109	11	120

Slide 17

Salmonellosis in mink and fox

Symptoms

Well bread animals

Stop eating prior to delivery

Uterus (pyometritis)

Death of unborn puppies

No cases outside the breeding season

Slide 18

Salmonellosis in mink and fox

Epidemiology

Affected farms had the same feed-stuff supplier

Disease not seen in farms receiving feed-stuff from other suppliers

Slide 19

Salmonellosis in mink and fox

Epidemiology

Only known relation to cattle was a blood product (Blood powder)

Slide 20

Pet animals

Number of pet animals examined/*Salmonella* positive

Species	1995	1996	1997	1998	1999	2000	Total	Pct. pos.
Dog	36 / 2	17 / 0	13 / 0	13 / 0	11 / 0	318 / 4	408 / 6	*1.5*
Cat	8 / 0	3 / 0	7 / 1	6 / 0	5 / 0	105 / 1	134 / 2	*1.5*
Total	44 / 2	20 / 0	20 / 1	19 / 0	16 / 0	423 / 5	542 / 8	*1.5*

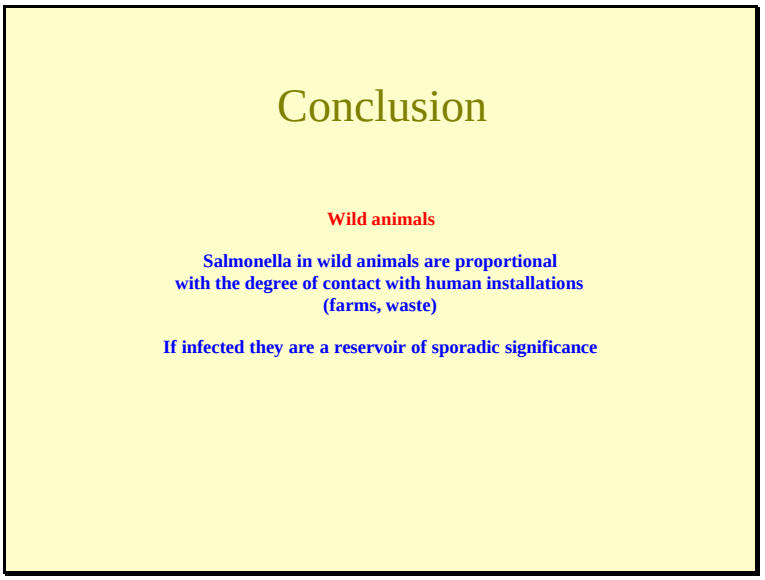
Slide 21

Pet animals

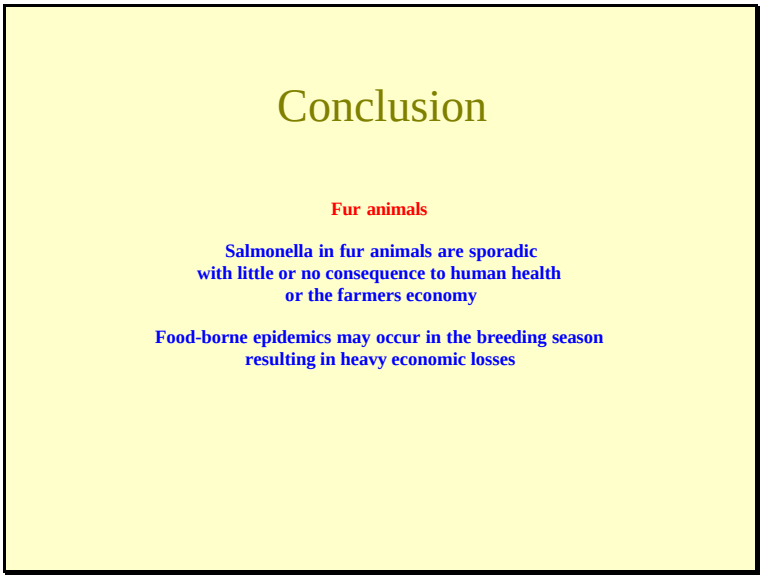
***Salmonella* serotypes in pet animals**

Serotype	Dog	Cat	Total
S.Krefeld	1	0	1
S.Indiana	1	0	1
S.Montevideo	2	0	2
S.Typhimurium	0	2	2
S.Unknown	2	0	2
Total	6	2	8

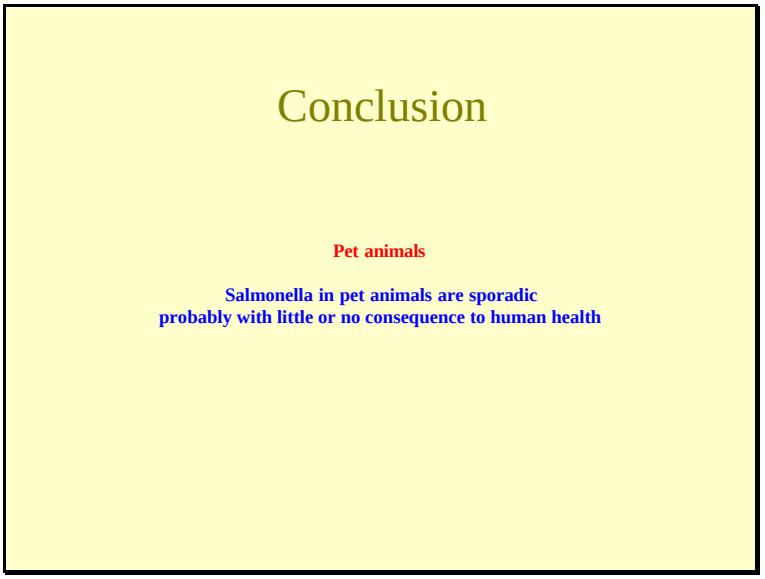
Slide 22



Slide 23

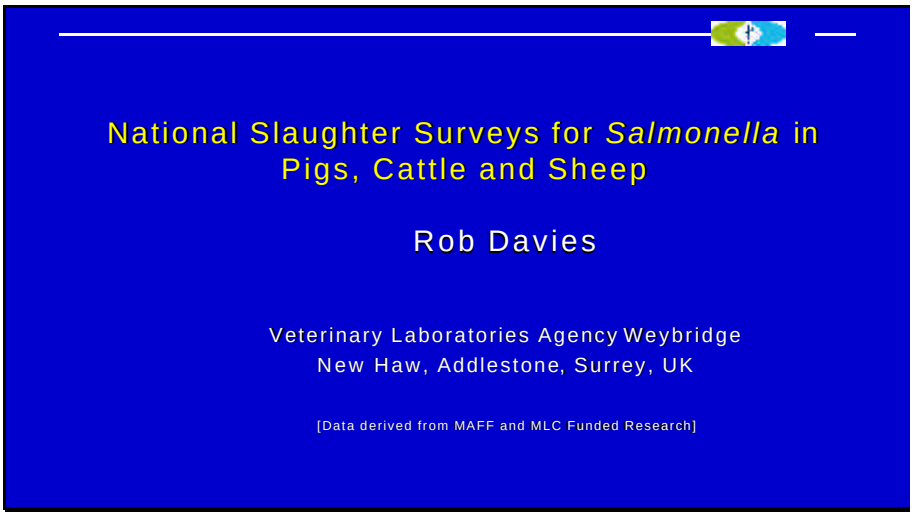


Slide 24

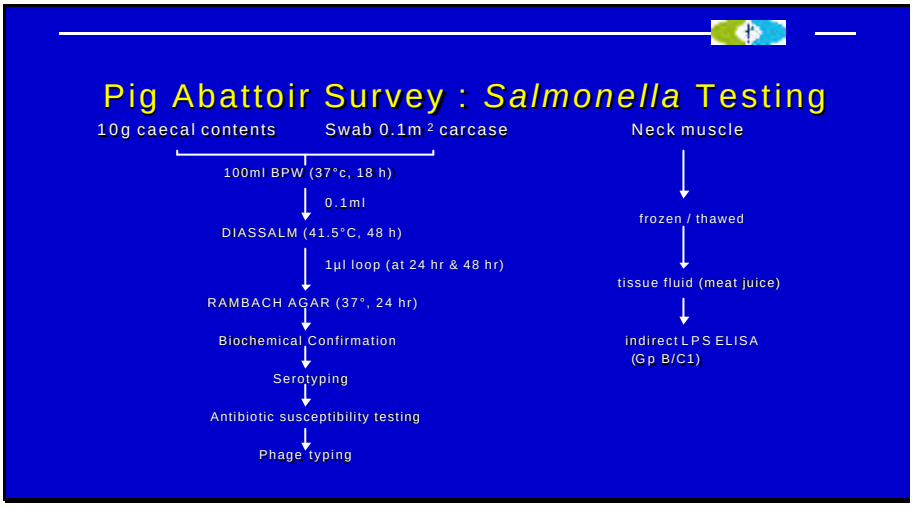


Appendix 9. Sheets of presentation 3.3

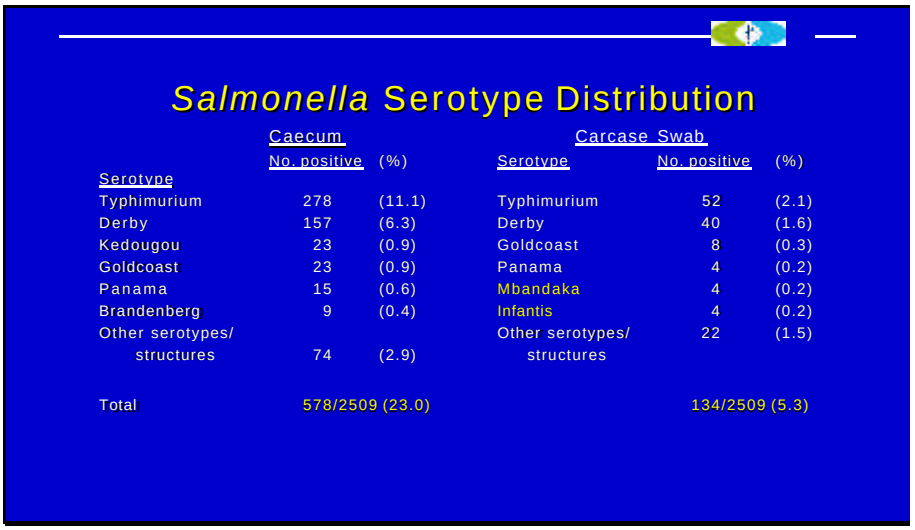
Slide 1



Slide 2



Slide 3



Slide 4

Salmonella Typhimurium Definitive Types Isolated from Caeca and Carcase Swabs

Definitive type	Caecum	%	Carcase swab	%
104/104B/104A	71	25.5	13	25.0
193	52	18.7	12	23.1
UNTY	49	17.6	11	21.2
208	37	13.3	7	13.5
U302	37	13.3	5	9.6
135	6	2.2	0	0.0
Others	26	9.3	4	7.6
Grand Total	278		52	

Slide 5

Pig Abattoir Survey : March 1999 - February 2000 Prevalences and 95% Confidence Intervals

a) All Abattoirs

Sample	No. positive	No. tested	Prevalence (%)	95% C.I.
Caecum	578	2509	23.04	21.39 - 24.68
Carcase swab	134	2509	5.34	4.46 - 6.22
Neck muscle (MJE >40% OD)	365	2403	15.19	13.75 - 16.62
Neck muscle (MJE >10% OD)	857	2403	35.66	33.75 - 37.58

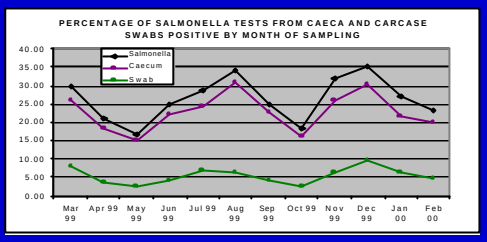
Slide 6

Percentage of Tests Positive for Each Organism/Sample – Comparison of Abattoirs by Annual Throughput

	Percent Positive				
Percentage positive	>15,000	>100,000	>150,000	>600,000	Total
Salmonella from caeca	19.4	20.8	18.7	27.9	23.0
Salmonella from carcase	16.7	6.2	5.2	4.3	5.3
Salmonella from neck muscle (Moderate = >40% OD)	10.0	16.5	15.5	14.7	15.2
Swab/Caecum Ratio	0.9	0.3	0.3	0.2	0.2

Slide 7

Percentage of *Salmonella* Tests from Caeca and Carcase Swabs Positive by Month of Sampling



Slide 8

Positive Association between Yersinia, Enterococcus and *Salmonella*

		Yersinia enterocolitica Positive	Yersinia enterocolitica Negative
Salmonella	Positive	88*	148
	Negative	162	462

(* p = 0.001)

		Enterococcus faecium Positive	Enterococcus faecium Negative
Salmonella	Positive	20**	216
	Negative	30	594

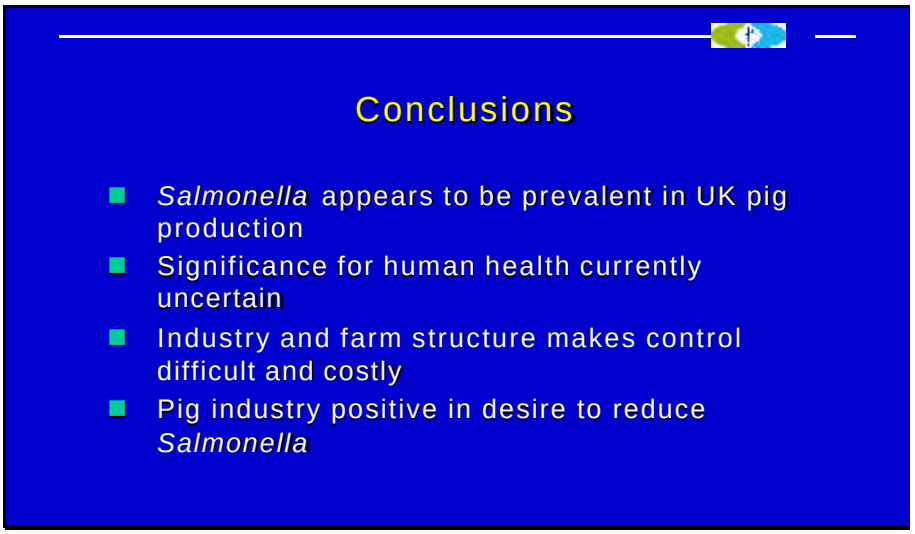
(**p = 0.032)

Slide 9

Cattle and Sheep Survey

CATTLE	SHEEP
2/891(0.2%)[CI95% 0-0.5]	1/973(0.1%)[CI95% 0-0.3]
STM DT193	STM DT41
STM DT12	

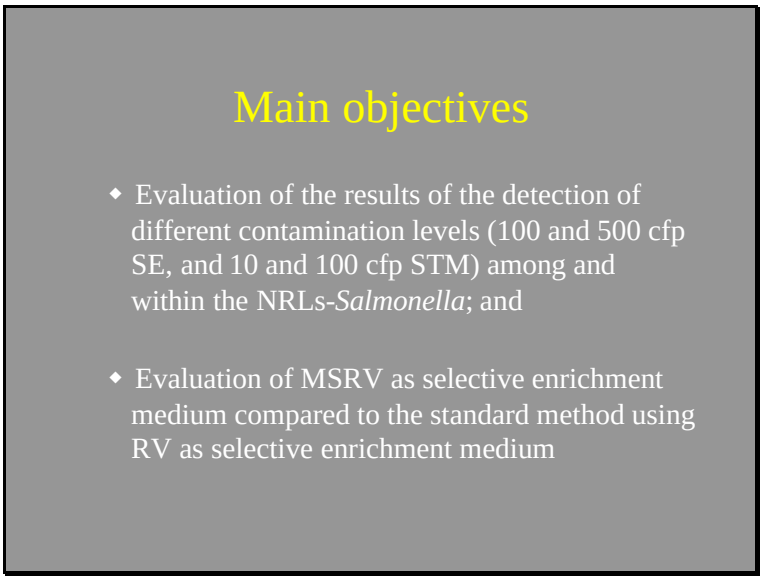
Slide 10


Conclusions
Salmonella appears to be prevalent in UK pig production
Significance for human health currently uncertain
Industry and farm structure makes control difficult and costly
Pig industry positive in desire to reduce *Salmonella*

Appendix 10. Sheets of presentation 3.4

Slide 1

Slide 2



Slide 3

Prescribed method

- Pre-enrichment in BPW (pre-heated 37°C)
- Selective enrichment in RV and MSRV
  - optionally PCR, own selective enrichment medium
- Isolation on BGA and XLD
  - optionally own isolation medium
- Biochemical confirmation using Urea, TSI and LDC

Slide 4

Samples tested

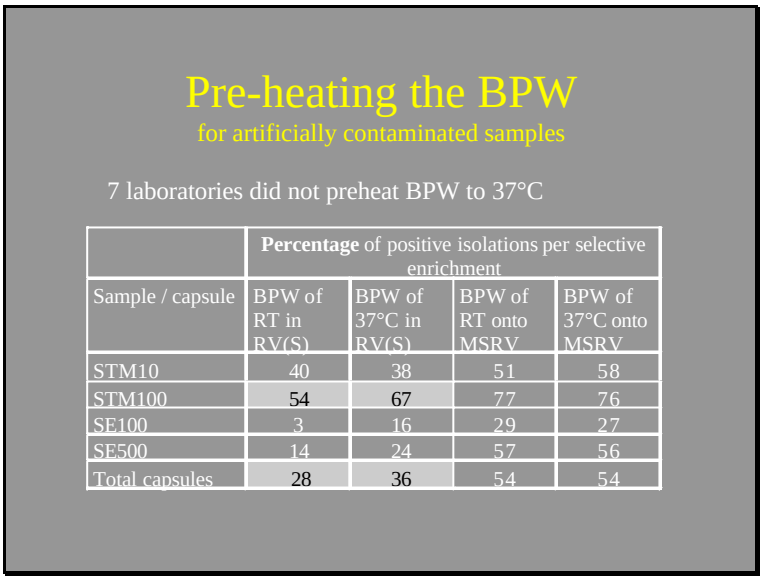
content	Number of capsules, faeces added
STM10	5
STM100	5
SE100	5
SE500	5
Blank	5
	Number of capsules, No faeces added
STM10	3
SE100	3
S. Panama	2
Blank	2
	Naturally contaminated
Several serotypes	20

Slide 5

Naturally contaminated samples

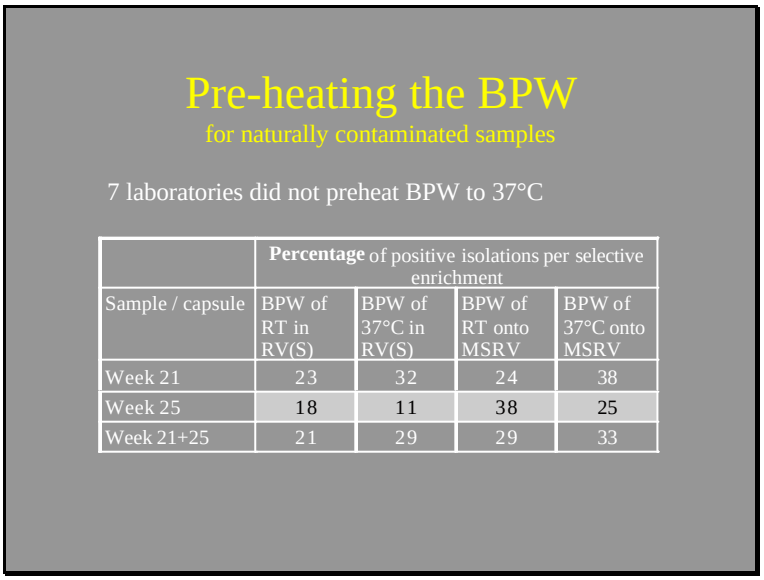
Batch faeces	Serotypes	Group (K&W)
Week 21 (n=12)	*S.* Ruiri	C1
	S. Tennessee	G
	S. Cubana	L
Week 25 (n=8)	*S.* Enteritidis PT4, PT35	D
	S. Livingstone	C1

Slide 6



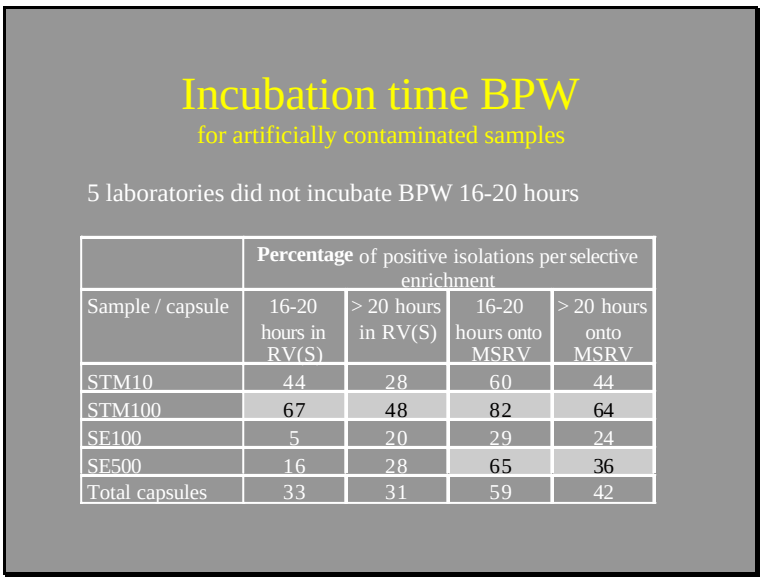
	Percentage of positive isolations per selective enrichment			
Sample / capsule	BPW of RT in RV(S)	BPW of 37°C in RV(S)	BPW of RT onto MSRV	BPW of 37°C onto MSRV
STM10	40	38	51	58
STM100	54	67	77	76
SE100	3	16	29	27
SE500	14	24	57	56
Total capsules	28	36	54	54

Slide 7



	Percentage of positive isolations per selective enrichment			
Sample / capsule	BPW of RT in RV(S)	BPW of 37°C in RV(S)	BPW of RT onto MSRV	BPW of 37°C onto MSRV
Week 21	23	32	24	38
Week 25	18	11	38	25
Week 21+25	21	29	29	33

Slide 8



	Percentage of positive isolations per selective enrichment			
Sample / capsule	16-20 hours in RV(S)	> 20 hours in RV(S)	16-20 hours onto MSRV	> 20 hours onto MSRV
STM10	44	28	60	44
STM100	67	48	82	64
SE100	5	20	29	24
SE500	16	28	65	36
Total capsules	33	31	59	42

Slide 9

Incubation time BPW

for naturally contaminated samples

5 laboratories did not incubate BPW 16-20 hours

	Percentage of positive isolations per selective enrichment			
Sample / capsule	16-20 hours in RV(S)	> 20 hours in RV(S)	16-20 hours onto MSRV	> 20 hours onto MSRV
Week 21	30	23	33	27
Week 25	18	5	40	10
Week 21 + 25	25	16	35	20

Slide 10

Selective enrichment media

1) RV: Rappaport Vassiliadis broth
2) RVS: Rappaport Vassiliadis Soya broth
3) MSRV: Modified Semi-solid Rappaport Vassiliadis
4) TBG: Tetrathionate-Brilliant-green Bile broth
5) MSRV+: MSRV with bromcresolpurpur and saccharose
6) SC: Selenit / Cystine broth
7) DIASALM: Diagnostic Semi-solid *Salmonella* medium (DIA)
8) MK: Muller Kauffmann broth
9) Rap: Rappaport broth

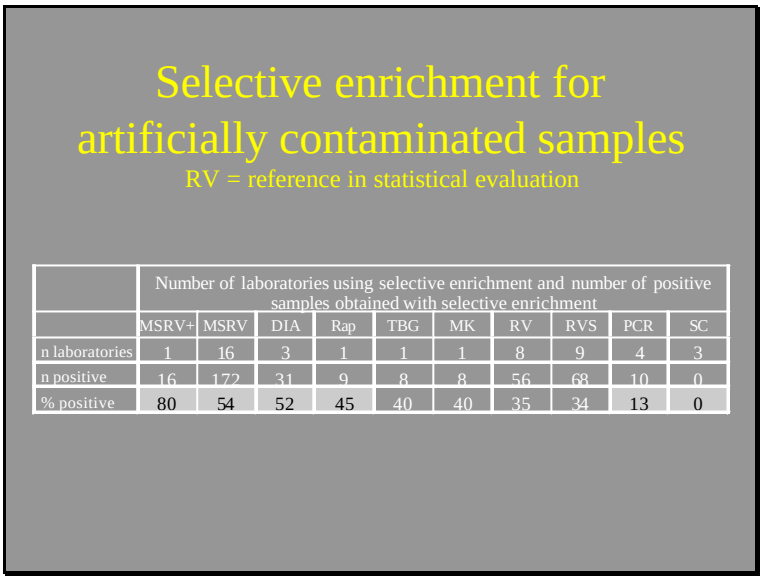
10) PCR: Polymerase Chain Reaction

Slide 11

Volumes used for PCR

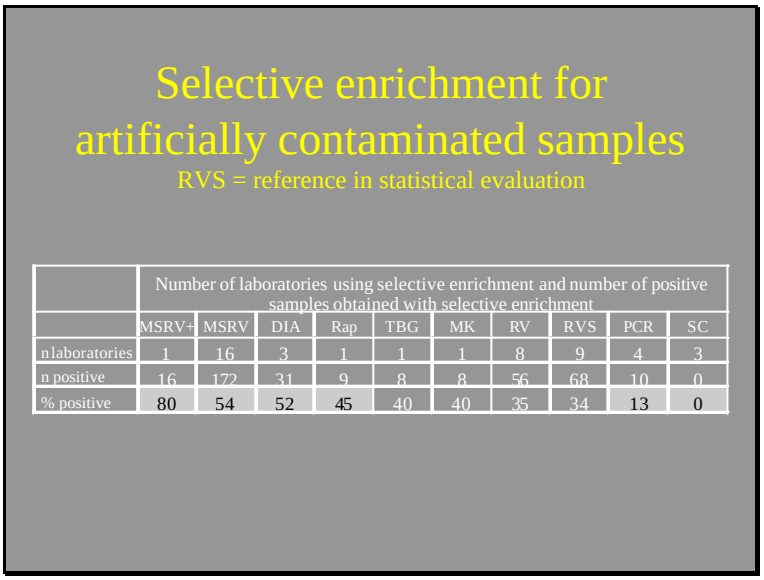
Volume of BPW (ml)	Volume of DNA extract (ml)	Volume used in PCR reaction (µl)	Actual volume of BPW tested in PCR (µl)
1	1	2	2
1	0.3	5	17
1	0.1	5	50
2	0.05	5	200

Slide 12



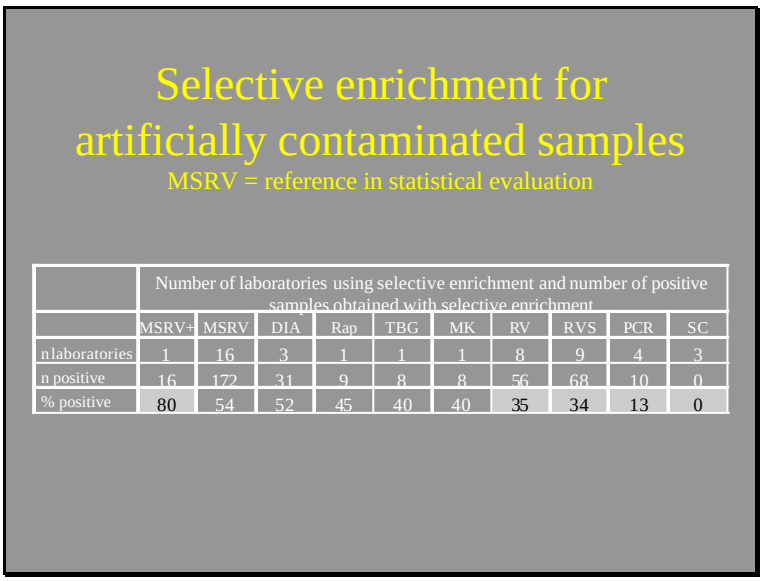
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	MSRV+	MSRV	DIA	Rap	TBG	MK	RV	RVS	PCR	SC
n laboratories	1	16	3	1	1	1	8	9	4	3
n positive	16	172	31	9	8	8	56	68	10	0
% positive	80	54	52	45	40	40	35	34	13	0

Slide 13



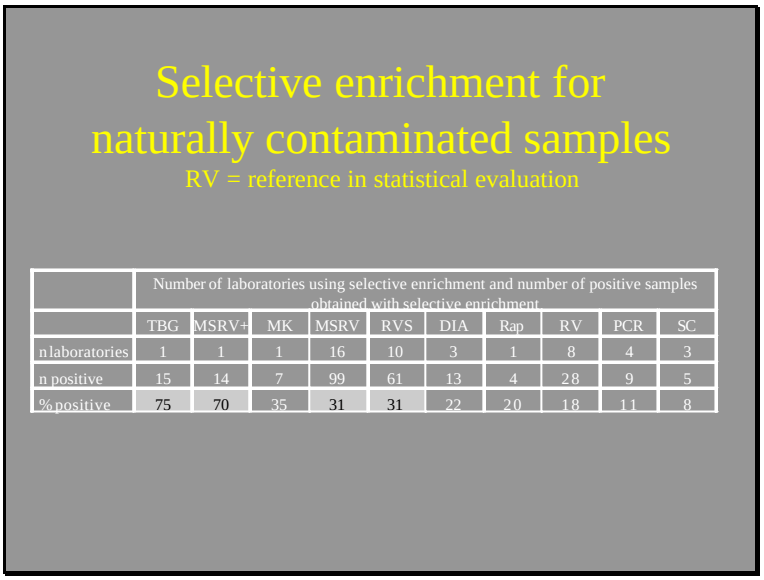
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	MSRV+	MSRV	DIA	Rap	TBG	MK	RV	RVS	PCR	SC
n laboratories	1	16	3	1	1	1	8	9	4	3
n positive	16	172	31	9	8	8	56	68	10	0
% positive	80	54	52	45	40	40	35	34	13	0

Slide 14



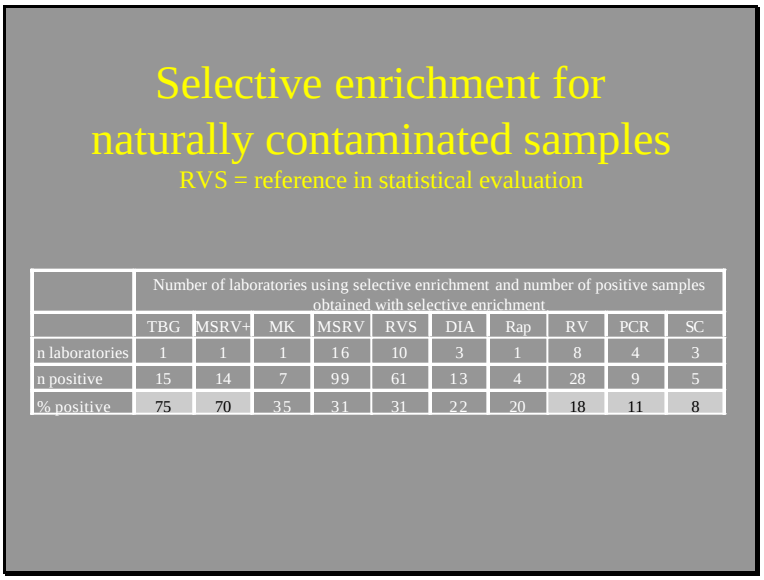
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	MSRV+	MSRV	DIA	Rap	TBG	MK	RV	RVS	PCR	SC
n laboratories	1	16	3	1	1	1	8	9	4	3
n positive	16	172	31	9	8	8	56	68	10	0
% positive	80	54	52	45	40	40	35	34	13	0

Slide 15



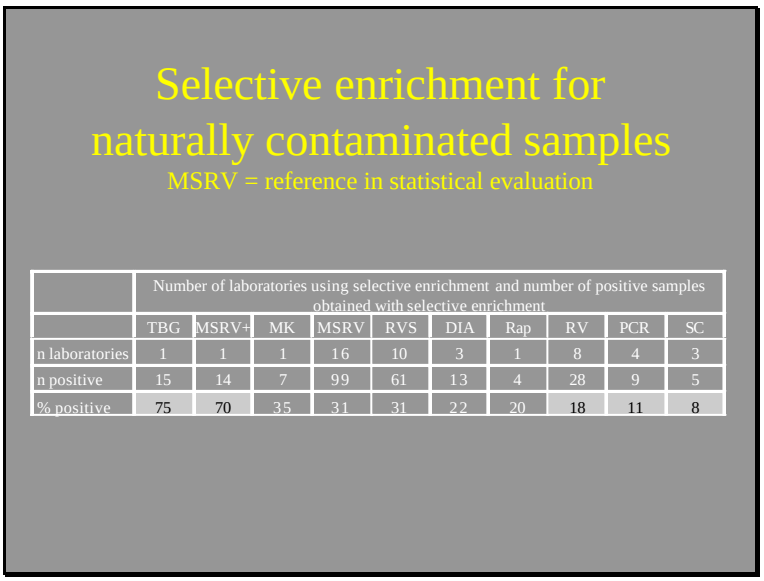
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	TBG	MSRV+	MK	MSRV	RVS	DIA	Rap	RV	PCR	SC
n laboratories	1	1	1	16	10	3	1	8	4	3
n positive	15	14	7	99	61	13	4	28	9	5
% positive	75	70	35	31	31	22	20	18	11	8

Slide 16



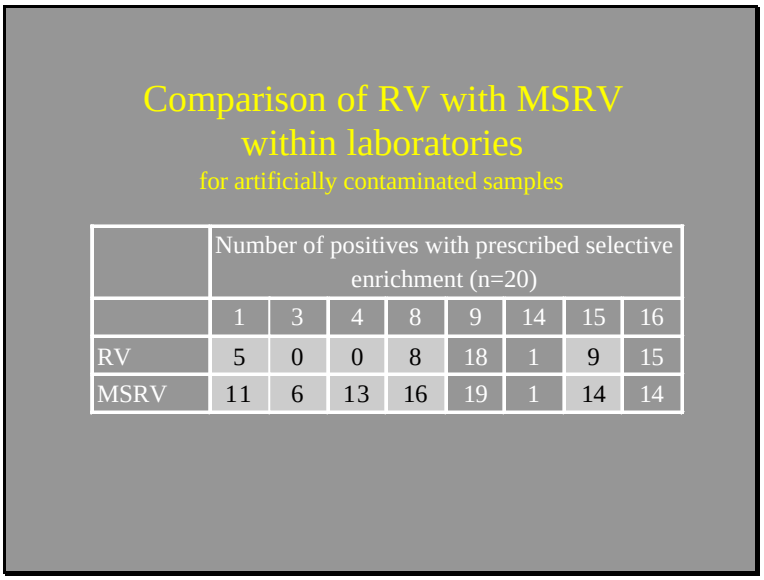
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	TBG	MSRV+	MK	MSRV	RVS	DIA	Rap	RV	PCR	SC
n laboratories	1	1	1	16	10	3	1	8	4	3
n positive	15	14	7	99	61	13	4	28	9	5
% positive	75	70	35	31	31	22	20	18	11	8

Slide 17



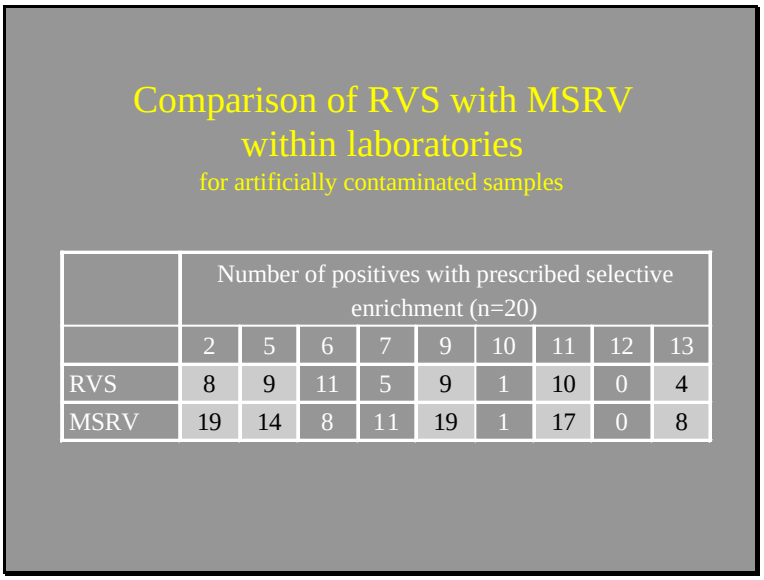
	Number of laboratories using selective enrichment and number of positive samples obtained with selective enrichment									
	TBG	MSRV+	MK	MSRV	RVS	DIA	Rap	RV	PCR	SC
n laboratories	1	1	1	16	10	3	1	8	4	3
n positive	15	14	7	99	61	13	4	28	9	5
% positive	75	70	35	31	31	22	20	18	11	8

Slide 18



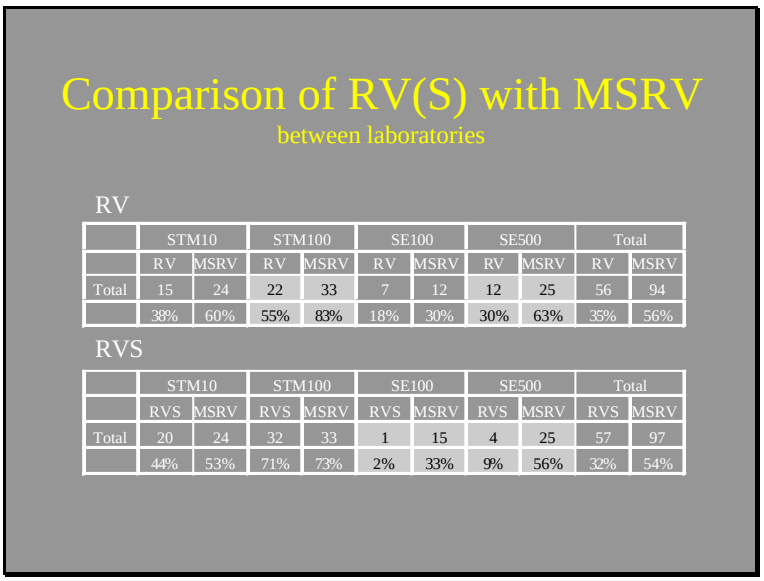
	Number of positives with prescribed selective enrichment (n=20)							
	1	3	4	8	9	14	15	16
RV	5	0	0	8	18	1	9	15
MSRV	11	6	13	16	19	1	14	14

Slide 19



	Number of positives with prescribed selective enrichment (n=20)								
	2	5	6	7	9	10	11	12	13
RVS	8	9	11	5	9	1	10	0	4
MSRV	19	14	8	11	19	1	17	0	8

Slide 20



RV

	STM10		STM100		SE100		SE500		Total	
	RV	MSRV	RV	MSRV	RV	MSRV	RV	MSRV	RV	MSRV
Total	15	24	22	33	7	12	12	25	56	94
	38%	60%	55%	83%	18%	30%	30%	63%	35%	56%

RVS

	STM10		STM100		SE100		SE500		Total	
	RVS	MSRV	RVS	MSRV	RVS	MSRV	RVS	MSRV	RVS	MSRV
Total	20	24	32	33	1	15	4	25	57	97
	44%	53%	71%	73%	2%	33%	9%	56%	32%	54%

Slide 21

Comparison of RV with MSRV within laboratories

for naturally contaminated samples

	Number of positives with prescribed selective enrichment (n=20)							
	1	3	4	8	9	14	15	16
RV	7	0	0	4	8	0	0	9
MSRV	11	2	4	11	9	0	0	10

Slide 22

Comparison of RVS with MSRV within laboratories

for naturally contaminated samples

	Number of positives with prescribed selective enrichment (n=20)									
	2	5	6	7	9	10	11	12	13	16
RVS	18	0	8	0	6	0	17	0	1	11
MSRV	20	0	7	0	9	0	17	0	8	10

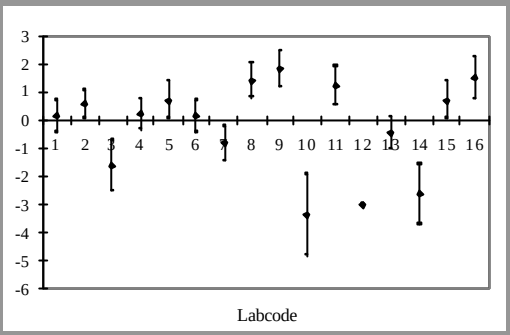
Slide 23

Comparison of media per batch of naturally contaminated faeces

Samples of	Serotypes found in faeces before freezing	Number of positive isolations with combination of	
		RV / MSRV (7 laboratories)	RVS / MSRV (9 laboratories)
Week 21 (12 samples / laboratory)	Ruiri Tennessee Cubana	27 / 34 (n=84) (32.1% / 40.5%)	27 / 26 (n=108) (25.0% / 24.1%)
Week 25 (8 samples / laboratory)	Enteritidis PT4 and 35 Livingstone	1 / 13 (n=56) (1.8% / 23.2%)	17 / 26 (n=72) (23.6% / 36.1%)

Slide 24

Comparison of overall results
(artificially contaminated samples)



Slide 25

Bacteriology compared to PCR

		Number of positives per laboratory			
capsules	Bacteriology	11	11	8	1
	PCR	1	0	8	0
naturally contaminated	Bacteriology	11	0	8	0
	PCR	0	0	9	0

Highest volume of BPW

Slide 26

Conclusion & Discussion (1)

- Preheating the BPW
  - significantly better for STM100 into RV(S), no significant difference for MSRV
  - significantly less for naturally contaminated with SE
- Incubation time BPW $\Rightarrow$ 16-20 hours
  - significantly better for STM100 and SE500 capsules
  - better for naturally contaminated (not significant)
- Capsules and difficulty to isolate *Salmonella*

Slide 27

Conclusion & Discussion (2)

- Selective enrichment for artificially contaminated samples
  - MSRV significantly better than RV(S) in 10 labs
- Selective enrichment for naturally contaminated samples
  - MSRV significantly better than RV(S) in 4 labs
- The use of PCR
- Faeces (problem?)

Appendix 11. Sheets of presentation 3.6

ISO/DIS 6579 (1999/2000)

Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp.

- **Pre-discussion on 5 February 2001 with Dk, B, D, NL, USA, Fr**

- **Discussion at ISO/TC34/SC9 meeting in Bern (Switzerland) on 19-21 June 2001**

Points of discussion (from 5/2 meeting):

➪ **Selective enrichment:**

- **RVS: Incubation time now 24 h; some prefer 2 x 24 h. Extra info will be sent to 'cie and discussed in Bern**
- **MKTTn:**
  - Selenite Cystine broth was deleted because of toxicity;
  - MKTTn is found difficult to prepare;
  - A small trial will be organised to compare MKKTn with TT (AOAC medium) at 37 °C, results will be discussed in Bern

➪ **Isolation agar (plating-out):**

- **XLD:**

  - Medium was originally prepared for detection of *Shigella* (not for *Salmonella*);

  - Not easy to prepare;

  - Some prefer BGA instead of XLD;

  - Additional data on isolation media will be sent to 'cie and discussed in Bern.

➪ **Biochemical confirmation:**

- **Prefer to keep TSI-agar**
- **Add interpretation table to the ISO of biochemical and serological tests**

➪ **Serological confirmation:**

- **Maintain only polyvalent sera in the ISO**

Advise from EU-validation study:
'To aim at defining a common AOAC and ISO method for the detection of *Salmonella* in foods.'

Appendix 12. Sheets of presentation 4.1

OVERVIEW

- 17 NRL (3 also ENL)
- 15 ENL
- 20 strains of *S. enterica*, of which

19x subsp. *enterica*
1x subsp. *Arizonae*

Table 1: Guidelines for evaluation

Result of laboratory	Evaluation
• Autoagglutination • Incomplete set of antisera (outside range of antisera)	Not typable (nt)
• Partly typable due to incomplete set of antisera • No name serovar • Part of the formula (for the name of the serovar)	Partly correct (+/-)
• Wrong serovar • Mixed sera formula	Incorrect (-)

Antigenic formulas of the 20 **Salmonella** *strains*

No.	Serotype	O antigens	H antigens	Origin of strains
1	S. Blockley	6, 8	k : 1, 5	Human faeces
2	S. Agona	1, 4, 12	f, g, s : [1, 2]	Human faeces
3	S. Rissen	6, 7, 14	fg : -	Human faeces
4	S. Brazzaville	6, 7	b : 1, 2	Human faeces
5	S. Kiambu	1, 4, 12	z : 1, 5	Chicken
6	S. Typhimurium 506	1, 4, [5], 12	i : 1, 2	Human faeces
7	S. Goldcoast	6, 8	r : l, w	Human faeces
8	S. Kottbus	6, 8	e, h : 1, 5	Human faeces
9	S. Blockley	6, 8	k : 1, 5	Human faeces
10	S. Yoruba	16	c : l, w	Animal feed
11	S. Grumpensis	1, 13, 23	d : 1, 7	Human faeces
12	S. Heidelberg	1, 4, [5], 12	r : 1, 2	Human faeces
13	S. spp.arizonae 41 : z4, z23 : -	41	z4, z23 : -	Human faeces
14	S. Enteritidis PT4b	1, 9, 12	g, m : -	Human faeces
15	S. Newport	6, 8, 20	e, h : 1, 2 : [z67]	Human faeces
16	S. Dublin	1, 9, 12	g, p : -	Human faeces
17	S. Muenchen	6, 8 : d	1, 2 : [z67]	Human faeces
18	S. Lexington	3, 10	z10 : 1, 5	Environmental sample
19	S. Waycross	41	z4, z23 : [e, n, z15]	Human faeces
20	S. Llandoff	1, 3, 19	z29 : [z6]	Animal feed

- **Level of difficulty next study**

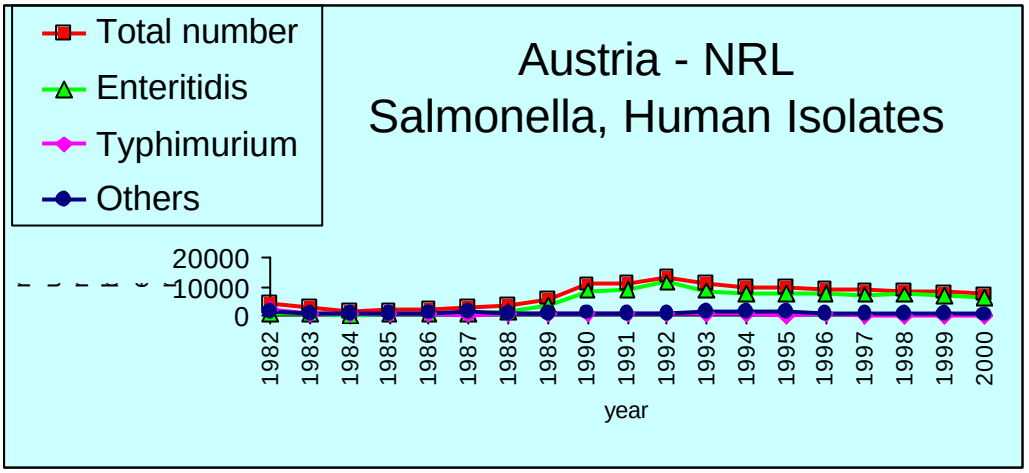
- **Follow-up lesser performing labs:**

    1. inventory media / antisera
    2. training / course at CRL ?
    3. small-scale ring trial ?
    4. audit ?

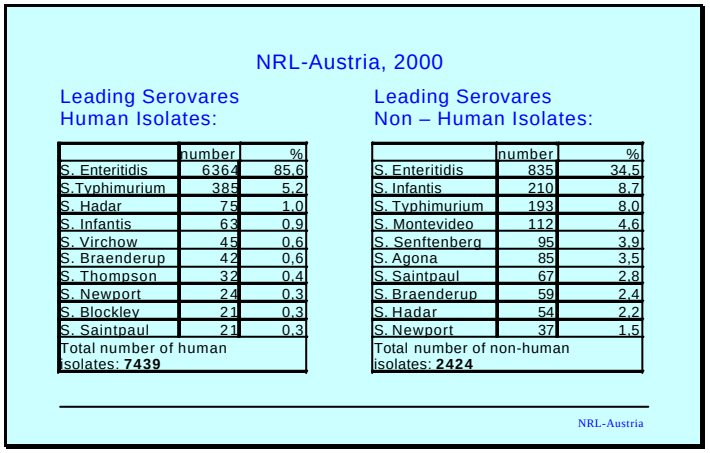
- **Antibiotic panel (ARBAO ?)**

Appendix 13. Sheets of presentation 4.2

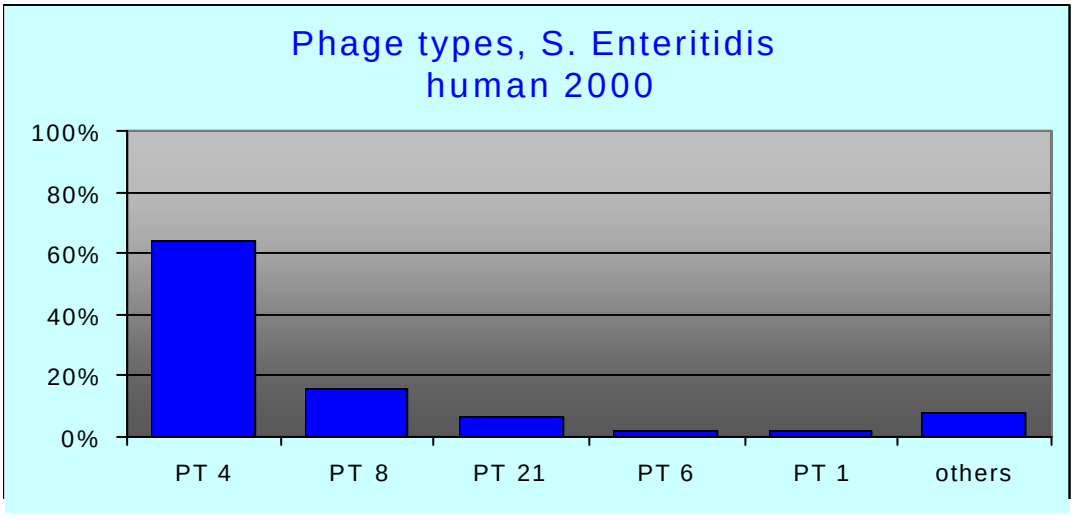
Slide 1



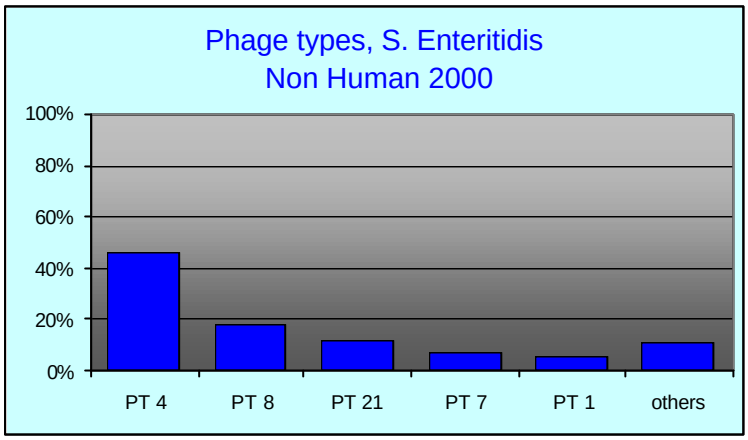
Slide 2



Slide 3



Slide 4



Slide 5

Resistance of Salmonella
Human (Primary Isolates)

antibiotic	1997	1999	2000
	%	%	%
ampicillin	4,6	3,8	4,0
chloramphenicol	1,7	1,9	2,0
streptomycin	5,0	4,3	4,5
sulfonamide	3,6	3,6	3,8
tetracycline	5,8	5,3	4,9
trimethoprim	2,3	1,4	1,3
gentamycin	0,1	0,4	0,3
kanamycin	0,6	1,2	0,7
nalidixic acid	**4,6**	**7,9**	**6,0**
ciprofloxacin	0	<0,0 (2)	<0,0 (1)
cefotaxim			<0,0 (1)
total number	8921	8165	7439

NRL-Austria

Slide 6

Resistance S. Hadar
Human, 2000

ampicillin	57.3
chloramphenicol	1,3
streptomycin	90,7
sulfonamide	12,0
tetracycline	92,0
trimethoprim	12,0
gentamicin	0,0
kanamycin	4,0
Nalidixic acid	85,3
ciprofloxacin	0,0
cefotaxim	0,0
total number	**75**

NRL-Austria

Slide 7

Resistance S. Enteritidis
Human 2000

antibiotics	%
ampicillin	1,2
chloramphenicol	0,0
streptomycin	0,7
sulfonamide	0,9
tetracycline	0,9
trimethoprim	0,5
gentamicin	0,1
Kanamycin	0,1
nalidixic acid	4,6
ciprofloxacin	0,0
cefotaxim	0,0
total number	**6364**

NRL-Austria

Slide 8

Resistance S. Enteritidis PT1
Human 2000

antibiotics	%
ampicillin	0,7
chloramphenicol	0,0
streptomycin	0,7
sulfonamide	0,7
tetracycline	0,7
trimethoprim	0,0
gentamicin	0,7
kanamycin	0,0
nalidixic acid	33,8
ciprofloxacin	0,0
cefotaxim	0,0
total number	**145**

NRL-Austria

Slide 9

Resistance, S. Enteritidis PT 4
Human 2000

antibiotics	%
ampicillin	0,6
chloramphenicol	0,0
streptomycin	0,9
sulfonamide	1,1
tetracycline	0,6
trimethoprim	0,2
gentamicin	0,1
kanamycin	0,0
nalidixic acid	5,2
ciprofloxacin	0,0
cefotaxim	0,0
total number	**4080**

NRL-Austria

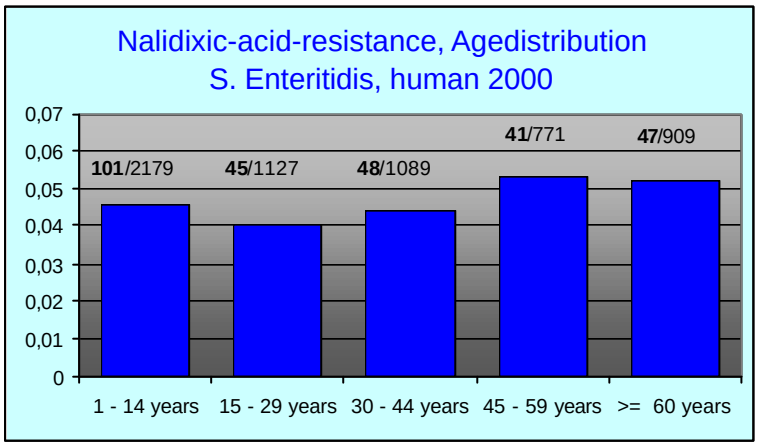
Slide 10

Resistance S. Enteritidis PT 8
Human 2000

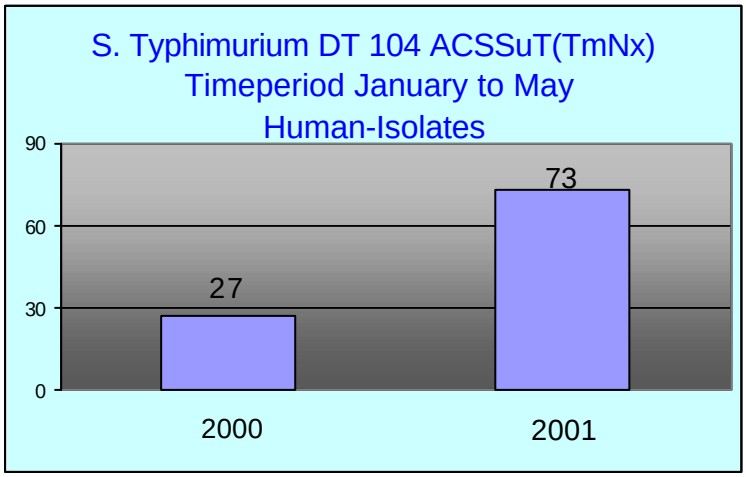
antibiotics	%
ampicillin	0,0
chloramphenicol	0,0
streptomycin	0,0
sulfonamide	0,1
tetracyclin	0,1
trimethoprim	0,0
gentamicin	0,0
kanamycin	0,0
nalidixic acid	0,0
ciprofloxacin	0,0
cefotaxim	0,0
total number	**1021**

NRL-Austria

Slide 11



Slide 12



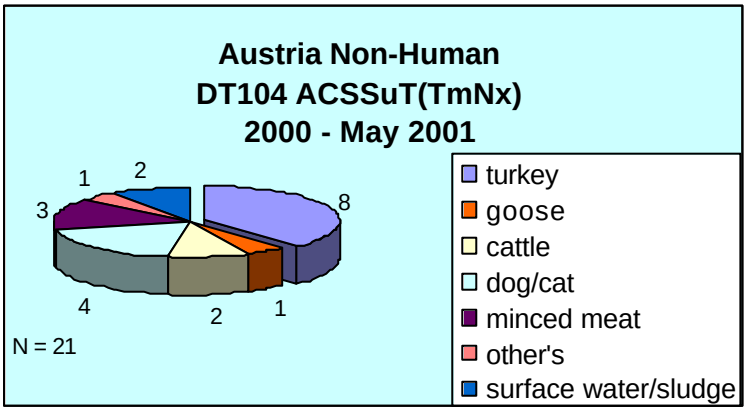
Slide 13

S. Typhimurium DT 104
Human, Austria

	fully sensitive	others	ACSSuT(TmNx)
1997	39	20	75
1998	49	13	75
1999	13	2	76
2000	23	9	90
(I-V) 2001	1	0	73

NRL-Austria

Slide 14



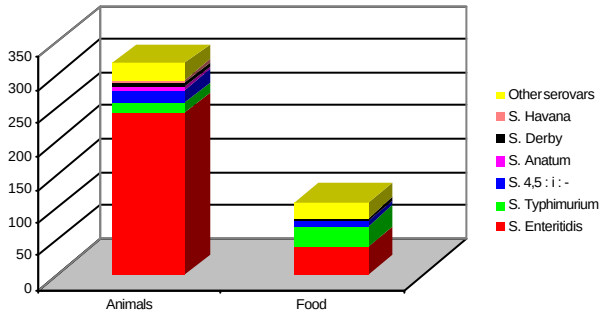
Appendix 14. Sheets of presentation 4.3

Slide 1

Studies performed on *Salmonella* isolates at LNIV on 2000

Slide 2

Salmonella serovars isolated in animals and food



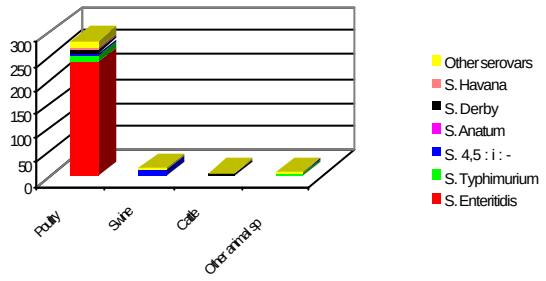
Slide 3

Salmonella serovars isolated in animals and food

- From the 419 *Salmonella* strains typed at LNIV on 2000, 106 were from food origin.
- In both, animals and food, *Salmonella* Enteritidis was the main serovar found, followed by *S.* 4,5:i:- in animals and *S*. Typhimurium in food.

Slide 4

Animal *Salmonella* serovars

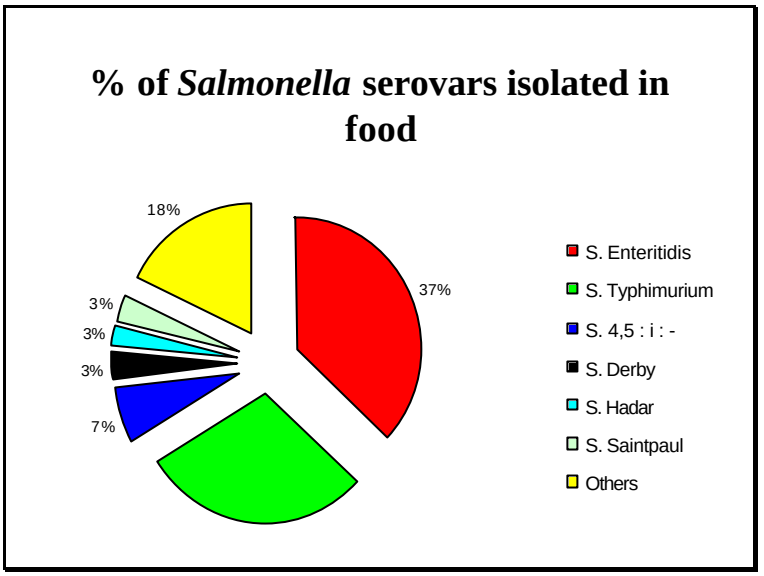


Slide 5

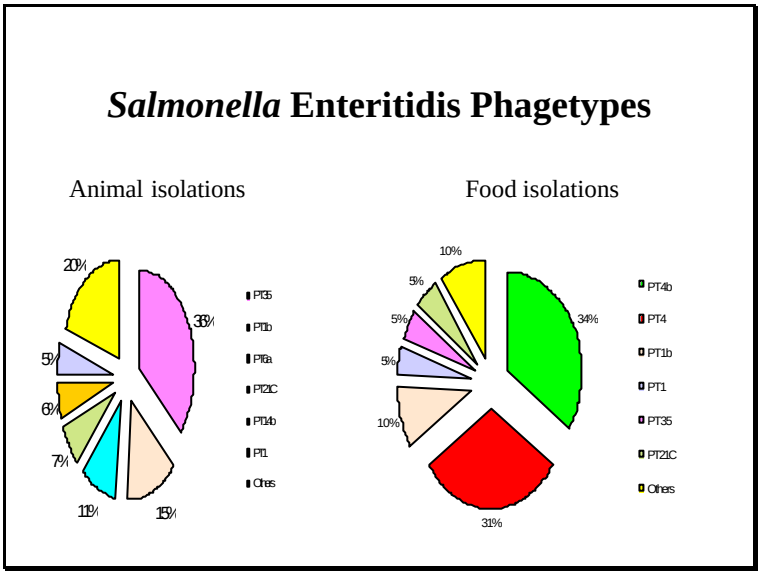
Animal *Salmonella* serovars (cont.)

- 90 % of the samples were isolated from poultry.
- The main serovar isolated in poultry was *S*. Enteritidis (84%), followed by *S.* Typhimurium (5%) and *S*. 4,5:i:- (2%).
- 63% of the swine isolates belong to serovar *S*. 4,5:i:-.

Slide 6



Slide 7



Slide 8

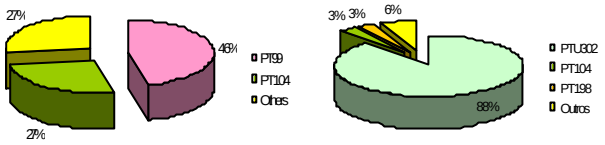
S. Enteritidis Phagetypes (cont.)

- PT35 is the most common phagetype found in animals (36%), because 60% of our poultry isolates come from DRABL hatcheries isolations.
- Still PT35, PT1b, PT21C are present in both animals and food, but in different percentages.
- PT1 is present in both at the same percentage (5%).
- The Human phagetypes more frequent are: PT1b (30%), PT6a (22%), PT1 (11%), PT4 (10%) and PT 4b (8%).

Slide 9

Salmonella Typhimurium Phagetypes

Animal isolations **Food isolations**



Slide 10

S.Typhimurium Phagetypes (cont.)

- PT99 was the more frequent phagetype in animals (46%).
- PTU302 was the most frequent phagetype in samples from food origin (88%). Most of the samples (26 of 27) came from Azores islands isolates.
- PTU302 is also the most common phagetype on Human isolates (54%), followed by PT104 (24%).

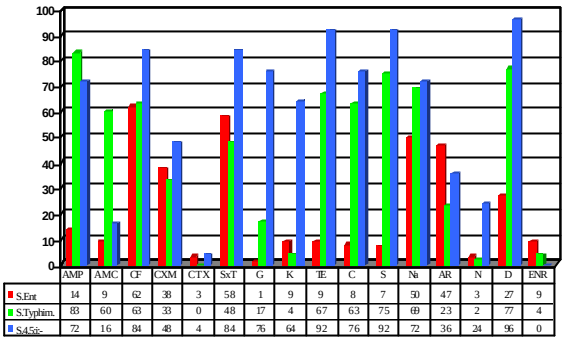
Slide 11

Resistance to antimicrobials

- The resistance to antimicrobials is performed by disk diffusion Method in Mueller Hinton plates.
- The antimicrobials tested are: AMP_{10}, AMC_{30}, CF_{30}, CXM_{30}, CTX_{30}, SxT_{25}, G_{10}, K_{30}, TE_{30}, C_{30}, S_{10}, NA_{30}, UB_{30}, N_{30}, D_{30}, ENR_5.
- The zone diameters are read, following NCCLS Vol.19 Nº1, January 99.

Slide 12

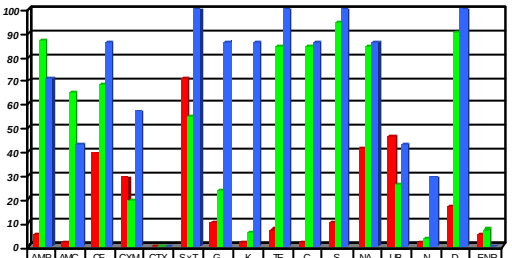
% of antimicrobials resistance in animals



	AMP	AMC	CF	CXM	CTX	SxT	G	K	TE	C	S	Na	AR	N	D	ENR
S.Ent	14	9	62	38	3	58	1	9	9	8	7	50	47	3	27	9
S.Typhim.	83	60	63	33	0	48	17	4	67	63	75	69	23	2	77	4
S.4,5:i:-	72	16	84	48	4	84	76	64	92	76	92	72	36	24	96	0

Slide 13

% of antimicrobials resistance in food



	AMP	AMC	CF	CXM	CTX	SxT	G	K	TE	C	S	NA	UB	N	D	ENR
S.Ent	5	2	39	29	0	71	10	2	7	2	10	41	46	2	17	5
S.Typh	87	65	68	19	0	55	23	6	84	84	94	84	26	3	90	7
S.4,5:i:-	71	43	86	57	0	100	86	86	100	86	100	86	43	29	100	0

Slide 14

Conclusions

- Further studies have to be done, concerning the antimicrobials resistance.
- PFGE will be performed on *S.* Typhimurium PTU302 human and food isolates, both originated from Azores island.
- PFGE will be performed on *S.* Enteritidis isolates belonging to the same PT and with the same antimicrobials resistance pattern, in humans, animals and food isolates.

Appendix 15. Sheets of presentation 4.5

Slide 1

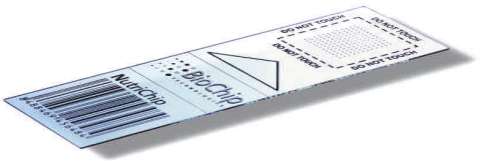
Principles and Applications of DNA-Chip Technology in Veterinary Science

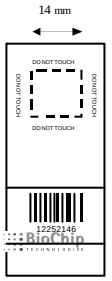
Burkhard Malorny, Reiner Helmuth

BgVV, NRL-Salm

Slide 2

What are DNA-Chips?





DNA-Chips are miniaturised solid supports, on which molecules of nucleic acids have been fixed in high number, density and in a defined order (Microarrays)

DNA-Chips generate information about the presence and expression of a DNA molecule

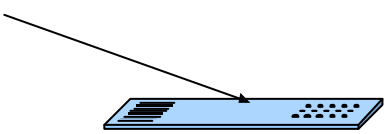
BgVV, NRL-Salm

Slide 3

What is on a DNA-Chip?

DNA Probes (single stranded):

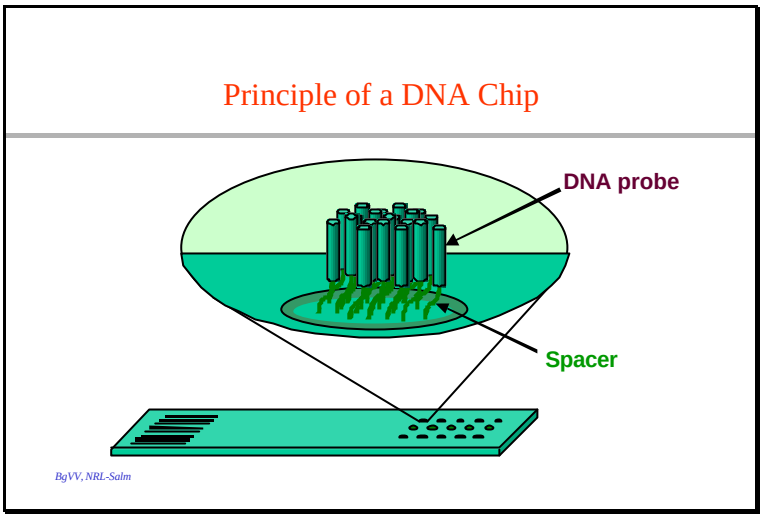
- Oligonucleotides
- PCR Products
- cDNAs
- Plasmids



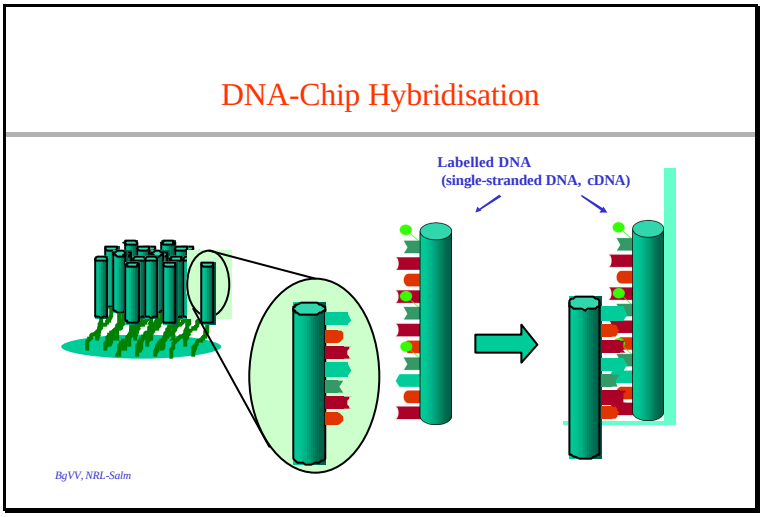
- DNA probes are applied in microscopic amounts (1-10 nl) at a well defined position.
- Complementary DNA of a sample binds to the probe

BgVV, NRL-Salm

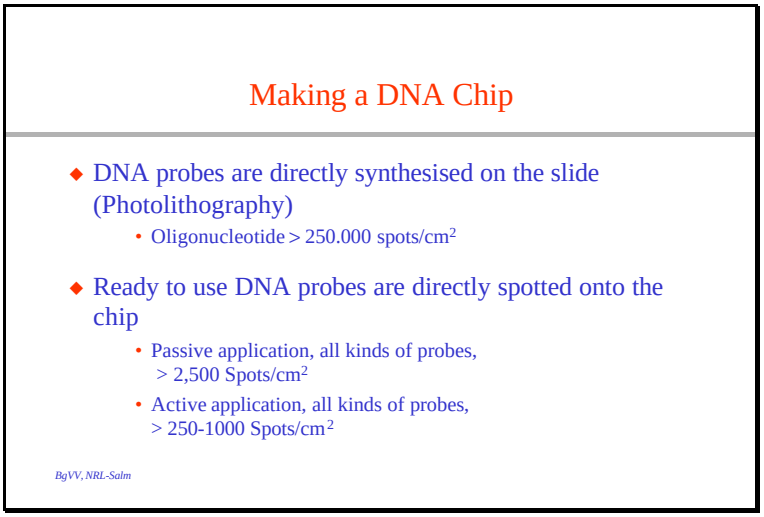
Slide 4



Slide 5

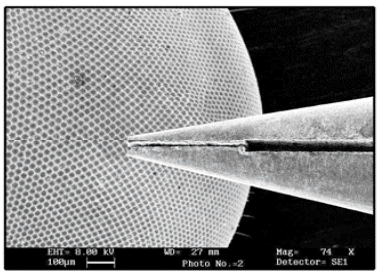


Slide 6



Slide 7

Size of a Spotter-Pin



Electron micrograph of MicroSpot 2500 pin and the eye of a house fly

BgVV, NRL-Salm

Slide 8

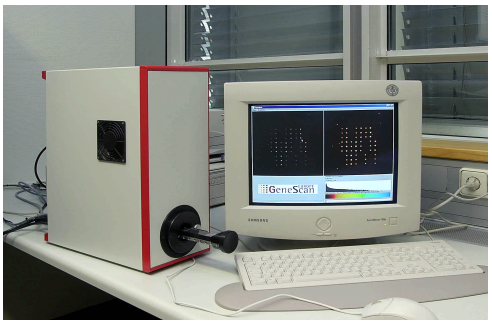
Steps in a DNA Chip-Analysis

1. **Probe Isolation** • genomic DNA • RNA
2. **Labelling** • directly during PCR • indirectly after PCR
3. **Hybridisation** • genomic DNA • PCR products • cDNA probes
4. **Detection** *via* Analyser
5. **Data-Analysis**

BgVV, NRL-Salm

Slide 9

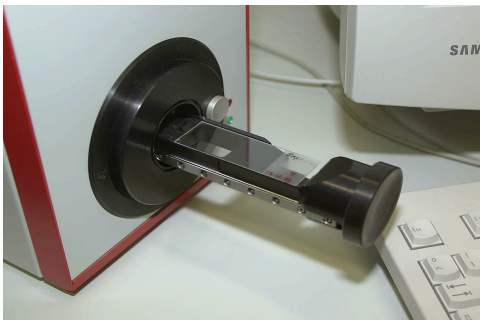
Chip-Reader BioDetect 645/4 (1)



BgVV, NRL-Salm

Slide 10

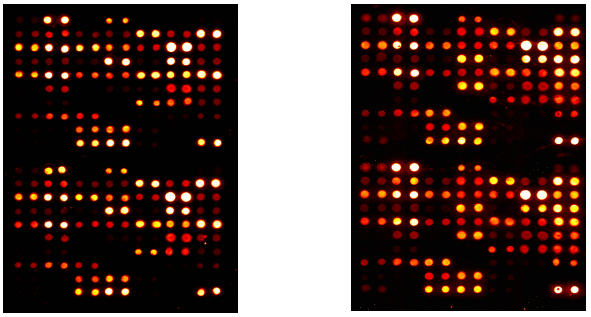
Chip-Reader BioDetect 645/4 (2)



BgVV, NRL-Salm

Slide 11

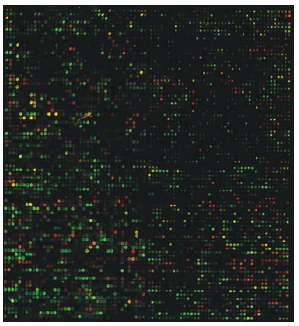
A Microarray after Hybridisation



BgVV, NRL-Salm

Slide 12

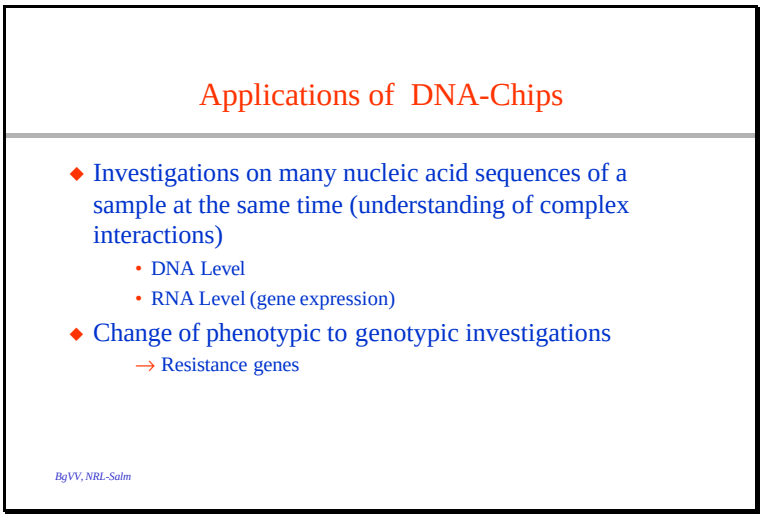
DNA Chip of Genes expressed in Yeast



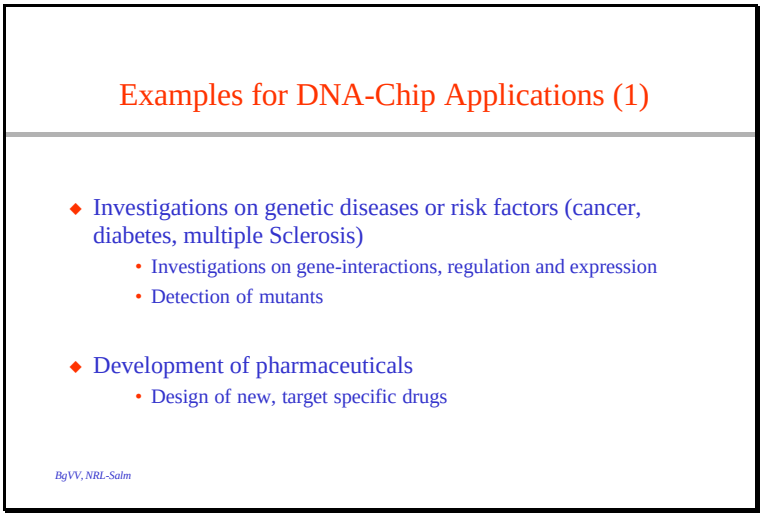
Ref: De Risi et al.
Science 278: 680-686

BgVV, NRL-Salm

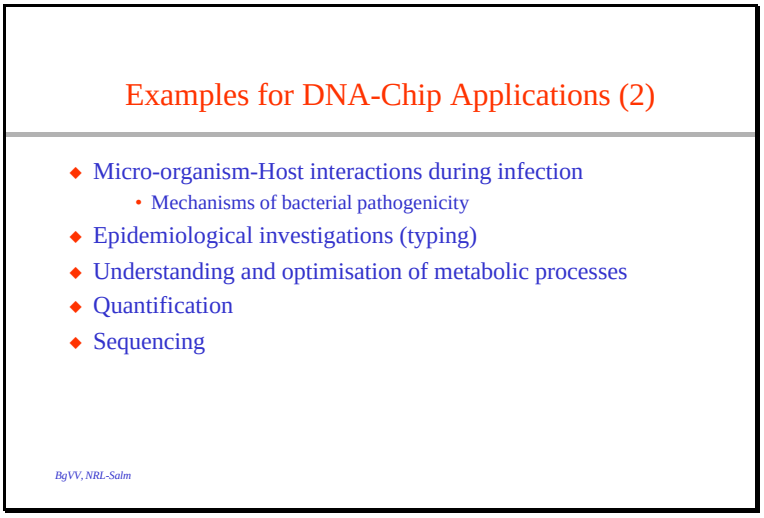
Slide 13



Slide 14



Slide 15



Slide 16

Public Health Applications of DNA-Chip Technologies

- Detection of foodborne pathogens
  - Bacteria, Viruses, Parasites, Fungi
- Genotyping of micro-organisms
- Food-safety (GMO-Analysis)
- Species-differentiation in food items
- Toxicology
- Animal experiments
- Veterinary medicine (Detection of pathogens, inheritable disease)
- Animal breeding

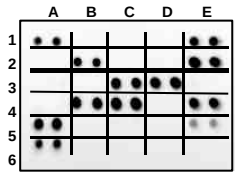
BgVV, NRL-Salm

Slide 17

Examples for DNA-Chip Applications : Genetically modified food items

DNA: Sample No. 26161:
(Sample from routine analysis)

Micro-array hybridization:



shown is one array (out of two)

Micro-array layout:

	A	B	C	D	E
1	CK				CK
2	Bt-S-WT	Bt-S-176/810	Bt-S-Bt11	Bt-S-BtXtra	Bt-S-Bt176
3		Bt-S-810/11	soy	canola	CaMV
4		35S-prom	maize		rice
5	bar	patsyn	nos		FK
6	CK				

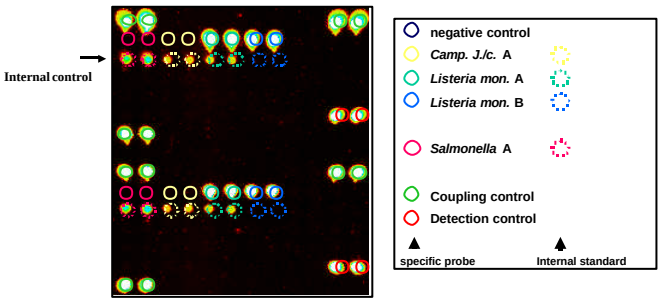
CK = coupling control
FK = detection control
positive hybrization signals aremarked in grey

BgVV, NRL-Salm

Slide 18

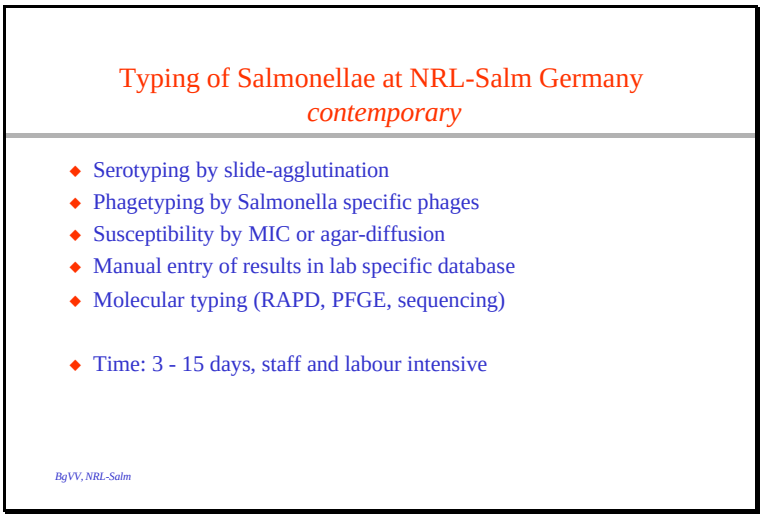
Examples for DNA-Chip Applications: Detection of foodborne pathogens

Multiplex PCR of a *Listeria monocytogenes* contaminated food sample



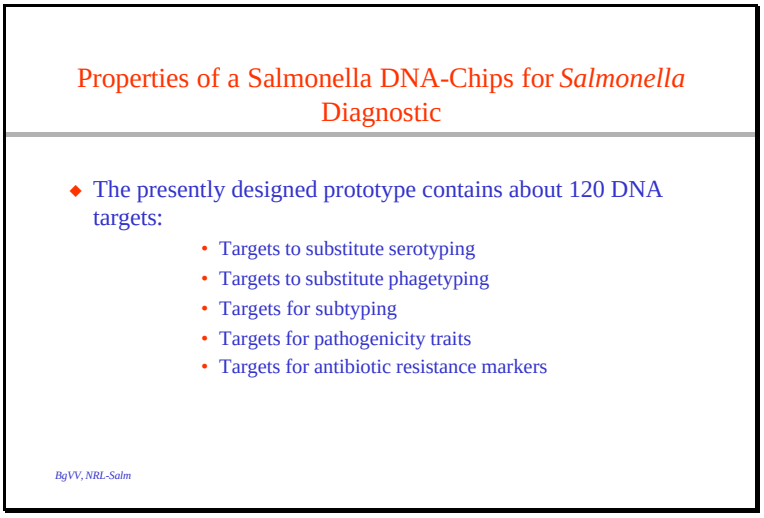
BgVV, NRL-Salm

Slide 19



Slide 20

Slide 21



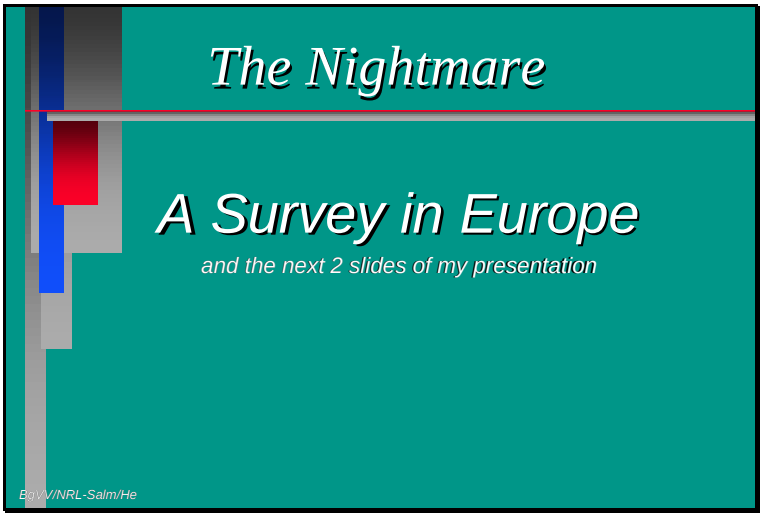
Slide 22


Summary
DNA chip technology is a powerful new technology which allows the simultaneous analysis of many gene targets in one experiment in order to learn their importance, interaction and regulation
DNA chip technology will be important for many institutions in many areas of public health, especially in food hygiene, microbiology, toxicology, and animal welfare
BgVV, NRL-Salm

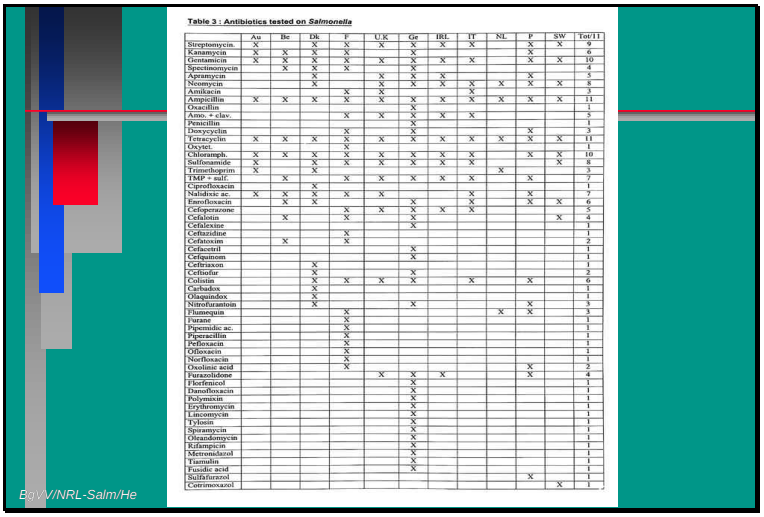
Appendix 16. Sheets of presentation 4.6

Slide 1

Slide 2



Slide 3



	Au	Be	Dk	F	U.K	Ge	IRL	IT	NL	P	SW	Tot/11
Streptomycin.	X		X	X	X	X	X	X		X	X	9
Kanamycin	X	X	X	X		X				X		6
Gentamicin	X	X	X	X	X	X	X	X		X	X	10
Spectinomycin		X	X	X		X						4
Apramycin			X		X	X	X			X		5
Neomycin			X		X	X	X	X	X	X	X	8
Amikacin				X	X			X				3
Ampicillin	X	X	X	X	X	X	X	X	X	X	X	11
Oxacillin						X						1
Amo. + clav.				X	X	X	X	X				5
Penicillin						X						1
Doxycycilin				X		X				X		3
Tetracyclin	X	X	X	X	X	X	X	X	X	X	X	11
Oxytet.				X								1
Chloramph.	X	X	X	X	X	X	X	X		X	X	10
Sulfonamide	X		X	X	X	X	X	X			X	8
Trimethoprim	X		X						X			3
TMP + sulf.		X		X	X	X	X	X		X		7
Ciprofloxacin			X									1
Nalidixic ac.	X	X	X	X	X			X		X		7
Enrofloxacin		X	X			X		X		X	X	6
Cefoperazone				X	X	X	X	X				5
Cefalotin		X		X		X					X	4
Cefalexine						X						1
Cefuzidine				X								1
Cefatoxim		X		X								2
Cefacetril						X						1
Cefquinom						X						1
Ceftriaxon			X									1
Ceftiofur			X			X						2
Colistin			X	X	X	X		X		X		6
Carbadox			X									1
Olaquindox			X									1
Nitrofurantoin			X			X				X		3
Flumequin				X					X	X		3
Furane				X								1
Pipemidic ac.				X								1
Piperacillin				X								1
Pefloxacin				X								1
Ofloxacin				X								1
Norfloxacin				X								1
Oxolinic acid				X						X		2
Furazolidone					X	X	X			X		4
Florfenicol						X						1
Danofloxacin						X						1
Polymixin						X						1
Erythromycin						X						1
Lincomycin						X						1
Tylosin						X						1
Spiramycin						X						1
Oleandomycin						X						1
Rifampicin						X						1
Metronidazol						X						1
Tiamulin						X						1
Fusidic acid						X						1
Sulfafurazol										X		1
Cotrimoxazol											X	1

Slide 4

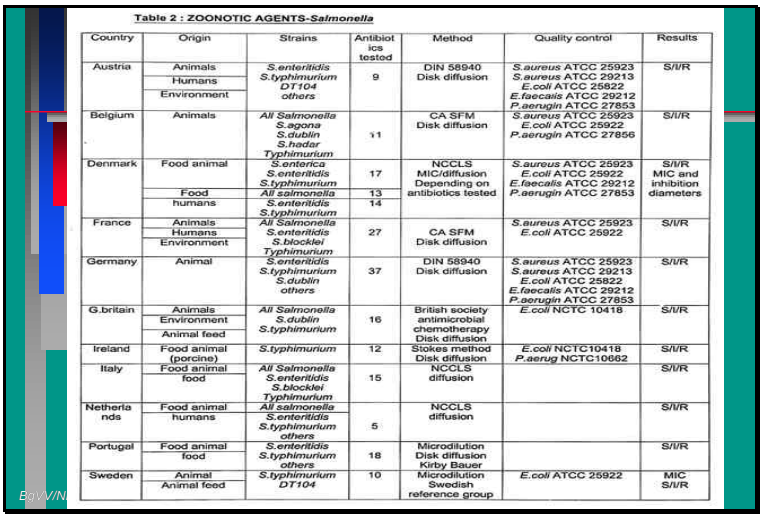
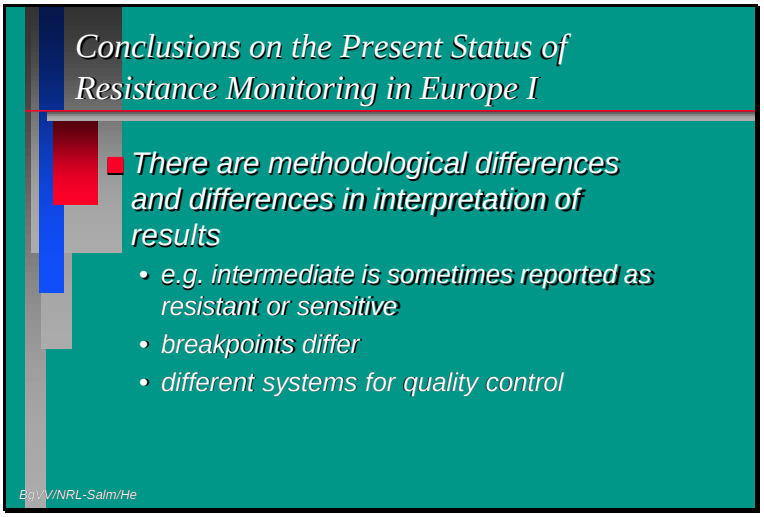


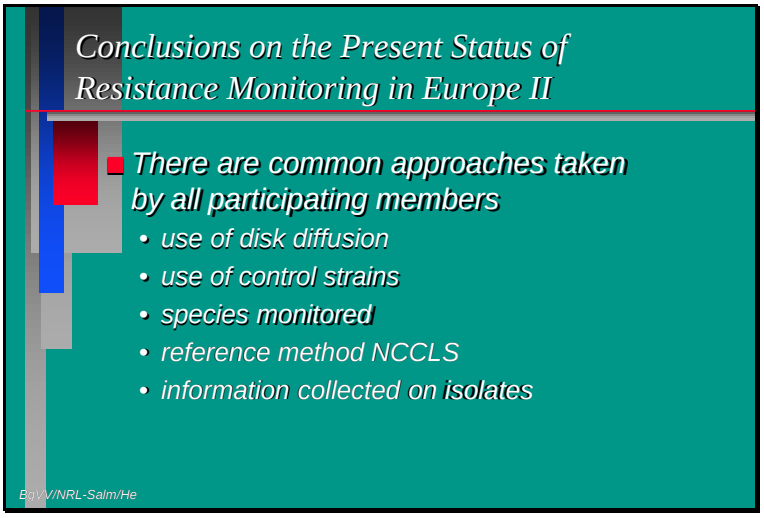
Table 2 : ZOONOTIC AGENTS-*Salmonella*

Country	Origin	Strains	Antibiotics tested	Method	Quality control	Results
Austria	Animals Humans Environment	*S.enteritidis* *S.typhimurium DT104* *others*	9	DIN 58940 Disk diffusion	*S.aureus* ATCC 25923 *S.aureus* ATCC 29213 *E.coli* ATCC 25822 *E.faecalis* ATCC 29212 *P.aerugin* ATCC 27853	S/I/R
Belgium	Animals	*All Salmonella* *S.agona* *S.dublin* *S.hadar* *Typhimurium*	11	CA SFM Disk diffusion	*S.aureus* ATCC 25923 *E.coli* ATCC 25922 *P.aerugin* ATCC 27856	S/I/R
Denmark	Food animal	*S.enterica* *S.enteritidis* *S.typhimurium*	17	NCCLS MIC/diffusion Depending on antibiotics tested	*S.aureus* ATCC 25923 *E.coli* ATCC 25922 *E.faecalis* ATCC 29212 *P.aerugin* ATCC 27853	S/I/R MIC and inhibition diameters
	Food	*All salmonella*	13			
	humans	*S.enteritidis* *S.typhimurium*	14			
France	Animals Humans Environment	*All Salmonella* *S.enteritidis* *S.blockley* *Typhimurium*	27	CA SFM Disk diffusion	*S.aureus* ATCC 25923 *E.coli* ATCC 25922	S/I/R
Germany	Animal	*S.enteritidis* *S.typhimurium* *S.dublin* *others*	37	DIN 58940 Disk diffusion	*S.aureus* ATCC 25923 *S.aureus* ATCC 29213 *E.coli* ATCC 25822 *E.faecalis* ATCC 29212 *P.aerugin* ATCC 27853	S/I/R
G.britain	Animals Environment Animal feed	*All Salmonella* *S.dublin* *S.typhimurium*	16	British society antimicrobial chemotherapy Disk diffusion	*E.coli* NCTC 10418	S/I/R
Ireland	Food animal (porcine)	*S.typhimurium*	12	Stokes method Disk diffusion	*E.coli* NCTC10418 *P.aerug* NCTC10662	S/I/R
Italy	Food animal food	*All Salmonella* *S.enteritidis* *S.blockley* *Typhimurium*	15	NCCLS diffusion		S/I/R
Netherlands	Food animal humans	*All salmonella* *S.enteritidis* *S.typhimurium* *others*	5	NCCLS diffusion		S/I/R
Portugal	Food animal food	*S.enteritidis* *S.typhimurium* *others*	18	Microdilution Disk diffusion Kirby Bauer		S/I/R
Sweden	Animal Animal feed	*S.typhimurium DT104*	10	Microdilution Swedish reference group	*E.coli* ATCC 25922	MIC S/I/R

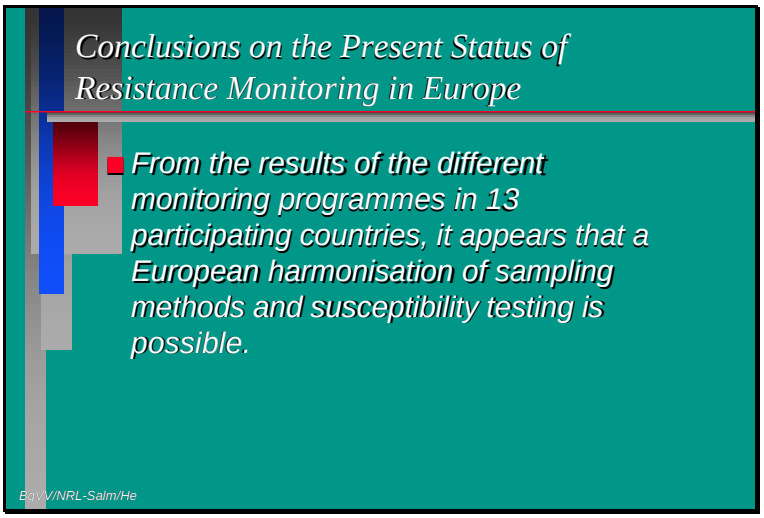
Slide 5



Slide 6

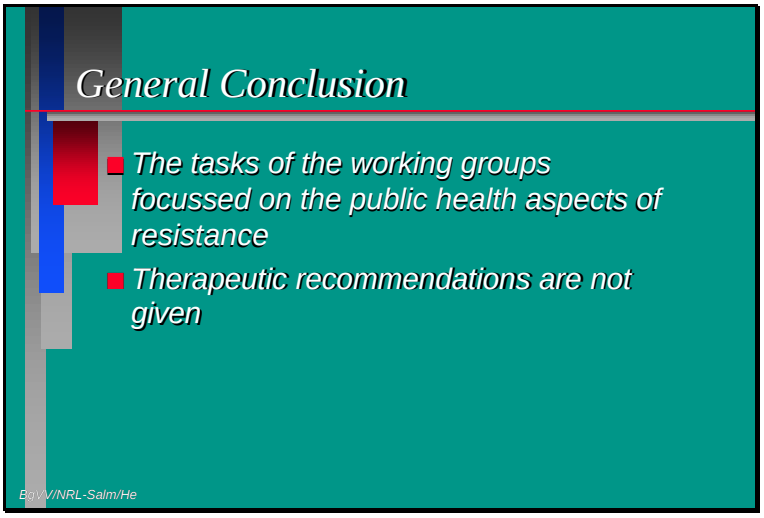


Slide 7

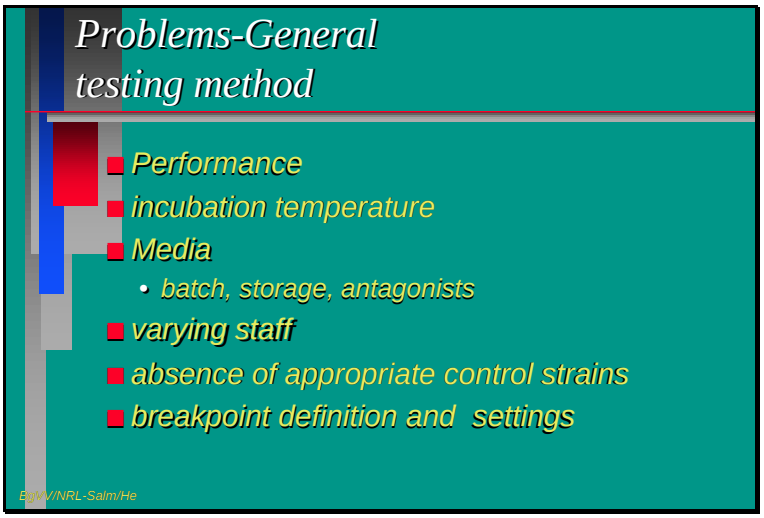


Slide 8

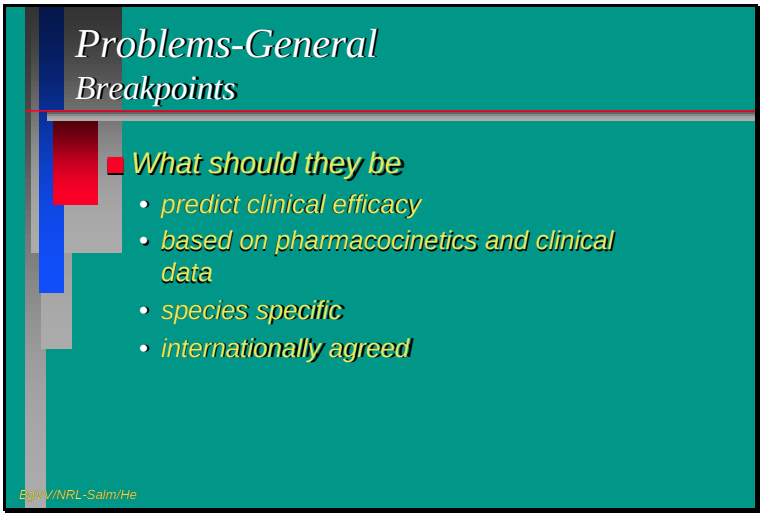
Slide 9



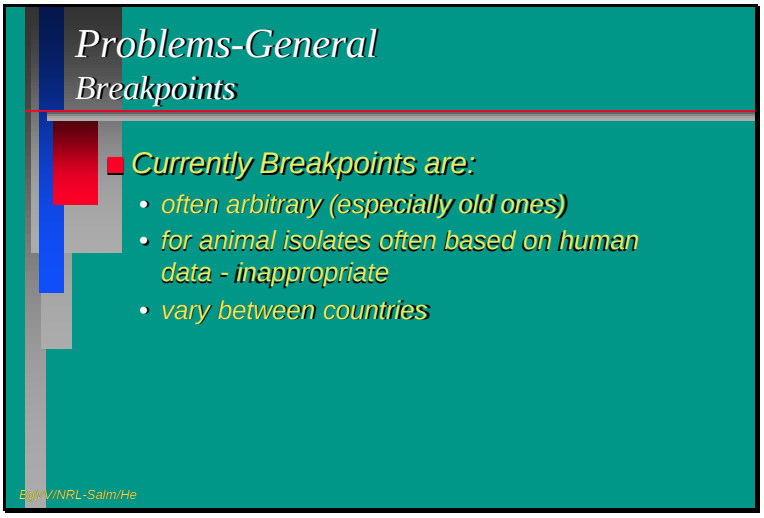
Slide 10



Slide 11

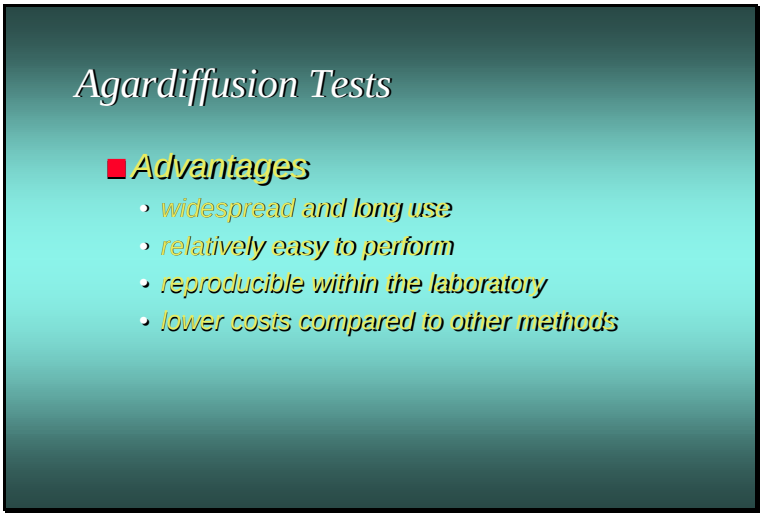


Slide 12

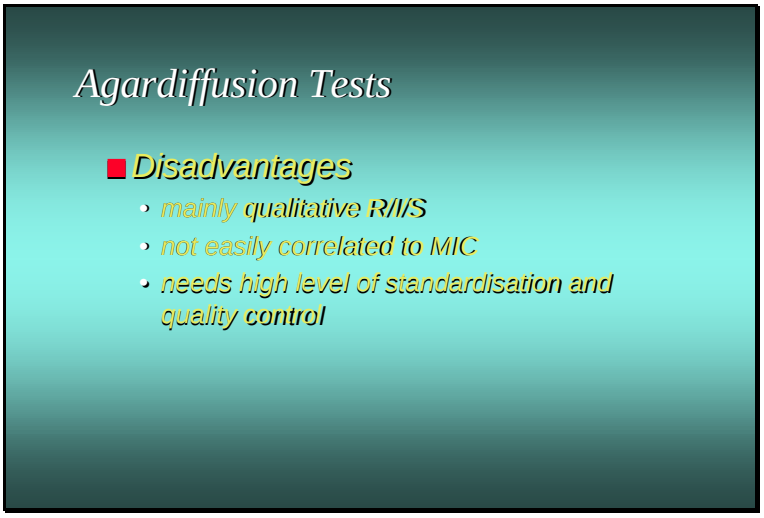


Slide 13

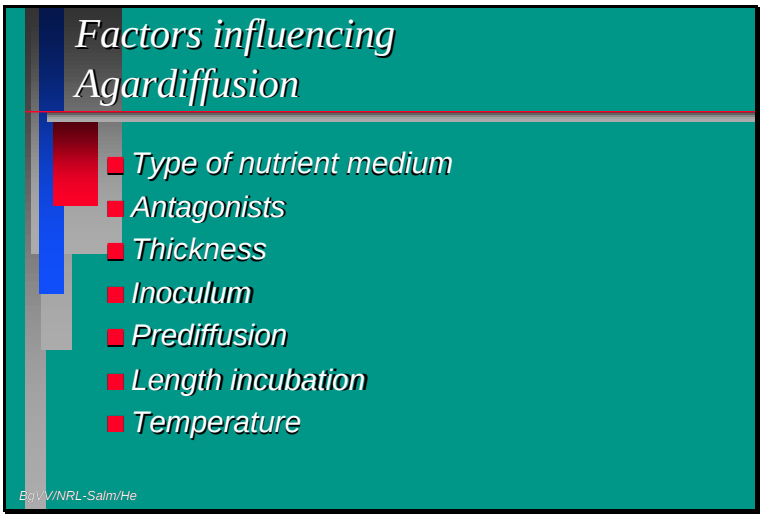
Slide 14



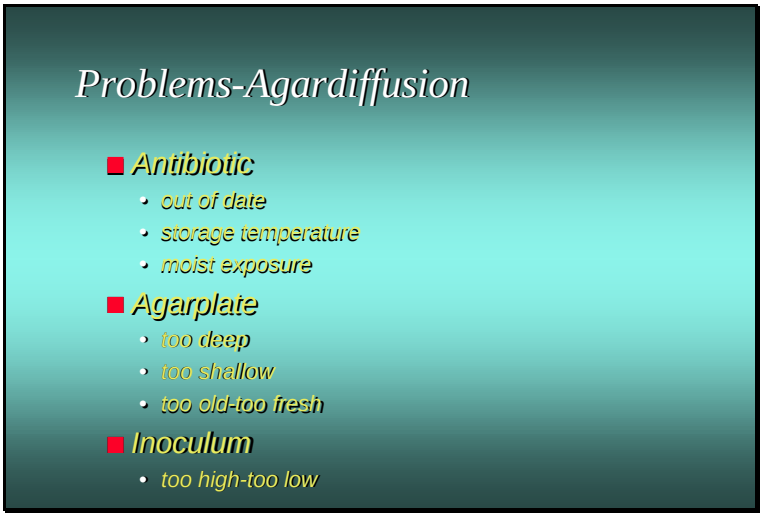
Slide 15



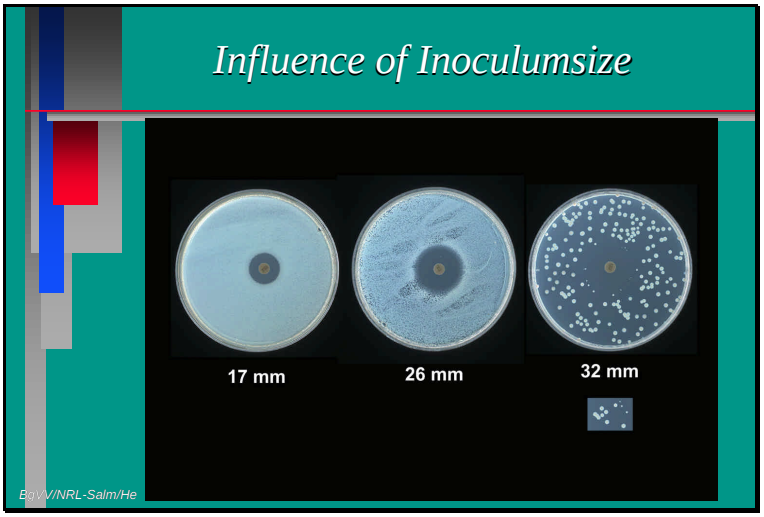
Slide 16



Slide 17



Slide 18



Slide 19

Broth Dilution

- Advantages
  - quantitative gives MIC
  - reproducible
  - automatization
- Disadvantages
  - lots of material are used
  - cost

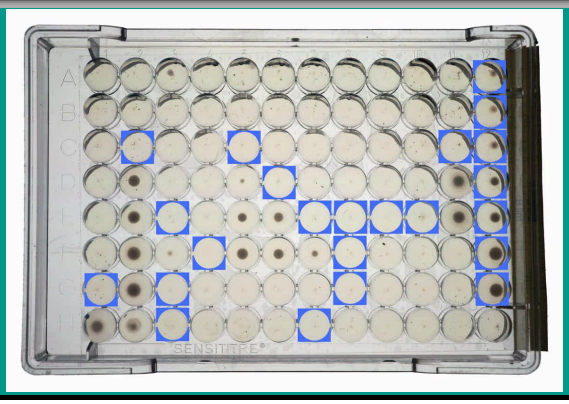
Slide 20

Problems Broth Dilution

- Inoculum
  - number of cfu
  - growth rate
- Culture Medium
  - Divalent Cations Ca++ Mg++
    - Tetracyclines, Aminoglycosides
  - pH
    - Aminoglycosides, MLS, Quinolones
  - Thymine, Thymidine
    - Sulfonamides, Trimethoprim

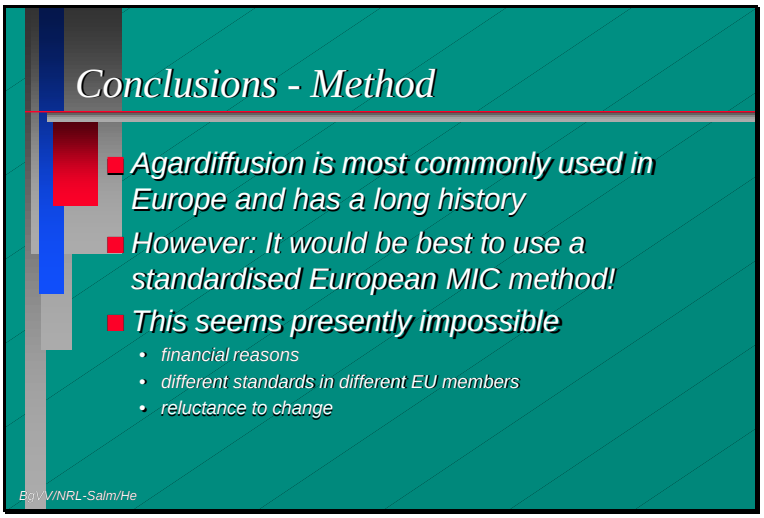
Slide 21

Broth Microdilution

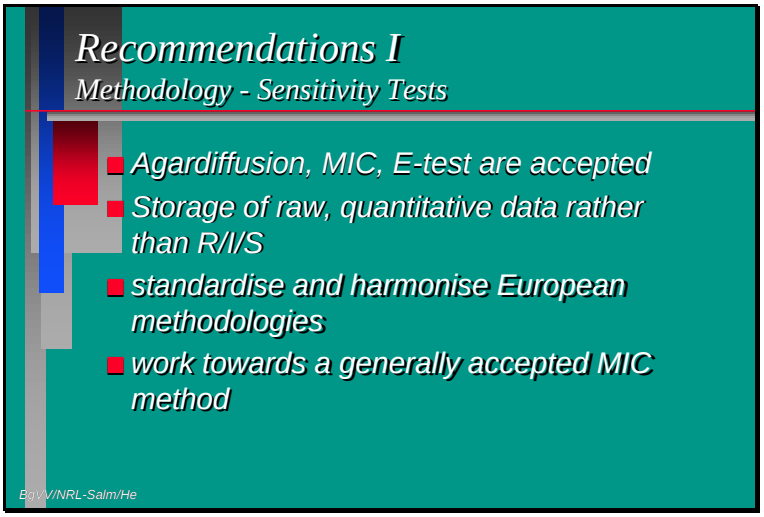


BuVV/NRL-Salm/He

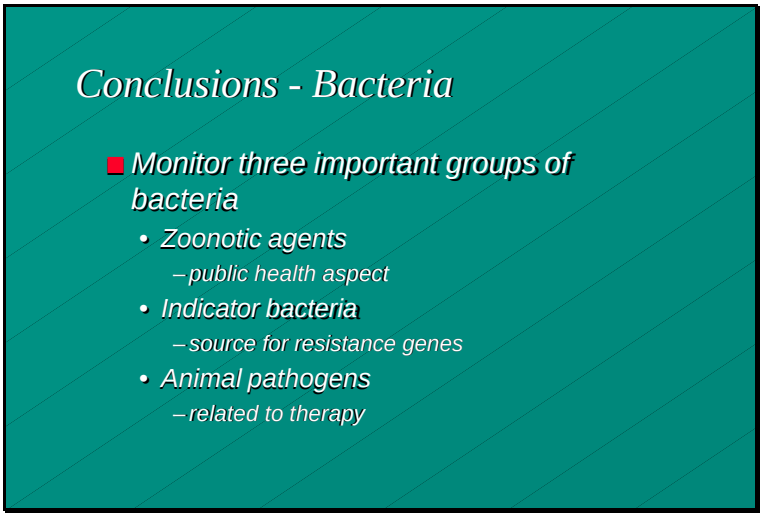
Slide 22



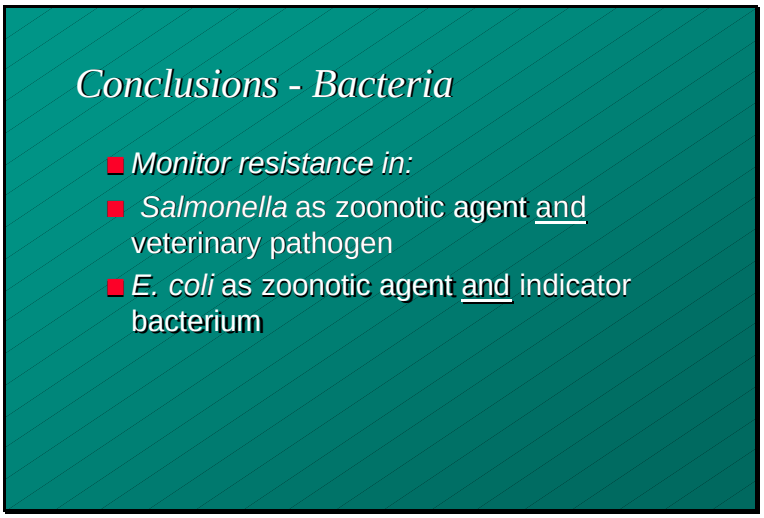
Slide 23



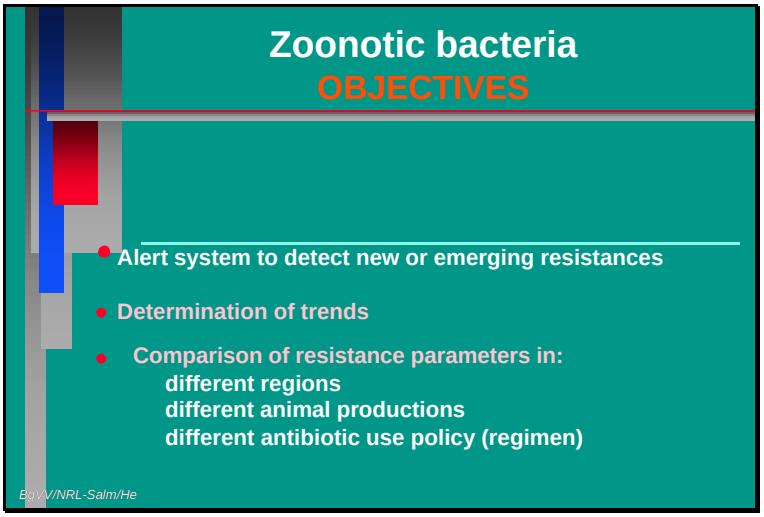
Slide 24



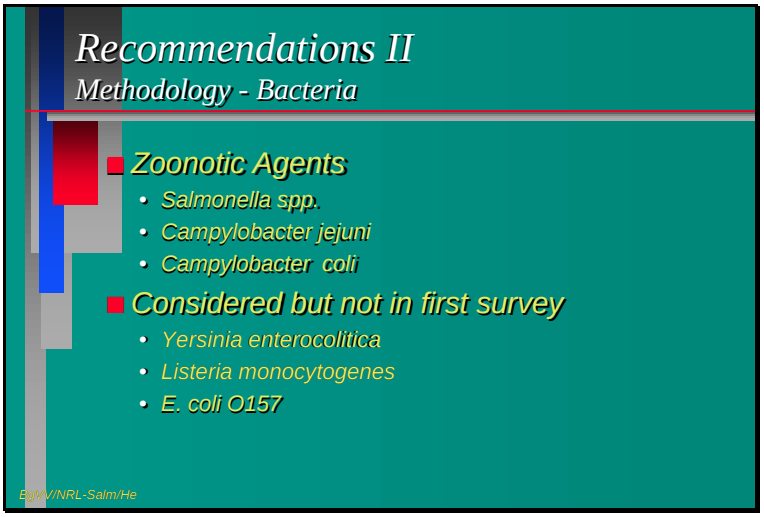
Slide 25



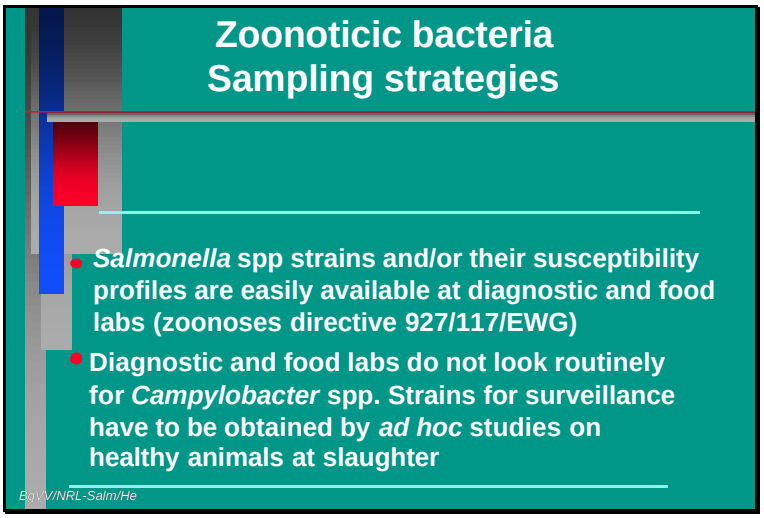
Slide 26



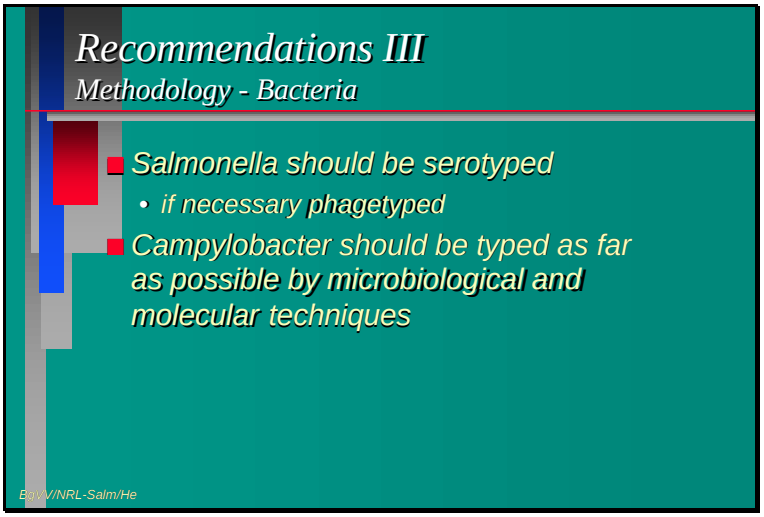
Slide 27



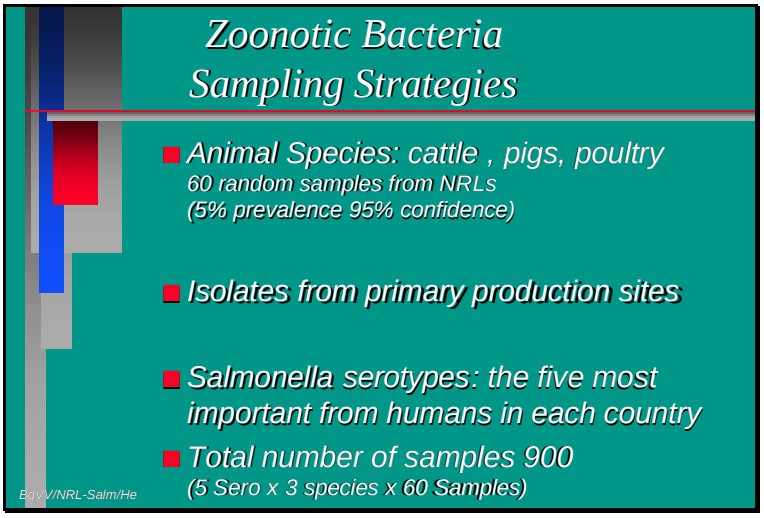
Slide 28



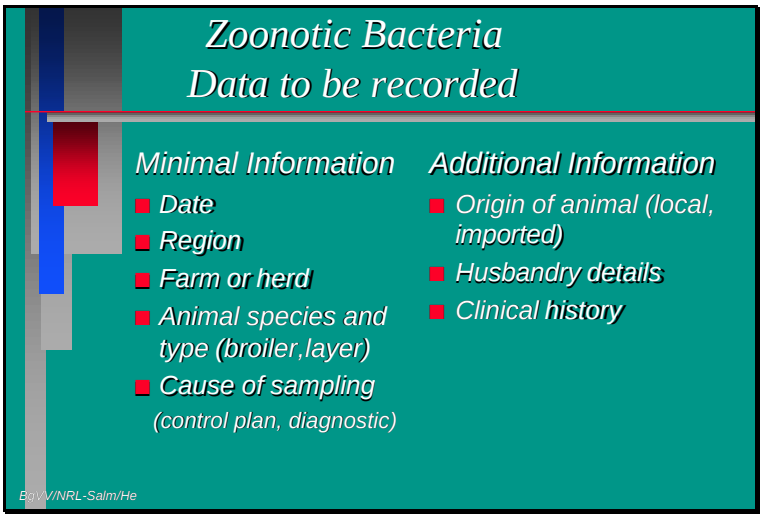
Slide 29



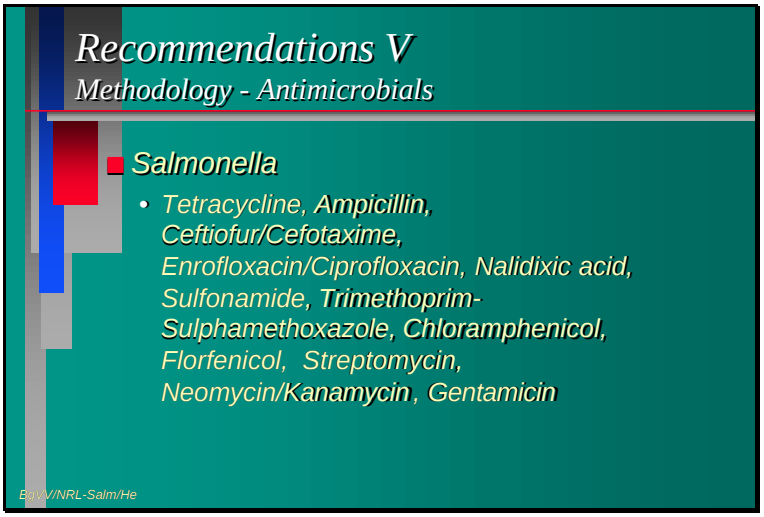
Slide 30



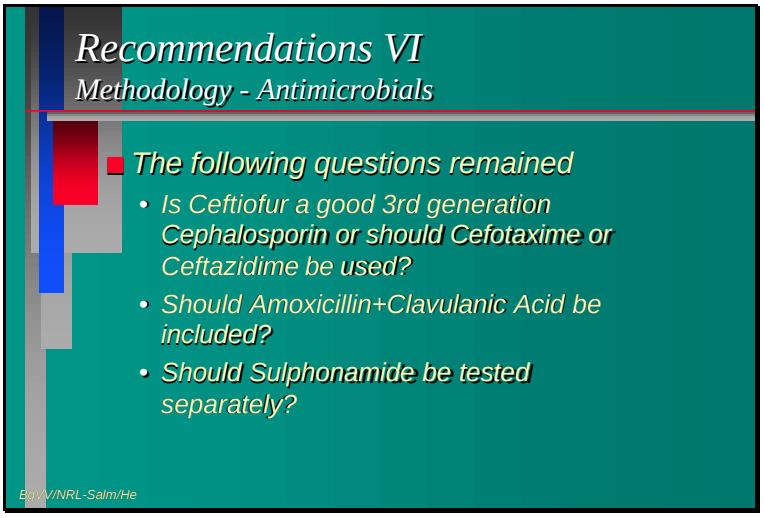
Slide 31



Slide 32



Slide 33



Slide 34

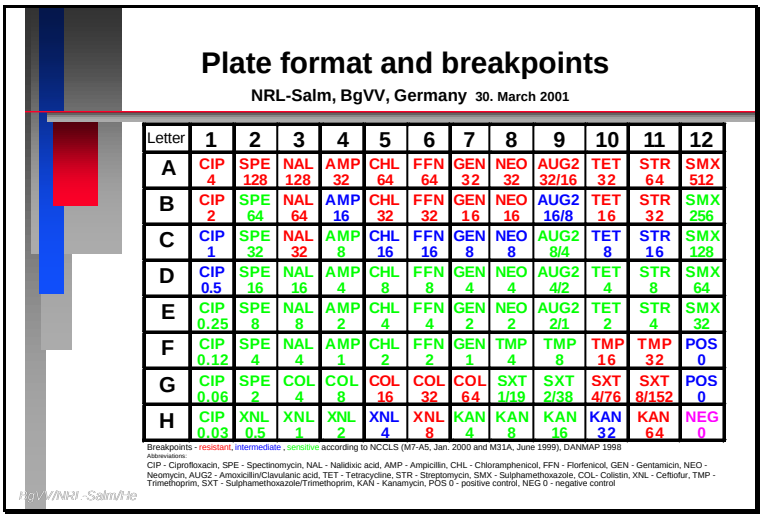


Plate format and breakpoints

NRL-Salm, BgVV, Germany 30. March 2001

Letter	1	2	3	4	5	6	7	8	9	10	11	12
A	CIP 4	SPE 128	NAL 128	AMP 32	CHL 64	FFN 64	GEN 32	NEO 32	AUG2 32/16	TET 32	STR 64	SMX 512
B	CIP 2	SPE 64	NAL 64	AMP 16	CHL 32	FFN 32	GEN 16	NEO 16	AUG2 16/8	TET 16	STR 32	SMX 256
C	CIP 1	SPE 32	NAL 32	AMP 8	CHL 16	FFN 16	GEN 8	NEO 8	AUG2 8/4	TET 8	STR 16	SMX 128
D	CIP 0.5	SPE 16	NAL 16	AMP 4	CHL 8	FFN 8	GEN 4	NEO 4	AUG2 4/2	TET 4	STR 8	SMX 64
E	CIP 0.25	SPE 8	NAL 8	AMP 2	CHL 4	FFN 4	GEN 2	NEO 2	AUG2 2/1	TET 2	STR 4	SMX 32
F	CIP 0.12	SPE 4	NAL 4	AMP 1	CHL 2	FFN 2	GEN 1	TMP 4	TMP 8	TMP 16	TMP 32	POS 0
G	CIP 0.06	SPE 2	COL 4	COL 8	COL 16	COL 32	COL 64	SXT 1/19	SXT 2/38	SXT 4/76	SXT 8/152	POS 0
H	CIP 0.03	XNL 0.5	XNL 1	XNL 2	XNL 4	XNL 8	KAN 4	KAN 8	KAN 16	KAN 32	KAN 64	NEG 0

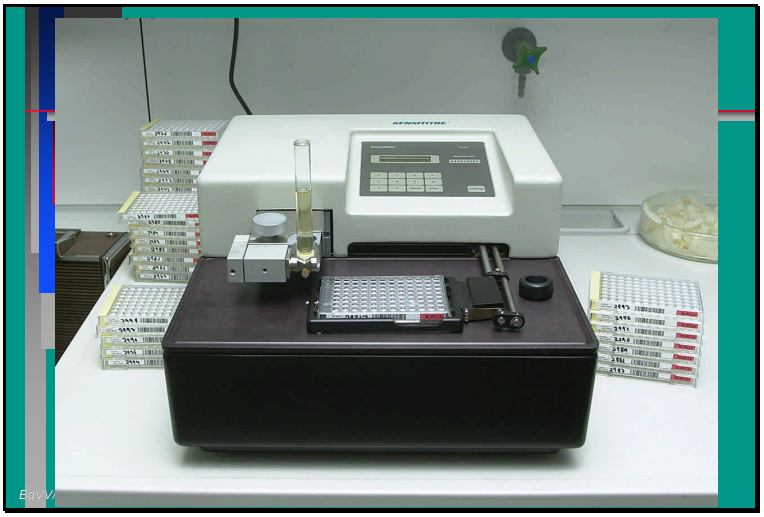
Breakpoints - resistant, intermediate , sensitive according to NCCLS (M7-A5, Jan. 2000 and M31A, June 1999), DANMAP 1998

Abbreviations:

CIP - Ciprofloxacin, SPE - Spectinomycin, NAL - Nalidixic acid, AMP - Ampicillin, CHL - Chloramphenicol, FFN - Florfenicol, GEN - Gentamicin, NEO - Neomycin, AUG2 - Amoxicillin/Clavulanic acid, TET - Tetracycline, STR - Streptomycin, SMX - Sulphamethoxazole, COL- Colistin, XNL - Ceftiofur, TMP - Trimethoprim, SXT - Sulphamethoxazole/Trimethoprim, KAN - Kanamycin, POS 0 - positive control, NEG 0 - negative control

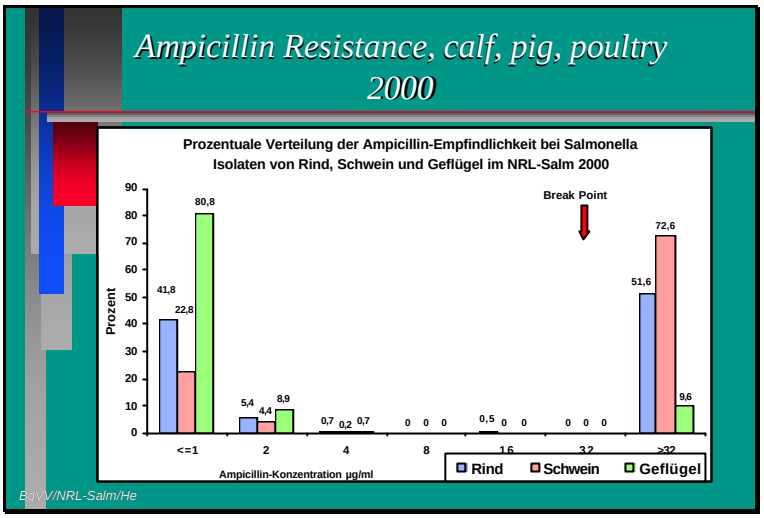
BgVV/NRL Salm/Fe

Slide 35

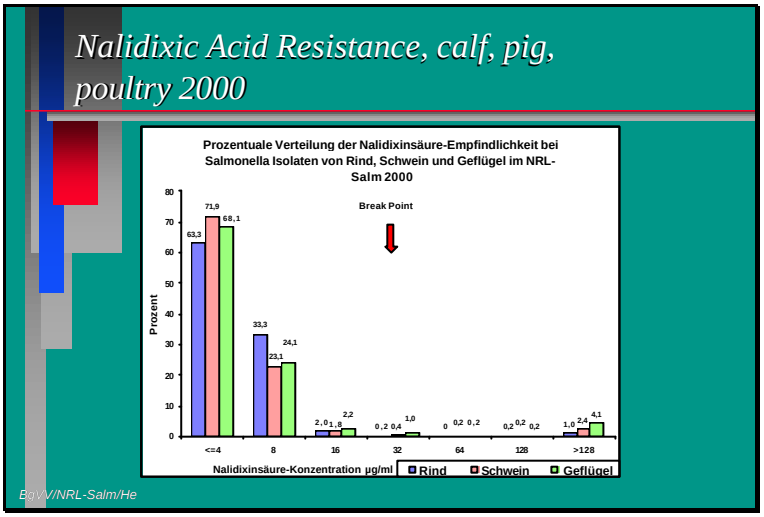


Slide 36

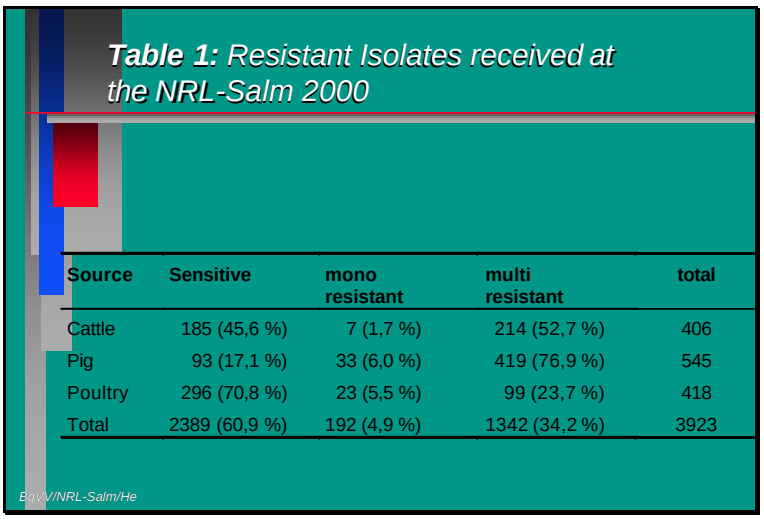
Slide 37



Slide 38

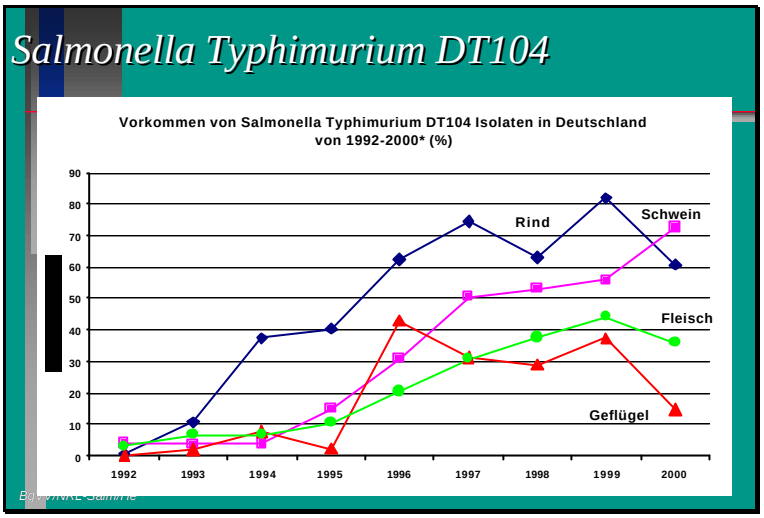


Slide 39

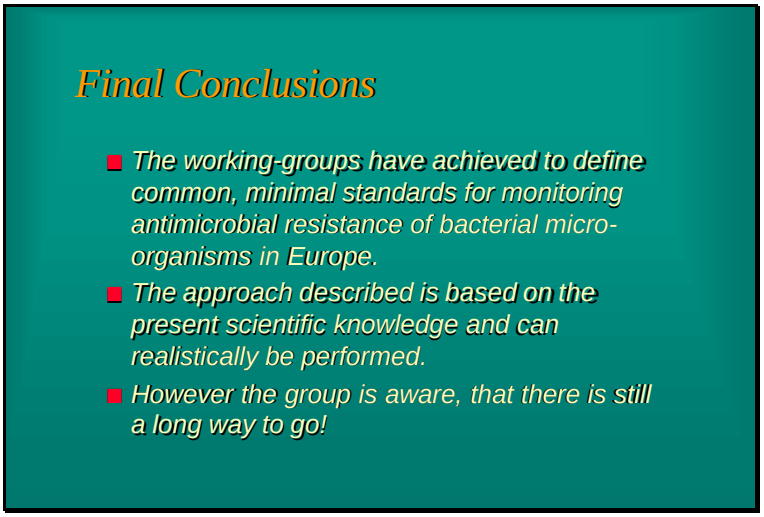


Source	Sensitive	mono resistant	multi resistant	total
Cattle	185 (45,6 %)	7 (1,7 %)	214 (52,7 %)	406
Pig	93 (17,1 %)	33 (6,0 %)	419 (76,9 %)	545
Poultry	296 (70,8 %)	23 (5,5 %)	99 (23,7 %)	418
Total	2389 (60,9 %)	192 (4,9 %)	1342 (34,2 %)	3923

Slide 40

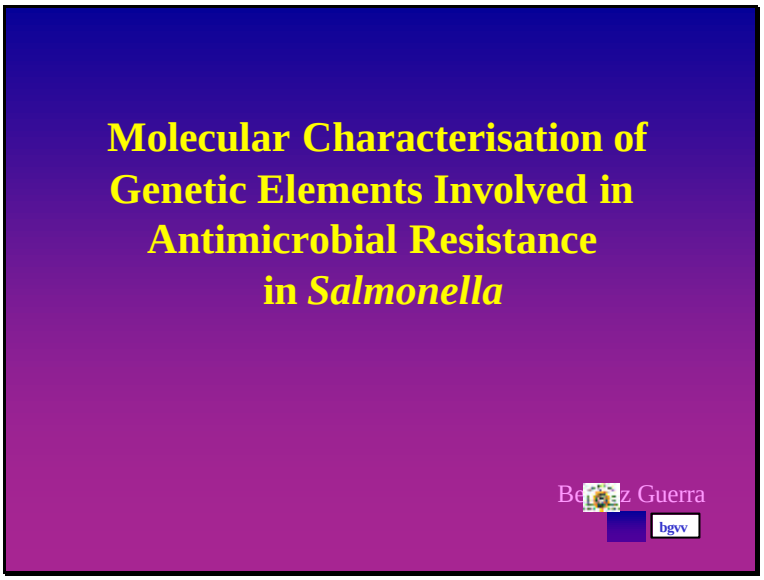


Slide 41

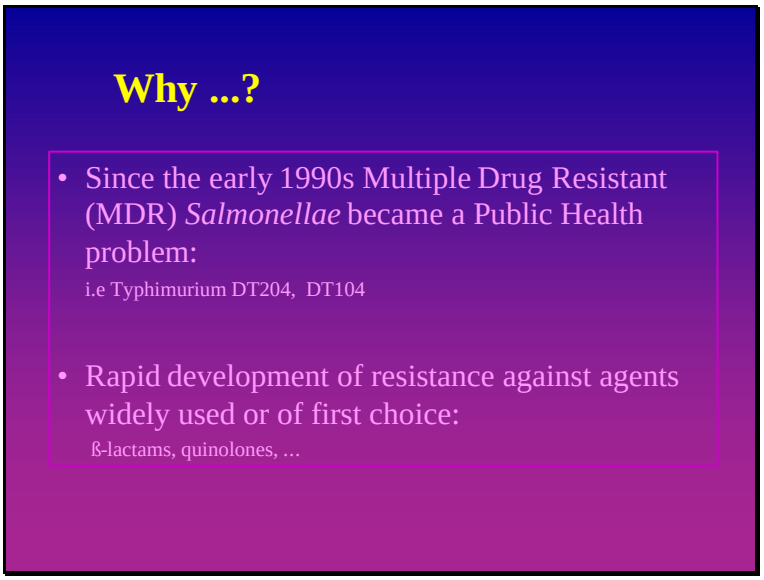


Appendix 17. Sheets of presentation 4.7

Slide 1



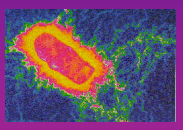
Slide 2



Slide 3

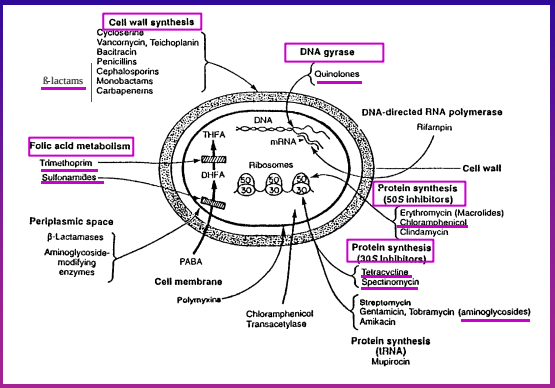
Antimicrobial Resistance

...



Slide 4

Targets of Antimicrobial Action



Slide 5

Resistance Mechanisms

- Modification of cell-permeability:
  - ↓ porins
- Active Excretion:
  - ↑ efflux pumps
- Enzymatic inactivation:
  - hydrolisis, introduction of chemical radicals
- Modification of the target

Slide 6

Resistance Mechanisms

Main bacterial resistance mechanisms against classes of antibiotics

Class of antibiotics	Cellular target	Modification of				Over-production
		bacterial cell permeability		antibiotic inactivation	target	
		Decreased influx	Increased efflux			
β-Lactams	Penicillin-binding proteins	+	-	+	+	-
Aminoglycosides	30S ribosomal subunit	+	-	+	+	-
Sulphonamides	Dihydropteroate synthetase	+	-	-	+	+
Trimethoprim	Dihydrofolate reductase	+	-	-	+	+
Macrolides and Lincosamides	50S ribosomal subunit	+	+	+	+	-
Streptogramins	50S ribosomal subunit	+	+	+	+	-
Tetracyclines	30S ribosomal subunit	-	+	+	+	-
Chloramphenicol	50S ribosomal subunit	+	+/-	+	-	-
Quinolones (Fluoroquinolones)	DNA gyrase Topoisomerase IV	+	+	-	+	-
Glycopeptides	Peptidoglycan precursor	-	-	-	+	-

Slide 7

Important Resistance Genes in *Salmonella*

ß-lactamases:

Important families: TEM, OXA, SHV, CARB, etc.

Classification of Bush et al. 1995 (Antimicrob. Agents Chemoth.)
www.lahey.org/studies/webt.htm

Salmonella: *pse1* (*carb-2*) in DT104, *oxa-1*, *tem-1* like

Slide 8

Important Resistance Genes in *Salmonella*

Aminoglycosides:

- Adenyl (nucleotidyl) transferases AAD (ANT)
- Acetyl transferases: AAC
- Phosphotransferases: APH

Shaw et al. 1998 (Methods. Mol.).

Salmonella: *aadA2* (DT104), *aadA1a* (integron): S-Sp
aac (3)-IV: Gm, etc.

Slide 9

Important Resistance Genes in *Salmonella*

Quinolones:

Mutations in the QRDR of
• gyrase (*gyrA* and/or *B*) and
•Topoisomerase IV (*parC* and *parE*).

Vet. Res. (Special Issue). Jul 2001

Salmonella:
1° mutation high Nal, increased FQ
2° mutation high FQ resistance

Slide 10

Table 6: MIC for Nalidixic acid and Fqs and Pointmutations in QRDR of GyraseA.

Strain.	Source	MIC CIP[1] (µg/ml)	MIC NAL[2] (µg/ml)	Codon 83 (cod. Nuleotide)	Codon 87 (cod. Nukleotide)
00-2992	human	0,03	8	Ser (TTC)	Asp (GAC)
00-2627	poultry	0,03	≤ 4	Ser (TTC)	Asp (GAC)
00-2987	human	0,5	≥ 128	Tyr (TAC)	Asp (GAC)
00-832	eg	0,5	≥ 128	Tyr (TAC)	Asp (GAC)
00-2864	Tiramisu	0,5	≥ 128	Ser (TTC)	Tyr (TAC)
00-2977	human	0,5	≥ 128	Ser (TTC)	Tyr (TAC)

[1]: Ciprofloxacin
[2]: Nalidixic acid

Slide 11

Important Resistance Genes in *Salmonella*

Chloramphenicol:
Acetyl transferases (*cat*),
Efflux pumps: *floR* (DT104), *cmlA*

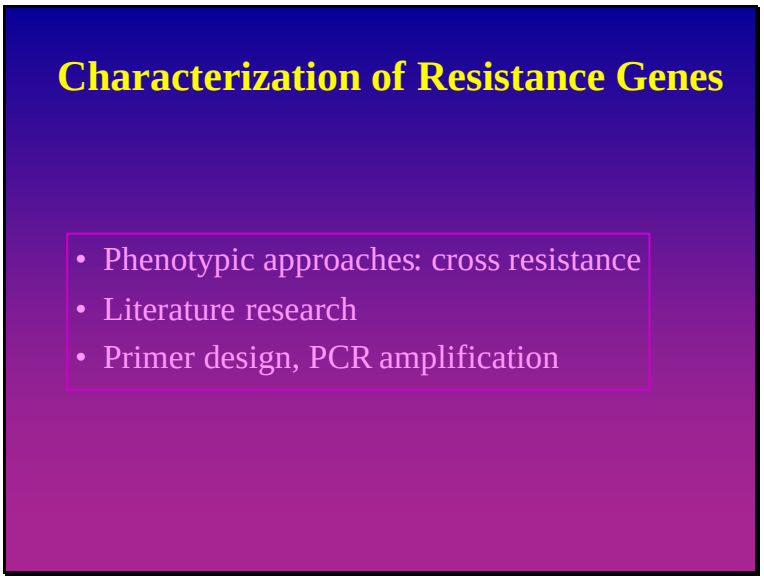
Tetracycline: 61 resistance genes in 32 classes
Efflux pumps: *tet (G)*: *tetR*, *tetA* (DT104); *tet(B); tet (A)*

Sulphonamides: low affinity dihydropteroate synthetase
sul1 (class 1integrons), *sul2*

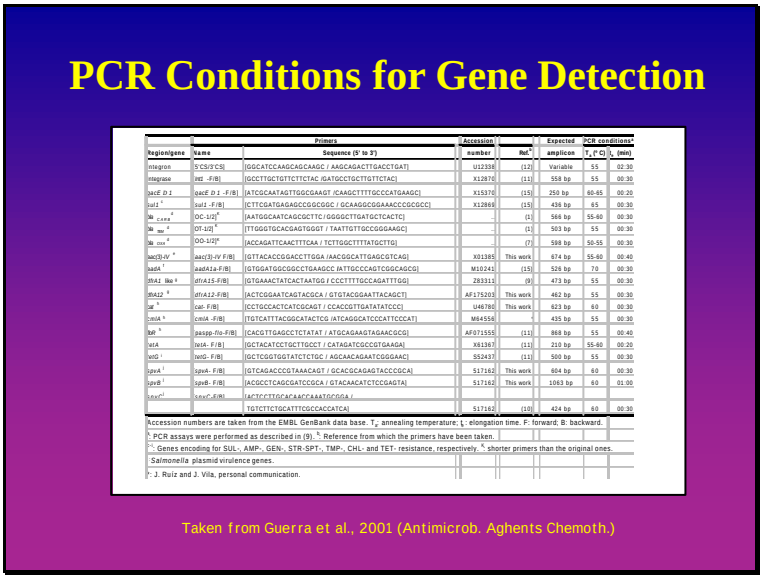
Trimethoprim: low affinity dihydrofolate reductase
dfr(dhfr)A14, *dfrA*1, *dfrA12*, etc.

Slide 12

Slide 13



Slide 14

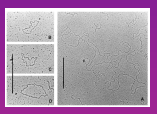


Region/gene	Primers Name	Primers Sequence (5' to 3')	Accession number	Ref.	Expected amplicon	PCR conditions T_a (°C)	PCR conditions t_e (min)
integron	5'CS/3'CS	[GGCATCCAAGCAGCAAGC / AAGCAGACTTGACCTGAT]	U12338	(12)	Variable	55	02:30
integrase	*int* -F/B]	[GCCTTGCTGTTCTTCTAC /GATGCCTGCTTGTTCTAC]	X12870	(11)	558 bp	55	00:30
qacE D 1	*qacE D 1* -F/B]	[ATCGCAATAGTTGGCGAAGT /CAAGCTTTTGCCCATGAAGC]	X15370	(15)	250 bp	60-65	00:20
sul 1 [a]	*sul1* -F/B]	[CTTCGATGAGAGCCGGCGGC / GCAAGGCGGAAACCCGCGCC]	X12869	(15)	436 bp	65	00:30
tet A	OC-1/2[d]	[AATGGCAATCAGCGCTTC/ GGGGCTTGATGCTCACTC]	-	(1)	566 bp	55-60	00:30
tet B	OT-1/2[d]	[TTGGGTGCACGAGTGGGT / TAATTGTTGCCGGGAAGC]	-	(1)	503 bp	55	00:30
tet C	OO-1/2[d]	[ACCAGATTCAACTTTCAA / TCTTGGCTTTTATGCTTG]	-	(7)	598 bp	50-55	00:30
aac(3)-IV [e]	*aac(3)-IV* F/B]	[GTTACACCGGACCTTGGA /AACGGCATTGAGCGTCAG]	X01385	This work	674 bp	55-60	00:40
aadA [f]	*aadA1a*-F/B]	[GTGGATGGCGGCCTGAAGCC /ATTGCCCAGTCGGCAGCG]	M10241	(15)	526 bp	70	00:30
dfrA1 like [g]	*dfrA15*-F/B]	[GTGAAACTATCACTAATGG / CCCTTTTGCCAGATTTGG]	Z83311	(9)	473 bp	55	00:30
dfrA12 [g]	*dfrA12*-F/B]	[ACTCGGAATCAGTACGCA / GTGTACGGAATTACAGCT]	AF175203	This work	462 bp	55	00:30
cat [h]	*cat*- F/B]	[CCTGCCACTCATCGCAGT / CCACCGTTGATATATCCC]	U40780	This work	623 bp	60	00:30
cmlA [h]	*cmlA* -F/B]	[TGTCATTTACGGCATACTCG /ATCAGGCATCCCATTCCCAT]	M64556	-	435 bp	55	00:30
floR [h]	*paspp-flo*-F/B]	[CACGTTGAGCCTCTATAT / ATGCAGAAGTAGAACGCG]	AF071555	(11)	868 bp	55	00:40
tetA	*tetA*- F/B]	[GCTACATCCTGCTTGCCT / CATAGATCGCCGTGAAGA]	X61367	(11)	210 bp	55-60	00:20
tetG [i]	*tetG*- F/B]	[GCTCGGTGGTATCTCTGC / AGCAACAGAATCGGGAAC]	S52437	(11)	500 bp	55	00:30
spvA [j]	*spvA*- F/B]	[GTCAGACCCGTAAACAGT / GCACGCAGAGTACCCGCA]	517162	This work	604 bp	60	00:30
spvB [j]	*spvB*- F/B]	[ACGCCTCAGCGATCCGCA / GTACAACATCTCCGAGTA]	517162	This work	1063 bp	60	01:00
invA[c]	*invA*-F/B]	[*ACTCCTTGCACAACCAAATGCGCGA* /					
		TGTCTTCTGCATTTCGCCACCATCA]	517162	(10)	424 bp	60	00:30

Accession numbers are taken from the EMBL GenBank data base. T_a: annealing temperature; t_e: elongation time. F: forward; B: backward.
[a]: PCR assays were performed as described in (9). [b]: Reference from which the primers have been taken.
[c]-[i]: Genes encoding for SUL-, AMP-, GEN-, STR-SPT-, TMP-, CHL- and TET- resistance, respectively. [k]: shorter primers than the original ones.
[j]: *Salmonella* plasmid virulence genes.
[l]: J. Ruiz and J. Vila, personal communication.

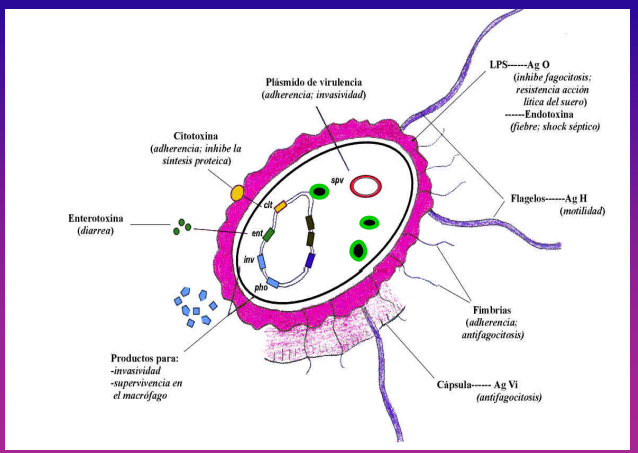
Slide 15

Vehicles of Resistance Transmission ...



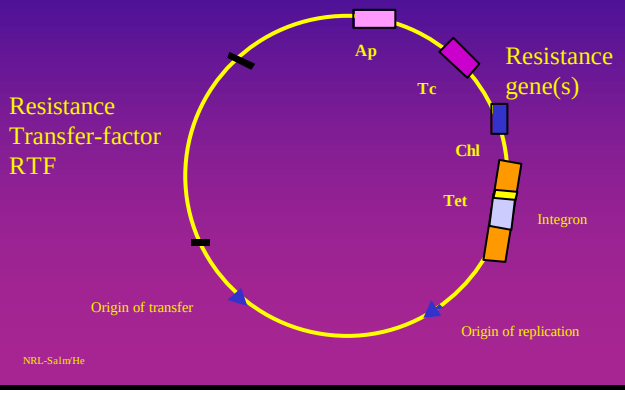
Slide 16

Resistance Plasmids in Salmonella



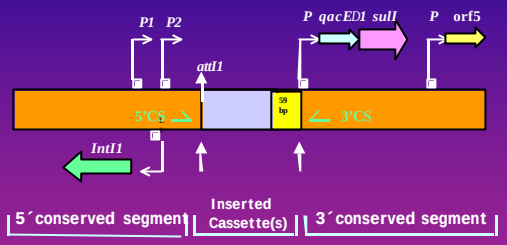
Slide 17

Scheme of a Conjugative Resistance-factor



Slide 18

Class 1 Integrons: Structure

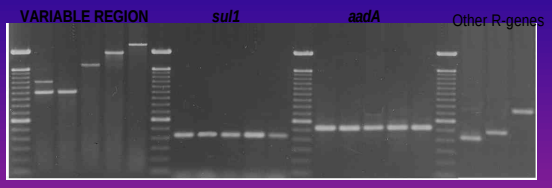


5´conserved segment | Inserted Cassette(s) | 3´conserved segment

Taken from Lévesque et al., 1996

Slide 19

INTEGRONS and R-*GENES* in *Salmonella*



Taken from Guerra et al., 2000 (Antimicrob. Aghents Chemoth.)

- PCR amplification with specific primers
- RFLP of PCR products
- Sequencing of PCR products

Slide 20

Mapping of Resistance Structures (genes, integrons) by...

- Hybridization with specific probes
- Mating experiments (Conjugation)
- Curing experiments

Slide 21

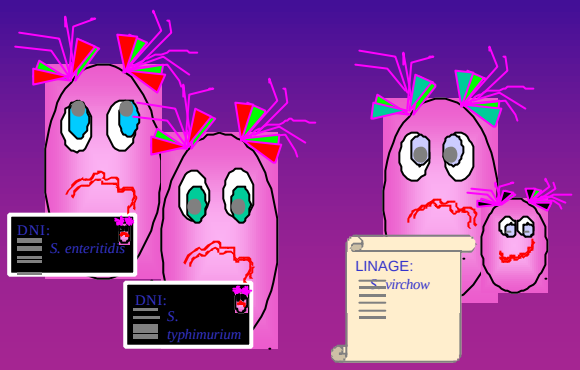
Clones / lineages characterization

...

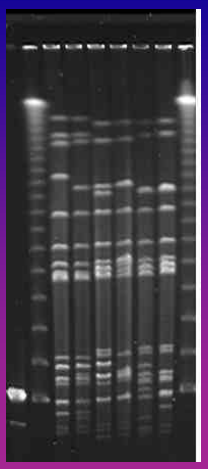
Molecular typing

Slide 22

Strain / Clone / Lineage Characterization

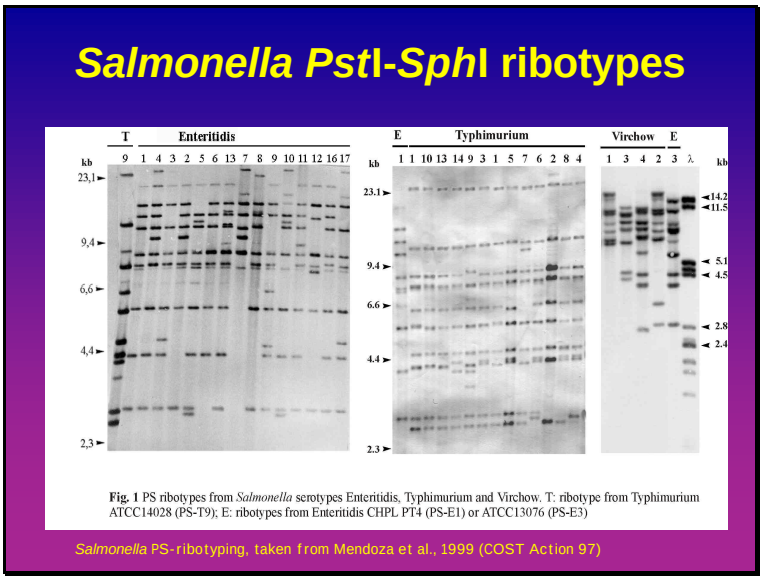


Slide 23

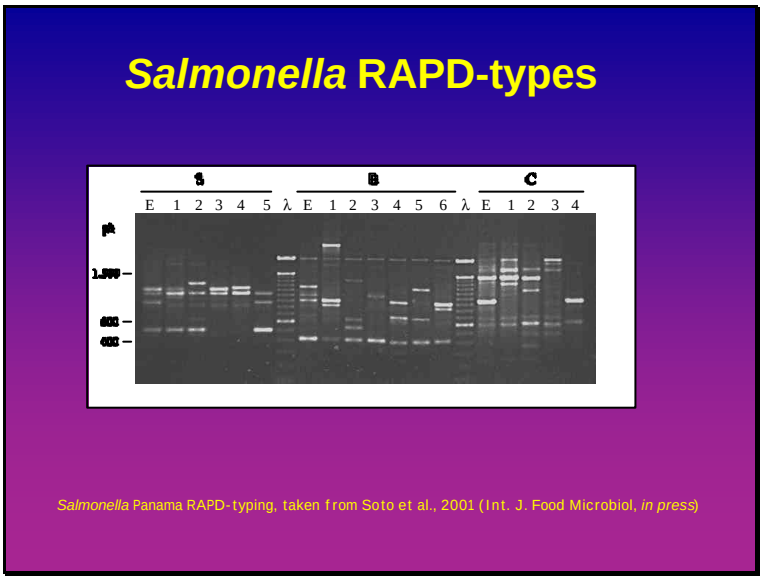


*Salmonella Xba*I-PFGE types (PFP)

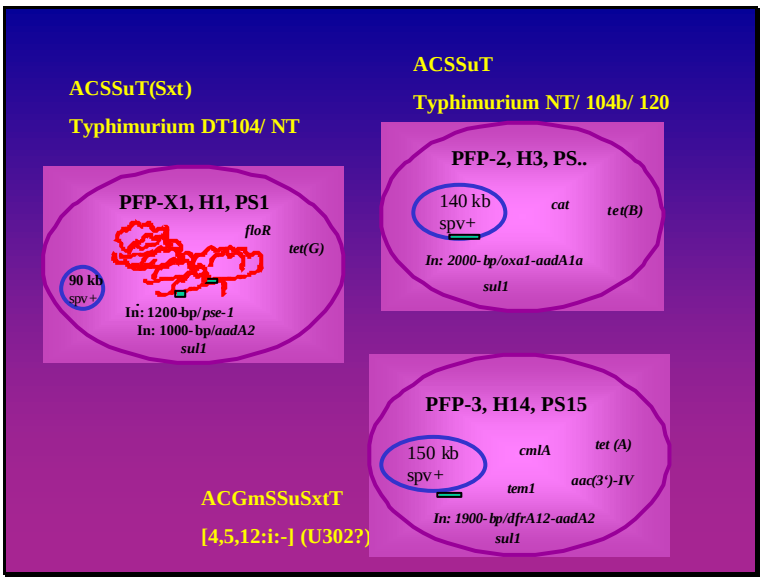
Slide 24



Slide 25



Slide 26



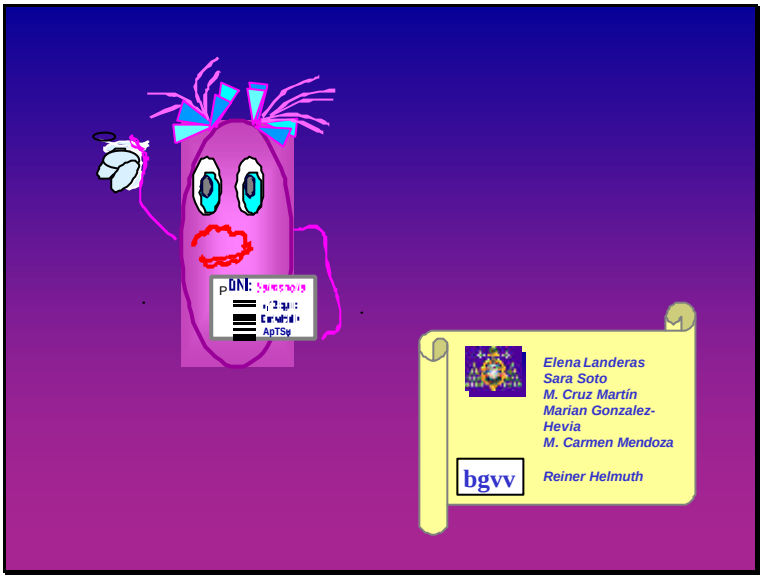
Slide 27

CONCLUSION: To identify the sources and dissemination of drug resistance genes in MDR *Salmonella*...

- ... the identification of the **organisms** harboring such genes
- ... the characterization of the genetic **vehicles** involved
- ... the characterization of the **genes** themselves

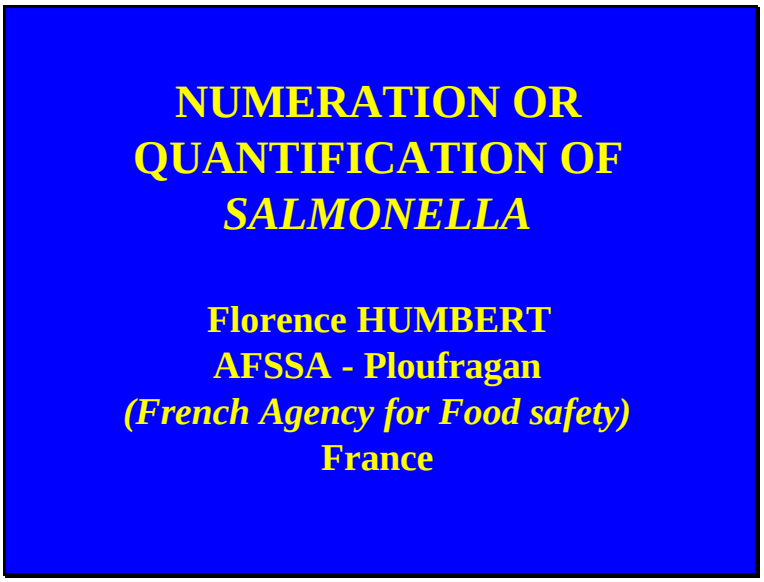
is needed.

Slide 28



Appendix 18. Sheets of presentation 5.1

Slide 1



Slide 2

Slide 3

Naturally/artificially contaminated samples or animals

- When artificial contaminations are tested, the principle is
  - to use antibiotic(s) resistant strains **(nalidixic acid, chloramphenicol+tetracyclin, rifampicin...)**
  - to use a selective isolation media added with the corresponding antibiotic
- The detection limit may be lowered, theoretically
  - 20 Salm/g if 0.25 ml/plate - 2 plates (surface)
  - 10 Salm/g if 1 ml/plate (poured)

➡ **In practice 50 *Salmonella*/g**

Slide 4

Problems related to artificial contaminations experiments

- *Salmonella* cells are not used under "natural physiological state" but directly from an overnight broth culture
- Levels of contamination used are in excess compared to natural ones (except for animals inoculations)
- An other alternative is to sterilise the matrix

➡ **No ideal model to follow *Salmonella* change/state in a complex environment**

Slide 5

There are 3 main strategies

- Direct count
  - Immuno-fluorescence
- Culture techniques
  - Direct isolation (without ATBresist.)
  - Most probable number method (MPN)

Slide 6

Immuno-fluorescence

- Critical points
  - filtration of the sample
  - choice of the antibody (all serovars ?)
  - choice of fluorochrome
- Official method in AOAC and BAM (FDA)
- For meat samples : 30-50% of false negative results
  (Thomason, 1981)

Slide 7

More recent applications of immuno-fluorescence

- Application to the detection of micro-colonies (after 6 hours incubation) instead of individual cells
  - always need filtration (Iso-grid method)
  - enhances sensitivity
  - no false positive (dead cells) : culture step
    (Rodrigues and Kroll, 1988 and 1989)

Slide 8

More recent applications of immuno-fluorescence

- Combined with flow cytometry
  - research state
  - large volume analysed : analyse of a population instead of individual cells
  - always related to the specificity of the antibody
    (McClelland and Pinder, 1994)

Slide 9

Personal experience with immuno-fluorescence

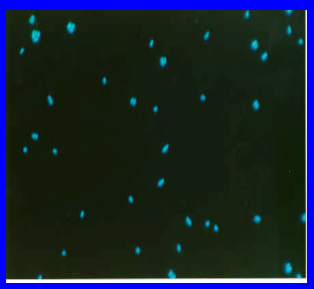
- Pure cultures
- Evaluation of survival after stress, starvation, dessiccation…: VNC (viable but not cultivable) cells
  - DAPI : total cells
  - V6 Chemchrome : esterase activity

➡ Difference between total cells (dead and viable) and those showing a physiological activity

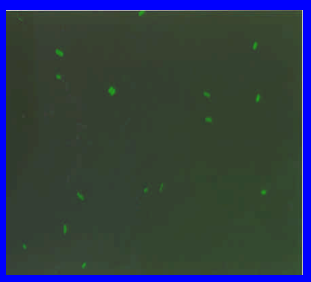
(J. Lesne and coll., IJFM, 2000, 60 : 195-203)

Slide 10

Pictures of immuno-fluorescence measures



Total cells count (DAPI)



Esterase positive cells (Chemchrom V6)

Slide 11

Results from 9 weeks of experimental dessication

- Total cell count (DAPI) and esterase activity remained stable but DAPI fluorescence intensity decreased slowly
- Total loss of culturability on nutrient agar after 9 weeks
- Loss of one day old chicken infectivity and mice virulence within 3 weeks

➡ Problems to relate results from immuno-fluorescence with epidemiological implications

Slide 12

Direct isolation

Feasible under **3 conditions** :

- if the number of *Salmonella* cells exceeds 100-1000 per g or ml
- if the ratio of *Salmonella* cell number to that of competitive flora is not too low
- if each *Salmonella* cell can develop into a colony on a solid selective agar

Slide 13

Direct isolation

- Need a media which is :
  - selective enough to impair growth of competitive micro-organisms
  - sensitive enough to allow growth of injured *Salmonella*
- Only if *Salmonella* > 10^3/g of solid sample
  or 10^2/g of liquid sample
  - compatible with levels in medical samples
  - "too much" for food or environmental samples

➡ Need different strategies to concentrate bacteria in a lower volume or space ➔ spread on a plate

Slide 14

Direct isolation needs strategies to concentrate samples

- Non selective concentration
  - Filtration
  - Centrifugation
- Selective concentration
  - Immuno-concentration

➡ **None gives total satisfaction**

Slide 15

Direct isolation on Rambach agar without concentration procedure

- On 620 caecal samples from chicken at French slaughter-houses :
  - 104 positive (16.8 %) by the 4 steps qualitative technique
  - 5 positive (0.8 %) by direct isolation of the 10^{-3} dilution onto Rambach agar (detection limit : 10^{4})
- A large majority (95 %) of animals are not heavily contaminated
- *Salmonella* is not a dominant digestive flora (total intestinal flora = 10^{11}/g)

Slide 16

Media to numerate *Salmonella* by direct isolation

- Selective media have to be developed just to be used in the particular case of direct plating
  ex : Dulcitol-Bile-Novobiocin (DBN)
  (Hawa, Morrison and Fleet, J. Food Protec., 1984, 47:932-936)
- DBN method applied to half carcass rinses
  - pre-enrichment (BPW) : 2 hours at 20°C
  - first centrifugation (10 000g, 15 min)
  - resuspension of the pellet in potassium-Ph buffer
  - second centrifugation (10 000g, 15 min)
  - resuspension and plating on DBN (0.1ml/15 cm plate)

Slide 17

Results of DBN direct isolation/MPN method

- Sensitivity (presence/absence) : not too bad
  For 30 carcasses

	MPN		DBN	
	PS	DW	PS	DW
Nb. Salm positive samples	6	8	10	0
% positive samples	20	27	33	0

- Quantification : all results < 1 Salm./ml of caracss rince ➔ difficult to statistically compare these results
  2 half carcasses are not positive at the same time

Slide 18

Personal experience with direct isolation and the DBN procedure

- Hard to manage large number of samples
- Difficult to work with successive centrifugation and overall resuspension
- Labour intensive to confirm "typical colonies"
- Less sensitive than the incubation of the whole carcass rinse volume in BPW (16-20h)
- May be transitorily used for risk evaluation studies on poultry carcasses or pieces

(Humbert and coll, 1987, published only in French)

Slide 19

Direct isolation combined with the overlay procedure

- Recommended for detection of injured coliforms
- Applied for *Salmonella* enumeration
  - inoculate TSA and allow to stand at room temperature for 4 hours
  - and then overlaid with different selective media (XLD, HE or SS)
  - incubation at 37°C, 24 hours
- Enhance recovery of injured *Salmonella*

(Strantz and Zottola, JFP, 1989, 52, 10 : 712-714)

Slide 20

Most Probable Number (MPN)

- Since *Salmonella* are typically found in very low number in food and environmental samples
  this is the method of choice
- Require addition of replicate volumes of a serially diluted sample into separate flasks or tubes (500, 50, 5 or 100, 10, 1 or 1, 0.1, 0.01)
- Each sample ➔ 9 : 3 repetitions-3 dilutions
  12 : 3 repetitions-4 dilutions
  25 : 5 repetitions-5 dilutions
  30 : 10 repetitions-3 dilutions

Slide 21

The theory assumes

❶ that the organisms are randomly distributed throughout the solution

❷ that each sample from the solution when incubated in the culture medium is certain to exhibit growth whenever the sample contains one or more organisms

Both hypothesis false (overall for *Salmonella*), but this underlines the need to perfectly homogenise sample before MPN analysis and to use the most sensitive detection technique

Slide 22

Calculation of the MPN

- The numbers of positive samples at each dilution are recorded

➡ characteristic number (ex. 321, 3311, 555)

- By reference to MPN tables (or computer files) using statistical assumptions, each characteristic number is related to a MPN of bacteria **with confidence limits**

Slide 23

MPN table for 3 x 1, 3 x 0.1 and 3 x 0.01 g (ml)

Number of positive tubes			MPN	Confidence limits > 95 %		Confidence limits > 99 %	
0	0	0	< 0.30	0.00	0.94	0.00	1.40
0	0	1	0.30	0.01	0.95	0.00	1.40
0	1	0	0.30	0.01	1.00	0.00	1.60
0	1	1	0.61	0.12	1.70	0.05	2.50
0	2	0	0.62	0.12	1.70	0.05	2.50
0	3	0	0.94	0.35	3.50	0.18	4.60
1	0	0	0.36	0.02	1.70	0.01	2.50
3	2	1	15	3	38	2	52
3	2	2	21	3	40	2	56
3	2	3	29	9	99	5	152
3	3	0	24	4	99	3	152
3	3	3	> 100				

Slide 24

Problem : confidence limits

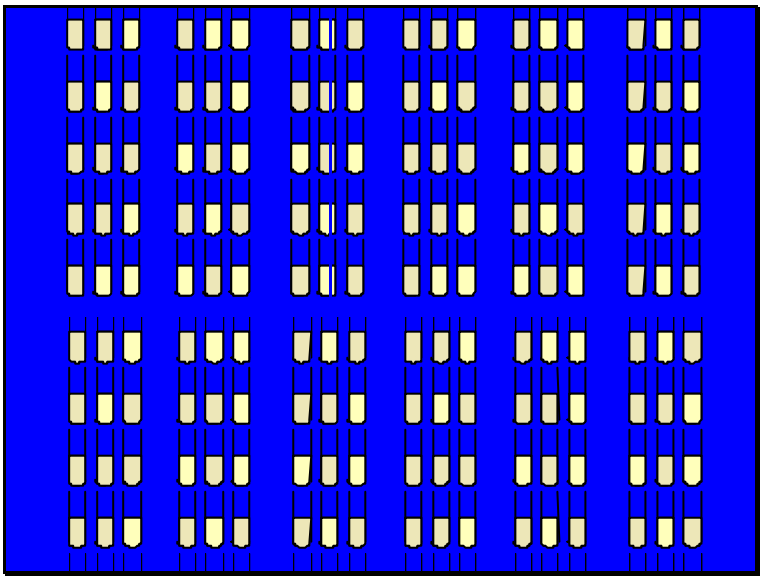
It is not worth to produce so much work to obtain such uncertain results !!!

➡ Solutions related to number of repetitions

– to have almost the same level of contamination in each sample, and to focus repetitions on the right dilutions (10 x 3)

– if it is not the case : to make more repetitions for a large range of dilutions...

Slide 25



Slide 26

Other solutions are technical ones

- To use "rapid method" at the final detection step instead of plating
  - 1-2 test *(Dickinson, JFP, 1989, 52:388-391)*
  - DNA colorimetric probe *(Pumfrey and Nelson, Poultry Sc., 1991, 70:780-784)*
  - ELISA *(Blais and Yamazaki, IJFM, 1991, 14:45-50)* VIDAS *(Bella and Tam, Wat.Res., 2000, 34:2397-2399)*
  - Luminescent phage assay *(Turpin and coll., Lett. Appl. Microbiol., 1993, 16:24-27)*

➡ **Does not offer great advantage = does not save so much time and labour**

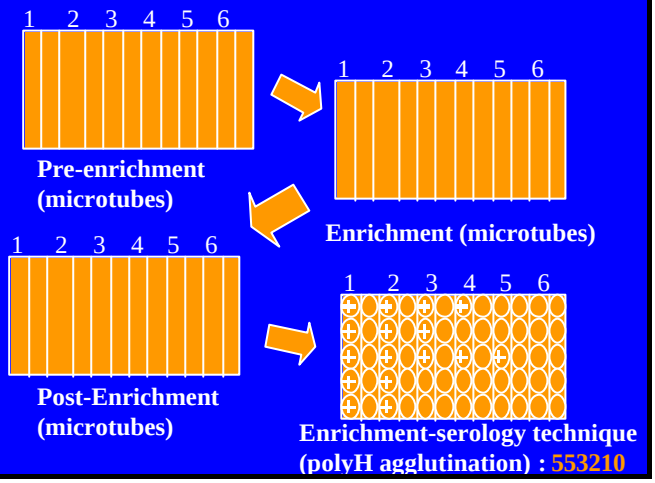
Slide 27

Miniaturized MPN

- To combine a centrifugation to concentrate bacteria in a lower volume in order to miniaturise the whole MPN procedure
- 5 g skin sample + 95 ml BPW
  - centrifugation (2500g, 15 min)
  - pellet resuspended in a final volume of 16 ml
  - divided in 8 replicates in the first row of minitubes plate
  - Serial 1/10 dilutions in minitubes (0.2 in 1.8 ml) only one out of two row used in an alternate way to ovoid cross contamination

Slide 28

The 4 steps of miniaturized MPN



Slide 29

Miniaturized MPN : results

- 26 skin samples from 2 abattoirs
- Miniaturized MPN : 12 Salm/g [0.9-5560]
- Traditionnal MPN : 57 Salm/g [1.8-95300]
- No statistically different
- Statistical difference between 2 abattoirs (mean numbers of 5800 and 10 Salm/g)
- No routinely applicable but usefull
  - to test decontamination treatment
  - to measure progress in application of prevention

➡ For all cases when 0 *Salmonella* is not expected

Slide 30

Some MPN results concerning chicken carcasses or cutting

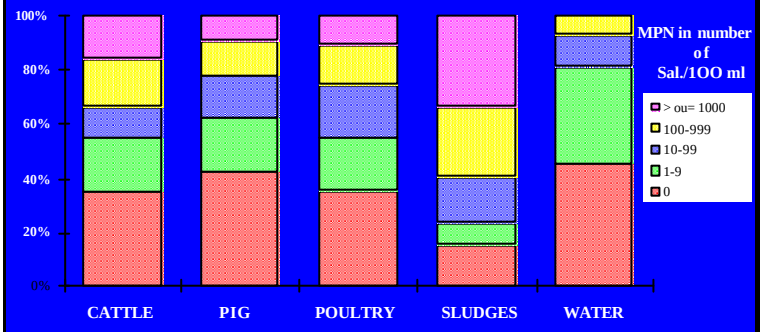
- Izat and coll., 1991 : 5-34/carcass
- Slavick and coll., 1995 : 3000-3200/carcass
- Whittemore, 1993 : < 1/carcass
- Tokumaru and coll., 1990 : 30-1000/100g

All results with 3 repetitions - 3 dilutions

➔ each sample = 9 analyses

Slide 31

Salmonella contamination of animal slurry, sludge and wastes



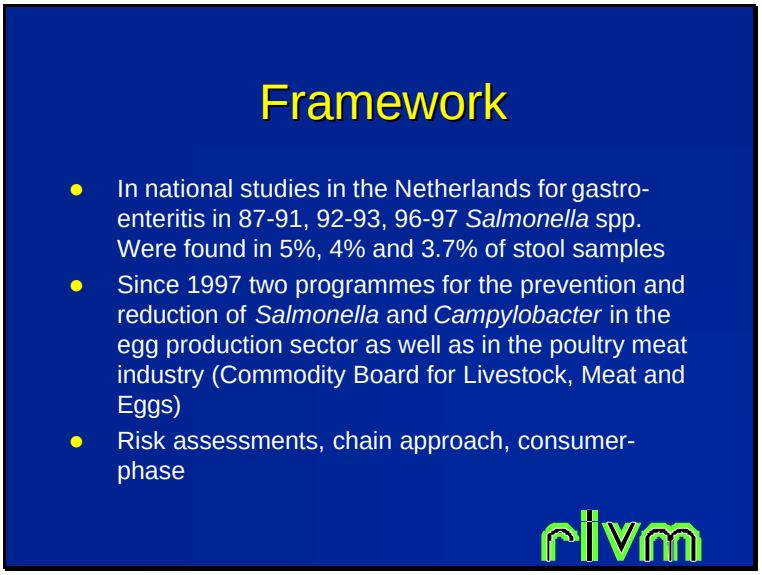
Slide 32

Today, the traditional, time consuming, labour intensive MPN is the only one method with the required sensitivity for as low level as 1-10 *Salmonella* in 100 g of sample

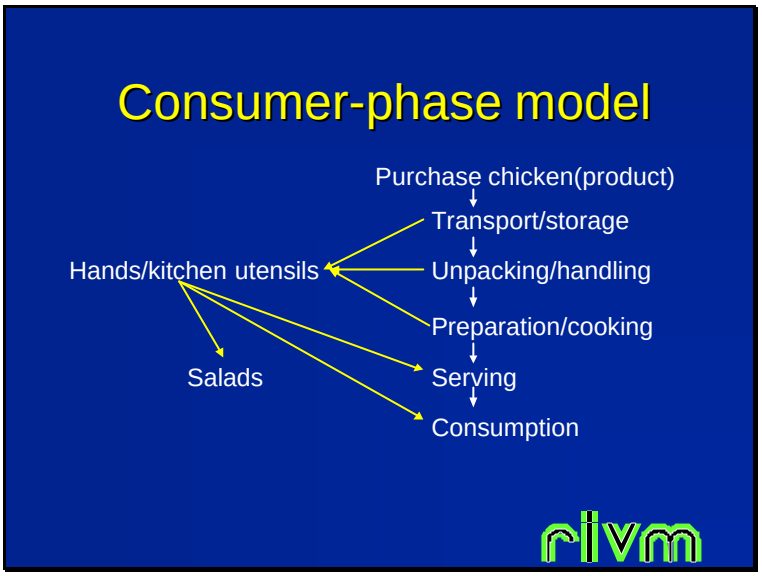
Appendix 19. Sheets of presentation 5.2

Slide 1

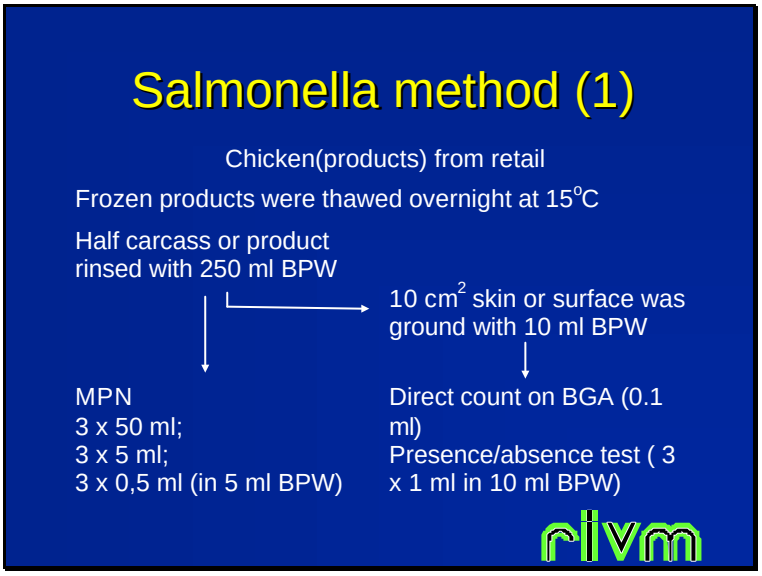
Slide 2



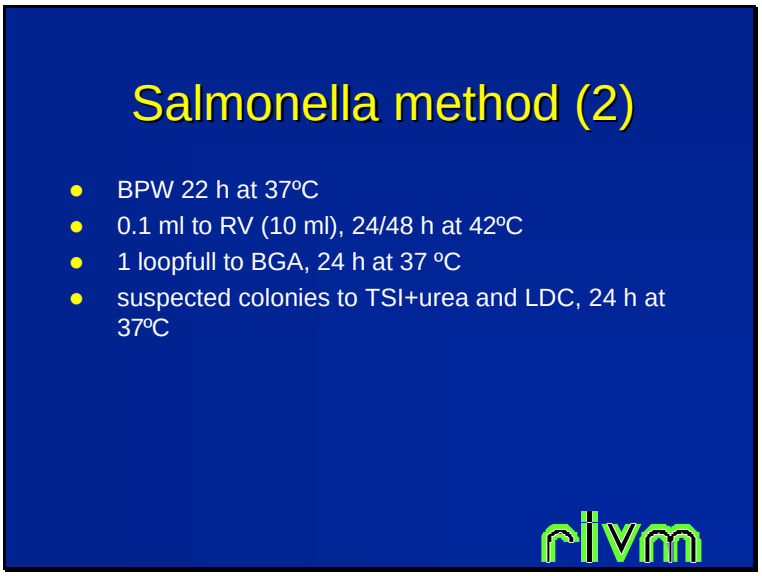
Slide 3



Slide 4

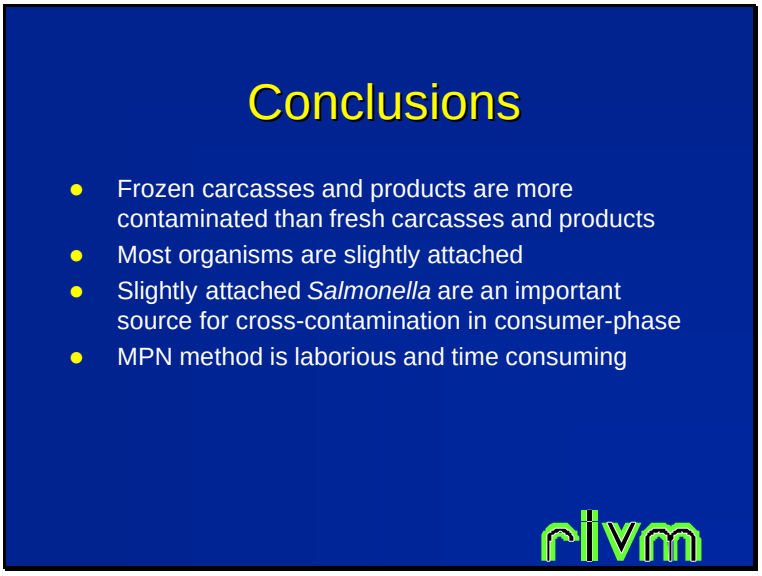


Slide 5



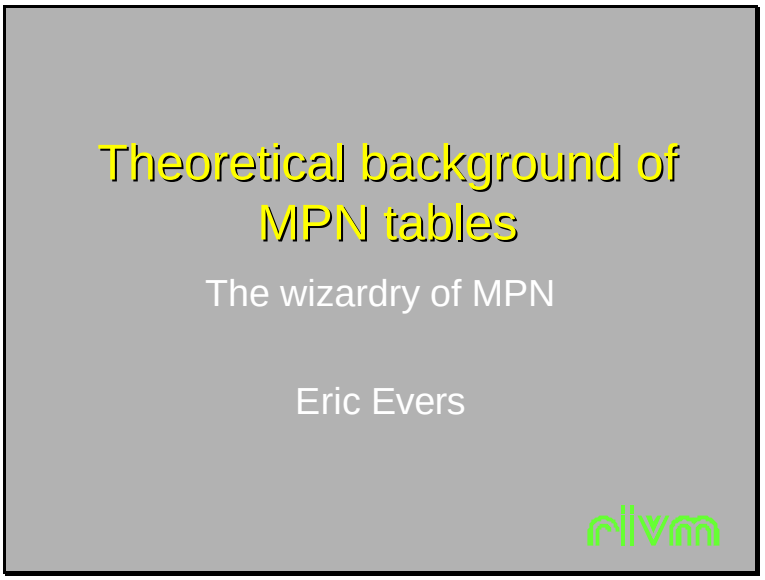
Slide 6

Slide 7

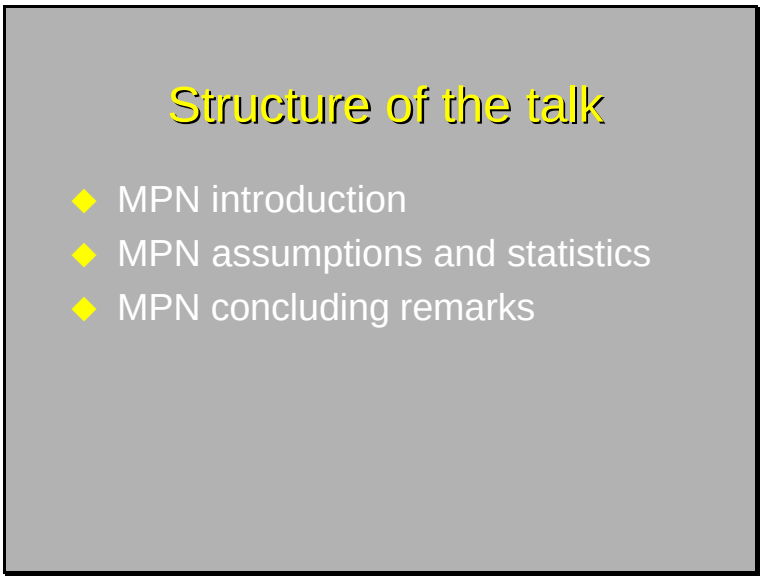


Appendix 20. Sheets of presentation 5.3

Slide 1



Slide 2



Slide 3

What is MPN?

- MPN = Most Probable Number
- Term established for a specific experimental set up

Slide 4

MPN example experimental set-up (1)

- Sample of 100 gram
- add 900 ml pre-enrichment medium (dilution 1:10)
- 1000 ml of solution A in jar (100 g sample)

Slide 5

MPN example experimental set-up (2)

- 3 culture jars each with 100 ml of solution A (10 g sample)
- 3 culture tubes each with 10 ml of solution A (1 g sample)
- 3 culture tubes each with 1 ml of sol. A and 9 ml pre-enrichment medium (0.1 g sample)
- pre-incubation for 18-22 h at 37 ºC

Slide 6

MPN example experimental set-up (3)

- 100 µl of each culture tube/jar in 10 ml sel. enrichment medium
- incubation for 24-48 h at 42 ºC
- 1 loop (30-50 µl) on a sel. plate
- incubation for 24 h at 37 ºC
- 9 observations of presence / absence on the plate

Slide 7

MPN example experimental set-up (summary for one dilution)

- Sample of 100 g
- pre-incubation of 10 g in culture jar
- incubation of 0.01 g in culture tube
- incubation of 0.00004 g on plate
- observation of presence/absence on the plate

Slide 8

Why MPN and not direct plating?

- Direct plating gives a more precise estimation of the concentration
- Concentration too low for direct plating
- Competitive flora

Slide 9

MPN Wizardry

Number of positive results from triplicates, for sample size:			MPN	lower 95% limit	upper 95 % limit
1 g	0.1 g	0.01 g			
2	0	0	0.92	0.15	3.50
2	0	1	1.4	0.4	3.5
2	0	2	2.0	0.5	3.8
2	1	0	1.5	0.4	3.8

Slide 10

Assumptions and statistics

- Poisson distribution
- High growth rate
- High detection probability
- Maximum likelihood

Slide 11

Poisson distribution (1)

- Theoretical probability distribution for sampling
- Probability of having 0, 1, 2, etc. micro-organisms in my sample
- Predicts the frequency of 0, 1, 2 in 1 million samples

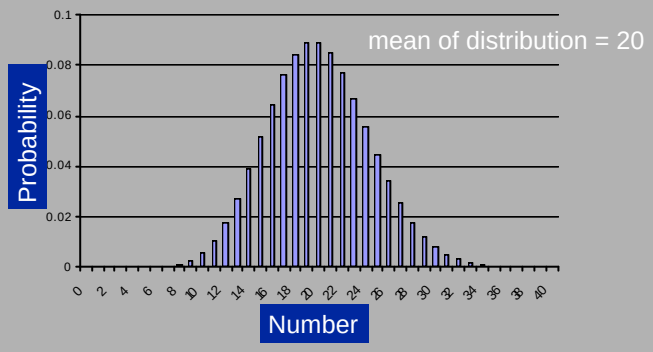
Slide 12

Poisson distribution (2)

- Describes the ‘natural’ or ‘inevitable’ variation in numbers in multiple sampling
- Variability
- Random distributed micro-organisms

Slide 13

Poisson distribution (3)



Slide 14

Poisson distribution (4)

- Examples of 5-plicates if mean of distribution = 20
  - 11, 15, 15, 21, 23
  - 13, 18, 19, 20, 27
  - 17, 19, 22, 22, 24
- Cause: random distributed micro-organisms, not experimental error!
- Unrealistic: 20, 20, 20, 20, 20

Slide 15

Poisson distribution (5)

$$f(x) = \frac{e^{-l}\, l^{x}}{x!}$$

- f(x) is the probability of x micro-organisms in the sample
- λ is the mean of the distribution (only one parameter!)

Slide 16

Assumptions and statistics

-
- High growth rate
- High detection probability
-

Slide 17

High growth rate and detection probability (1)

- MPN formulas assume that one single micro-organism in the sample is detected
- Close to this assumption if:
  - high growth rate during (pre)-incubation
  - high detection probability

Slide 18

High growth rate and detection probability (2)

- Otherwise the MPN Table values are an underestimation

Slide 19

High growth rate and detection probability (3): example

- Culture jar with 100 ml of solution A (10 g sample)
- pre-incubation for 18-22 h at 37 ºC (main growth)
- 100 µl of solution A in 10 ml selective enrichment medium
- 100 µl is 0.1 % of 100 ml

Slide 20

High growth rate and detection probability (4): example

- factor 1000 = 10 generations
- Incubation for 24-48 h at 42 ºC (limited growth)
- 40 µl on a selective plate
- 40 µl is 0.4 % of 10 ml
- factor 250 = 8 generations

Slide 21

High growth rate and detection probability (5): example

- Incubation for 24 h at 37 ºC
- Observation of presence/absence on the plate
- Detection probability on the plate

Slide 22

Assumptions and statistics

-
-
-
- Maximum likelihood estimation

Slide 23

Maximum likelihood estimation - illustration

- Illustration with measurements:
  - Direct plating
  - Absence/presence
  - MPN

Slide 24

MLE - Example Direct plating (1)

- Sample of 100 gram
- add 900 ml physiological saline (dilution 1:10)
- 1000 ml of solution A in jar (100 g sample)
- 100 µl on a plate

Slide 25

MLE - Example direct plating (2)

Possibly in addition another 1:10 dilution:

- A culture tube with 1 ml of solution A and 9 ml physiological saline
- 100 µl on a plate

Slide 26

Maximum likelihood estimation - principle

- Assume a distribution
- Given the measurements, what is the most probable value for the parameter(s) of this distribution

Slide 27

Direct plating-exercise 1 (1)

- Measurement:
  20 micro-organisms on a plate
- Distribution:

  Poisson
- Value for ? in 100 µl:

  20

Slide 28

Direct plating-exercise 1 (2)

- MLE formula:

  $$l = x$$
- x = measured no. of micro-organisms
- λ = the mean of the Poisson distribution

Slide 29

Direct plating-exercise 1 (3)

? in the original sample:

- 20 in 100 µl
- $2 \cdot 10^5$ in 1000 ml
- $2 \cdot 10^5$ in 100 g
- ? = 2000 per gram in the original sample

Slide 30

Direct plating-exercise 2 (1)

- Duplicate measurement: 20 and 25 micro-organisms on a plate
- Value for ? in 100 µl:

  22.5

Slide 31

Direct plating-exercise 2 (2)

MLE formula:

$$l = \frac{\sum_{i=1}^{n} x_i}{n}$$

x_i = measured no. of micro-organisms; n = number of measurements; λ = the mean of the Poisson distribution

Slide 32

Direct plating-exercise 2 (3)

$$l = \frac{20 + 25}{2} = 22.5$$

Slide 33

Direct plating-exercise 3 (1)

Two plate counts

- 100 µl of solution A: 90 CFU
- 100 µl of 1:10 dilution of solution A (= 10 µl of solution A): 20 CFU
- Value for ? in 100 µl of solution A:

  100

Slide 34

Direct plating-exercise 3 (2)

MLE formula: $$l = \frac{\sum_{i=1}^{n} x_i}{\sum_{i=1}^{n} v_i}$$

x_i = measured no. of micro-organisms; v_i = volume of sample; n = number of measurements; λ = the mean of the Poisson distribution

Slide 35

Direct plating-exercise 3 (3)

$$l = \frac{90+20}{1+0.1} = \frac{110}{1.1} = 100$$

Slide 36

Absence/presence-exercise 1 (1)

- Procedure includes incubation step(s) as in MPN
- Measurement: analysis of a 10 g (100 ml) sample gives absence on a plate
- Value for ? in 10 g:

  0

Slide 37

Absence/presence-exercise 1 (2)

MLE formula:

$$l = -\ln(neg)$$

neg = no. of negative plates; λ = the mean of the Poisson distribution

Slide 38

Absence/presence-exercise 1 (3)

$$l = -\ln(1) = 0$$

Slide 39

Absence/presence-exercise 2 (1)

- Measurement: analysis of a 10 g sample in duplicate gives 1 negative and 1 positive plate
- Value for ? in 10 g:

  0.69

Slide 40

Absence/presence-exercise 2 (2)

MLE formula:

$$l = \ln(tot) - \ln(neg)$$

neg = no. of negative plates; tot = total no. of plates; λ = the mean of the Poisson distribution

Slide 41

Absence/presence-exercise 2 (3)

$$l = \ln(2) - \ln(1) = 0.69$$

Slide 42

Absence/presence-exercise 3 (1)

- analysis of a 10 g sample gives a positive plate and analysis of a 1 g sample gives a negative plate
- Value for ? in 10 g:

  2.40

Slide 43

Absence/presence-exercise 3 (2)

MLE formula:

$$\sum_{i=1}^{n} tot_i w_i = \sum_{i=1}^{n} \left(\frac{pos_i w_i}{1 - e^{-w_i l}} \right)$$

tot_i = total no. of plates; w_i = sample weight; pos_i = no. of positive plates; λ = the mean of the Poisson distribution

Slide 44

Absence/presence-exercise 3 (3)

$$1 \times 1 + 1 \times 0.1 = \frac{1 \times 1}{1 - e^{-1 \times l}} + \frac{0 \times 0.1}{1 - e^{-0.1 \times l}}$$

$$l = -\ln\left(1 - \frac{1}{1.1}\right) = 2.40$$

Slide 45

MPN-exercise 1 (1)

Number of positive results from triplicates, for sample size:			MPN	lower 95% limit	upper 95 % limit
1 g	0.1 g	0.01 g			
2	0	0	0.92	0.15	3.50
2	0	1	1.4	0.4	3.5
2	0	2	2.0	0.5	3.8
2	1	0	1.5	0.4	3.8

Slide 46

MPN-exercise 1 (2)

MLE formula:

$$\sum_{i=1}^{n} tot_i w_i = \sum_{i=1}^{n}\left(\frac{pos_i w_i}{1-e^{-w_i l}}\right)$$

tot_i = total no. of plates; w_i = sample weight; pos_i = no. of positive plates; λ = the mean of the Poisson distribution

Slide 47

MPN-exercise 1 (3)

$$3\times1+3\times0.1+3\times0.01=\frac{2\times1}{1-e^{-1\times l}}+\frac{1\times0.01}{1-e^{-0.01\times l}}$$

$$3.33=\frac{2}{1-e^{-l}}+\frac{0.01}{1-e^{-0.01\times l}}$$

$$l=1.43$$

Slide 48

Concluding remarks

- Using MPN tables
- Confidence intervals
- Variability and uncertainty

Slide 49

Using MPN tables (1)

Number of positive results from triplicates, for sample size:			MPN	lower 95% limit	upper 95 % limit
1 g	0.1 g	0.01 g			
2	0	0	0.92	0.15	3.50
2	0	1	1.4	0.4	3.5
2	0	2	2.0	0.5	3.8
2	1	0	1.5	0.4	3.8

Slide 50

Using MPN tables (2)

- Be aware of the dimension of the MPN values in MPN tables
- Usually this is number per 1 g, being the highest amount tested
- Tables can also be used for other amounts, but be aware of the dimension

Slide 51

Using MPN tables(3): example

- Measurement: a test in triplicate of 10, 1 and 0.1 g gives 2, 0, 2 positives, resp.
- MPN table: a test in triplicate of 1, 0.1 and 0.01 g with the same result gives an MPN value of 2.0 per g
- The concentration in the sample is then 2.0 per 10 g = 0.2 per g

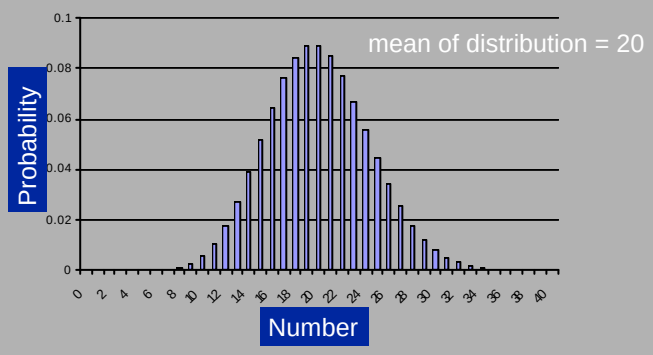
Slide 52

Confidence intervals

- For explanation additional statistics needed
- MPN confidence intervals are large
- Cause: estimation of a concentration using only absence/presence information

Slide 53

Variability and uncertainty (1)



Slide 54

Variability and uncertainty (2)

- The MPN confidence interval describes the uncertainty of ?, the mean of the Poisson distribution
- The actual variation in numbers between samples is larger, due to the variability of the Poisson distribution

Slide 55

Variability and uncertainty (3)

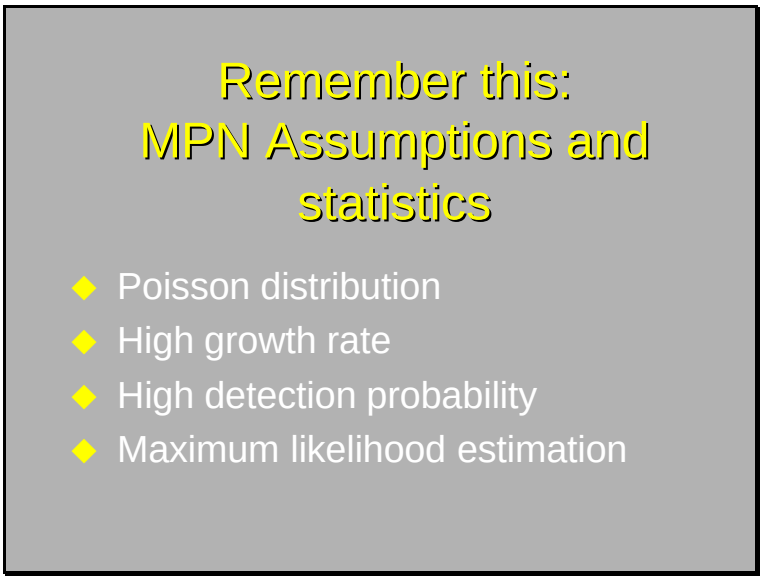
- Using the results of an MPN test, the estimated variation in numbers between samples consists of the uncertainty of ? and the variability of the corresponding Poisson distributions

Slide 56

Variability and uncertainty (4)

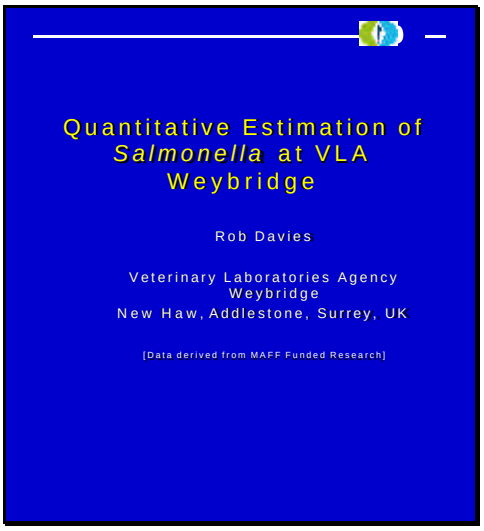
- The uncertainty of ? (the MPN confidence interval) can be reduced by additional measurements
- (Poisson) variability is a reality that cannot be reduced

Slide 57


Remember this:
MPN Assumptions and statistics
Poisson distribution
High growth rate
High detection probability
Maximum likelihood estimation

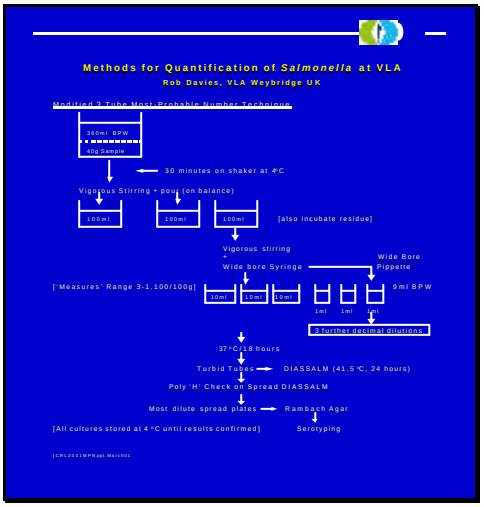
Appendix 21. Sheets of presentation 5.4

Slide 1

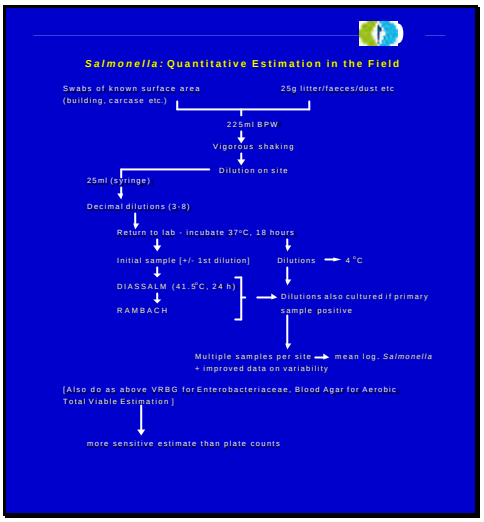


Slide 2

Slide 3



Slide 4



Slide 5

Appendix 22. Sheets of presentation 5.5

Slide 1

Quantification of DT104 in slurry from infected pig herds

Dorte Lau Baggesen, Danish Veterinary Laboratory, **Jakob Bagger**, **Vibeke Møgelmose**, **Bent Nielsen**, **Birgitta Svensmark**, Danish Bacon and Meat Counsil, **John Elmerdahl Olsen**, Royal Veterinary and Agricultural University

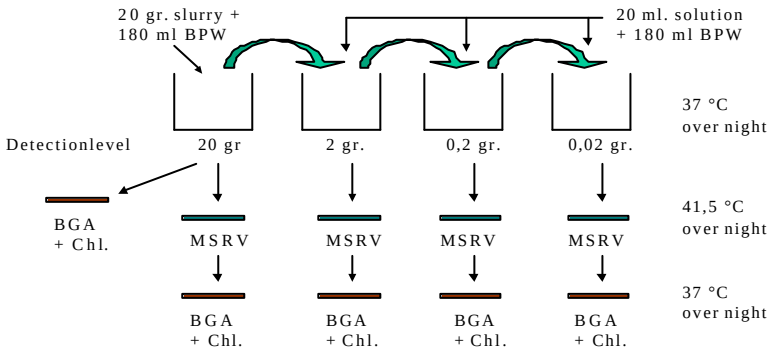
Slide 2

Aim of the study

- (Semi)quantification of MR DT104 i pig slurry from infected pig herds
- Evaluation of the risk by spread of DT104 contaminated slurry at landfill
- Correlation of contamination level of slurry with information from the national salmonella surveillance (serological and bacteriological examinations)

Slide 3

Method of quantification



Slide 4

Sampling

- One visit at each of 18 herds
- Sampling from one slurry tank at each farm
  - 3 samples from top layer
  - 3 samples from middle layer
  - 3 samples from bottom layer

Slide 5

Interpretation of results

- Only one direct isolation on BGA
- Each sample separately
  - Rough estimate
- Three samples together
  - "3-line MPN"
  - Precondition – bacteria's are equally distributed within each layer

Growth in	c.f.u.
20 g (-1)	> 0,05 per gr.
2 g (-2)	> 0,5 per gr.
0,2 (-3)	> 5 per gr.
0,02 (-4)	> 50 per gr.

Slide 6

Results – examples of three herds

		Top	Middle	Bottom	Top	Middle	Bottom
		Growth in –1, -2, -3 dilution			c.f.u. per gr. Slurry (95% CI)		
High level (97052)	1	+++	++	+++	5,5 (1,3 – 24)	2,3 (0,52 – 10)	>5,5 (>1,3 - >24)
	2	+++	+++	+++			
	3	++	++	+++			
Middle level (96831)	1		+	++	0,073 (0,021-0,26)	0,21 (0,052 – 0,88)	0,21 (0,52 – 0,88)
	2	++	+	+			
	3	+	++	+			
Low level (25291)	1		+		< 0,015	0,018 (0,0025 – 0,13)	< 0,015
	2						
	3						

Slide 7

Correlation between microbiological examination of slurry and informations from the national surveillance

	Serological and microbiological examiations, information on input to slurry tanks etc.		
Bact. Exam.	High risk	Middle risk	Low risk
High level > 1 c.f.u./gr.	4 herds	1 herd	
Middle level	2 herds	2 herds	1 herd
Low level < 0,1 c.f.u./gr.	2 herds*	2 herds	4 herds

* In two herds, pig slurry were diluted with non DT104 cattle slurry in the tank

Slide 8

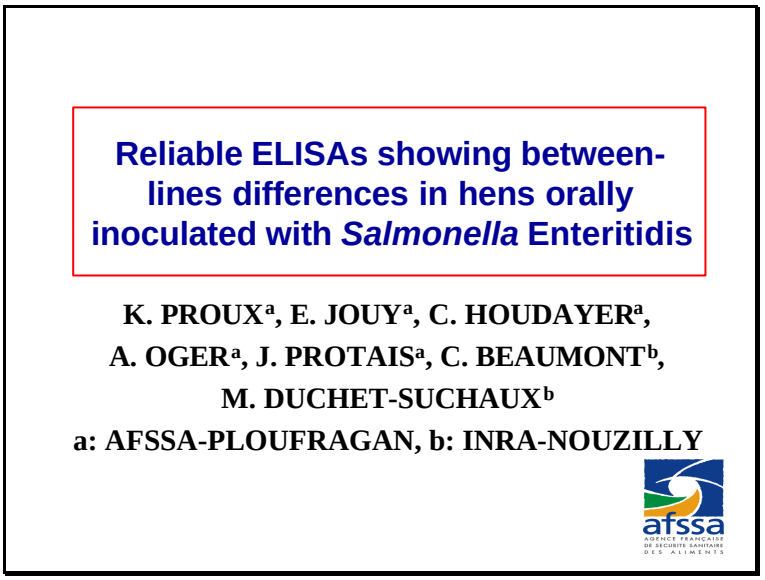
Risk of infection by spreading of slurry

- Normal use of slurry in Danish agriculture
- 40 ton slurry per 1 hectare (10.000 m^2)
- Contaminations level: ~ 1 c.f.u per gram
  $\Rightarrow$ 4 x 10^7 c.f.u. per ton
  $\Rightarrow$ **4000 c.f.u. per m^2 landfill**

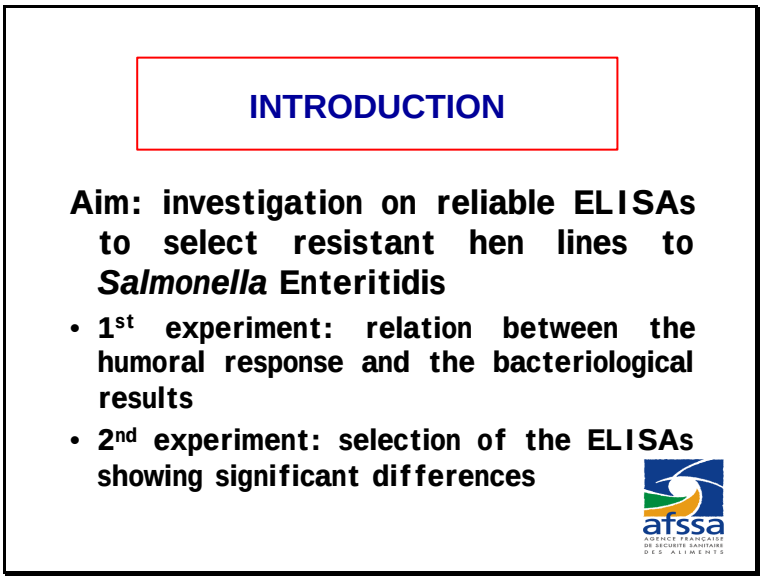
? What do this mean in relation to risk of infection
- to domestic animals
- to wild life

Appendix 23. Sheets of presentation 5.8

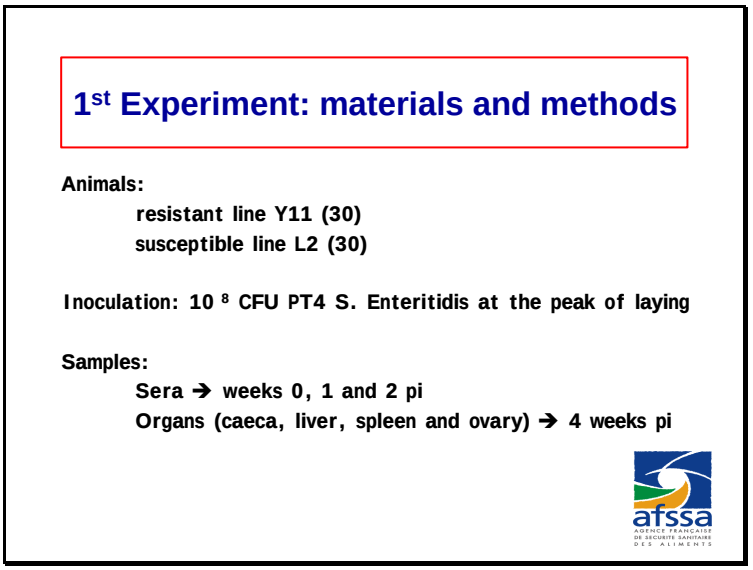
Slide 1



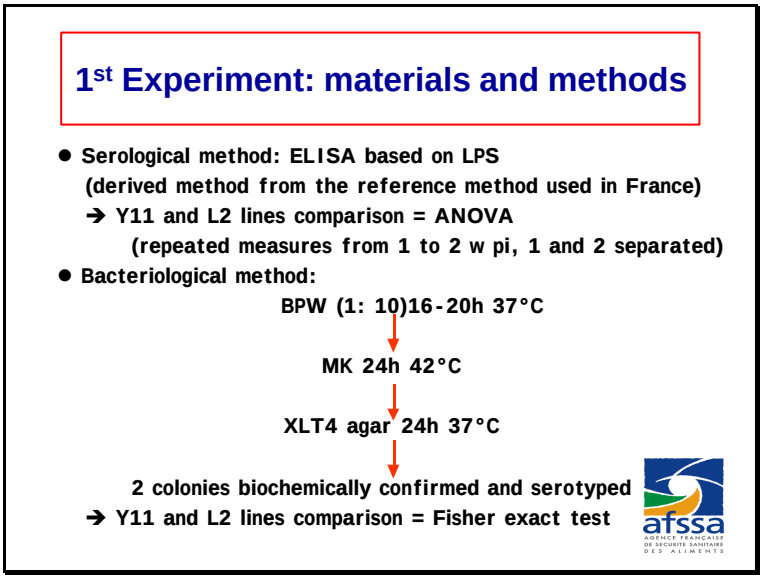
Slide 2



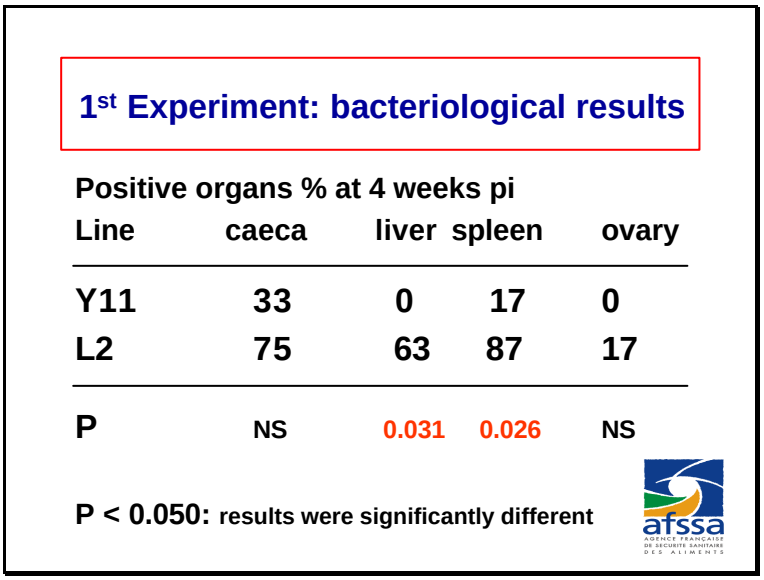
Slide 3



Slide 4

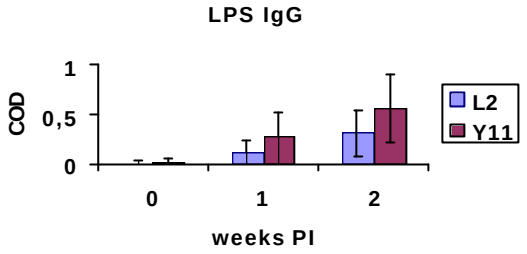


Slide 5



Slide 6

1st Experiment: serological results



➔ ANOVA: 1 to 2 w pi P=0.013

1 w pi P=0.006

2 w pi P=0.011

Slide 7

2nd Experiment: materials and methods

Animals:

resistant line Y11 (16)

susceptible line L2 (15)

➔ both lines kept in different rooms and each hen in individual cage

Inoculation: 1.7 x 10^8 CFU PT4 S. Enteritidis at the peak of laying / Y11 and L2 hens

Samples:

1 serum and 3 yolks per hen ➔ 1, 2, 4, 6, 8 and 10 weeks pi

Organs(caeca, liver, spleen and ovary) ➔ 10 weeks pi

Faeces / group of 4 cages

➔ 1 w bi and at 1, 2, 4, 6 and 8 weeks pi

Slide 8

2nd Experiment: materials and methods

Serological method:

antigens: LPS or flagella

sample: sera or yolks

immunoglobulins: IgG or IgM in sera, IgG in yolk

➔ between lines comparison = ANOVA

(repeated measures from 1 to 10 w pi and each week separated if the ELISA were significantly different on repeated measures)

For yolk: the mean COD were calculated for the 3 yolks

Bacteriological method:

the same as the one used in the 1st experiment

➔ Y11 and L2 lines comparison = Fisher exact test

Slide 9

2nd Experiment: bacteriological results

Positive organs % at 10 weeks pi

Line	caeca	liver	spleen	ovary
Y11	6.25	12.5	6.25	0
L2	13.3	0	0	13.3
P	NS	NS	NS	NS

P < 0.050: results were significantly different

Slide 10

2nd Experiment: bacteriological results

Positive faeces (4 pools: each week for each line)

	weeks pi				
Line	1	2	4	6	8
Y11	4	4	3	0	0
L2	4	4	1	0	0

➔ From 6 weeks pi *Salmonella* was no more excreted

Slide 11

2nd Experiment: serological results

ELISAs with significant between lines differences ?

- Sample ? / yolks: NS
- Antigens and immunoglobulins in sera ?

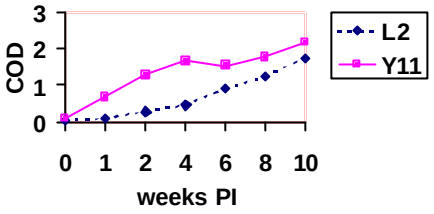
	Immunoglobulins	
Antigens	IgG	IgM
LPS	0.017	NS
flagella	0.017	<0.001

P < 0.05: significative difference

Slide 12

2nd Experiment: serological results

anti-LPS IgG



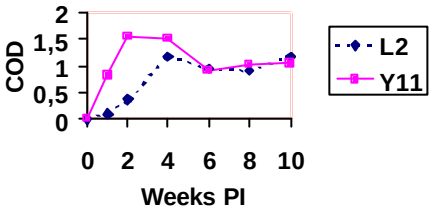
➔ Significative differences: 1, 2 and 4 wpi

p = 0.005, 0.001, 0.020 respectively

Slide 13

2nd Experiment: serological results

anti-FLAGELLA IgG



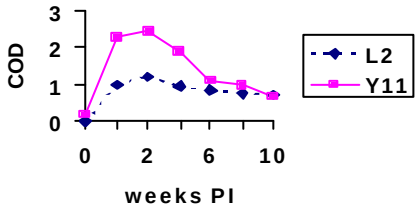
➔ Significative differences: 1 and 2 wpi

p = < 0.001, < 0.001 respectively

Slide 14

2nd Experiment: serological results

anti-FLAGELLA IgM



➔ Significative differences: 1, 2, 4 and 8 wpi

p = < 0.001, < 0.001, 0.003, 0.009 respectively

Slide 15

CONCLUSIONS

- **1st experiment: Use of serology ?**
  serological results: Y11 > L2
  bacteriological results: Y11 < L2
  (liver and spleen)
  ➔ ELISA seems to be reliable to select resistant hen lines
- **2nd experiment: Which ELISAs methods ? When ?**
  anti-LPS IgG ➔ 1, 2, 4 weeks pi
  anti-flagella IgG ➔ 1, 2
  anti-flagella IgM ➔ 1, 2, 4, 8

Slide 16

CONCLUSIONS

- **Bacteriological results: between-lines differences ?**
  at 4 weeks pi: Significative
  at 10 weeks pi: NS as *Salmonella* was almost completely eliminated from the organs
- **Further studies: look for differences between genes involved in the resistance to *Salmonella* in the selected lines.**

Slide 17

ACKNOWLEDGEMENTS

This work was supported by the EEC contract

FAIR 6-CT 98-4311

« Resistance genes to *Salmonella* carrier state in fowls »