



Midterm Status Report 2003 and Application for Continuation in 2004

For research projects financed by grants from
The Directorate for Food, Fisheries and Agro Business
under the Danish Ministry of Food, Agriculture and Fisheries

1. Research program

Research in organic farming 2000-2005 (DARCOF II)

2. Project title and number

Bacterial infection risk associated with outdoor organic pig production

- with special reference to *Salmonella* and *Campylobacter* infection
 - SaCaFree FØJO II project no. II.10
-

3. Head of project

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6. Project period (month, year)

Start of project:	1 st . July 2002
End of project:	30 th . June 2004

7. Midterm description of the project, its results and progress, and application for continuation in 2004

A. Project summary

The modern consumers are becoming increasingly critical of the quality of meat and other types of products of animal origin. Focus is put on the animal husbandry and the way of production concerning animal welfare. This leads to increasing interest for organic, free-range or other kinds of animal-friendly production systems where the animals benefit from a low animal density and good possibilities for expressing normal behaviour. In general, consumers also expect products from these kinds of systems to be of a higher microbiological quality compared to products from conventional production systems. However, today there is no documentation for a lower level of the most common zoonotic bacterial infections (e.g. *Salmonella*, *Campylobacter* and *Yersinia*) in organic or other alternative production systems.

The objective of this project is to improve the knowledge on the risk of outdoor pig production in relation to spread and persistence of *Campylobacter* and *Salmonella* infections. For *Salmonella* the specific objectives are to evaluate the survival of *Salmonella* Typhimurium in soil and grass of contaminated pastures used for outdoor pig production, measurement of the infectivity of naturally *S. Typhimurium* contaminated pastures in relation to time, and in the case of high infectivity, evaluation of the pathogen reducing effect of soil treatment. For thermophilic *Campylobacter*, the objectives are to describe the infection dynamics of natural *Campylobacter* infections over time in outdoor pigs, including time of colonisation, level of excretion in faeces, species distribution in the group and in the individuals, interaction with the environment, and to describe the possible changes in prevalence and species distribution in relation to time and environmental contamination.

Experimental pastures for production of outdoor organic piglets has been set up in 2003. Experimentally infected piglets were grouped together with uninfected tracer piglets in order to monitor the transmission of infection. In addition, soil samples was analysed to determine the level of contamination. After a period of 6 weeks the pastures were vacated. A new set of uninfected piglets was introduced in each pasture to monitor if these animals become infected due to their habitation in the *Salmonella* contaminated pastures. The pastures and animals were sampled and monitored by bacteriological (qualitatively and semi-quantitatively) and by serological examinations. Due to large variation of infection dynamics in the first two periods among "identical" pastures, the investigation of the ploughing effect was excluded. As an alternative, for further monitoring of the infectivity of the salmonella contaminated pasture environment, new tracer animals were introduced in one pasture (still contaminated), whereas the set-up from the first period was repeated in those three pastures with no or little contamination of the environment.

A method was developed and evaluated for determining the species composition (*C. jejuni* and *C. coli*) in faecal samples and subsequent isolation of the species in minority. The groups of pigs serving as controls in the experimental *Salmonella* infection study was also used for studying the dynamics of natural *Campylobacter* infection in outdoor pigs. The piglets were monitored for natural colonisation of thermophilic *Campylobacter* species, and the ratio between *C. jejuni* and *C. coli*. Likewise, environmental samples were analysed throughout the experimental periods. Isolates from animals and environment will be identified to the species level and serotyped to monitor the dynamics of the infection: number of different strains in each pig and persistence/exchange of strains over time.

The achievements obtained will be formulated in practical guidelines directly applicable for the organic pig producers in order to minimise the risk of zoonotic infection in organic pig herds. In addition, results will be available for the scientific community through publication in reviewed journals. The large amount of quantitative data obtained in this project will deliver the necessary information for use in quantitative risk assessment of zoonotic infection in organic pig production.

Table A.1: Work package list (from application)

No.	Work package title	Participants*	Budget** (1.000 DKK)	Start	End	Deliverable no(s):
WP1	Investigation of introduction and spread of <i>Salmonella</i> in outdoor pigs	<u>Dorte Lau Baggesen</u> ; Eva Møller Nielsen; Annette Nygård Jensen	1,325	2002	2004	1 – 6
WP2	Investigation of the dynamics of natural <i>Campylobacter</i> infection in outdoor pigs	<u>Eva Møller Nielsen</u> ; Dorte Lau Baggesen; Annette Nygård Jensen	675	2002	2004	7 – 11

* Responsible participants are underlined

** All expenses concerning the animal experiments (756,000 Dkr.) are covered by WP1. These experiments form the basis for both WP's.

B. Objectives and expected achievements (from application)

The overall objective of the project is to improve the knowledge on the dynamics behind environmental spread of two important zoonotic bacteria – *Salmonella* and *Campylobacter* – within outdoor pig production systems.

Good management procedures – good farming practice – based on scientific evaluation of the risks of introduction and persistence of zoonotic infections is essential for the redevelopment of the organic production. The present proposal focuses on studies to describe the dynamics of zoonotic infections in outdoors pigs and evaluate the risk of outdoor production in relation to spread and persistence of *Campylobacter* and *Salmonella* infections in pigs. This include

- evaluation of the survival of *Salmonella* Typhimurium in soil and grass of contaminated weaning and fattening pastures for outdoor pig production systems
- measurement of the infectivity of naturally *S. Typhimurium* contaminated pastures in relation to time, and
- in the case of high infectivity, evaluation of the pathogen reducing effect of soil treatment
- description of the prevalence of natural campylobacter infection over time in outdoor pigs including description of the species distribution in the group and in the individuals
- evaluation of the possible changes in prevalence and *Campylobacter* species distribution in relation to time and environmental contamination

Through the described project new important knowledge regarding salmonella and campylobacter infection in outdoor pig production systems will be obtained. This includes information on the infectivity of *Salmonella* under natural conditions, e.g. on the risk for healthy piglets to be in-

ected from infected animals or contaminated environment. The investigations will provide quantitative information on the infection and contamination levels, which is presently very limited. In the case of a very high contamination level in the pastures the study will provide additional information on the pathogen reducing effect of the ploughing pastures. In addition, new information on the infection dynamics of thermophilic campylobacters in outdoor pigs is expected especially in relation to the level of colonisation, distribution of different species and types, the possible co-infection of *C. coli* and *C. jejuni*, and the transmission between animals and the environment.

The achievements obtained will be formulated in practical guidelines directly applicable for the organic pig producers in order to minimize the risk of zoonotic infection in organic pig herds. In addition, results will be available for the scientific community through publication in reviewed journals. The large amount of quantitative data obtained in this project will deliver the necessary information for use in quantitative risk assessment of zoonotic infection in organic pig production. This achievement will be strength by the interaction with other projects carried out by the present group (Wildlife as a source of salmonella infection in food-animal production, FØSI00-SVS-6, which has been extended by *Campylobacter* investigations; ”Grundlag for rådgivning vedr. foderets betydning for salmonellainfektion i økologiske svinebesætninger” [The significance of feeding in relation to salmonella infections in organic pig herds] , J.nr. 93S-2462-Å01-00981).

C. Midterm results and progress

C.1 Description (summary) of main results and conclusions

A detection limit experiment for bacteriological and real-time PCR detection of Salmonella in faecal and soil samples, respectively, has been performed. Three different strains of salmonella, *S. Typhimurium* DT 12, *S. Typhimurium* DT 12 rifampicin resistant and *S. Dublin* were evaluated with pre-enrichment of ca. 5 CFU per 25 g sample. Beforehand, a method for preparation of a *Salmonella* culture with a known number of cells for spiking of non-infected sample material was established. The pre-enrichment step was performed in parallel in conventional buffered pepton buffer (BPW) and BPW added Novobiocin (22 mg/L), which previously has been shown to increase the number of salmonella after pre-enrichment. The best overall detection of *Salmonella* was obtained with the bacteriological method using pre-enrichment in BPW containing Novobiocin. This method was applied throughout the experimental study.

In addition a quantitatively method for enumeration of presumptive *E. coli* in soil and water samples were established. This organism serves as a faecal indicator, and would add to the information about the potential contamination load in the pasture, especially if detection of salmonella from the environmental samples was low.

Experimental pastures have been set up for organic pigs at Rørrendegård (research animal facility under the Danish Royal Veterinary and Agricultural University) with four pastures for 10 pigs each and two pastures for 8 pigs each. A total of 3 times 56 organic pigs, 7-8 weeks of age (post weaning) were purchased from an organic pig farmer in Sønderjylland.

In the first period of the study, 3 of the 10 pigs in each of the 4 pastures were given 10^7 *S. Typhimurium* DT 12 rifampicin resistant cells (oral) or 10^9 cells at day 1 to establish 2 low and 2 highly contaminated pastures. The establishment of different levels of contamination were adjusted by number of ingested cells instead of the number of infected animals, to maintain a similar high number of tracer animals in each pasture. Faecal and blood samples and environmental samples were collected weekly. The inoculum salmonella strain was not found in all of the inoculated pigs at 3 days post inoculation, however, salmonella were transmitted to some of the non-infected trace pigs. Varying numbers of salmonella-positive pigs within each pasture were found

each week throughout the 6-week period, but the transmission of salmonella was clearly higher in the two high dosis pastures. The semi-quantitative method indicated a rather low salmonella excretion level in most pigs <100 CFU/g.

In addition, 1 water and 6 soil samples (each pools of 5 samples) were collected from the surface of the environment of the pasture and tested bacteriological (qualitative) for *Salmonella* and presumptive *E. coli* (faecal qualitatively indicator). The salmonella inoculum-strain was recovered from some of the environmental samples already in the first week in the highly exposed pastures and later in the low exposed pastures.

Due to the results obtained within the first weeks of monitoring, it was decided to terminate the first period after 6 weeks instead of the proposed 2 months, in order to minimize the potential loss of contamination in the pasture environment, which is necessary for the evaluation of the infectivity of the environment in a second period with new tracer animals. This decision was also influenced by the possible delivery of pigs, which was only possible at 3 weeks interval due to the farmers post weaning procedure. Thus the pigs were killed after 6 weeks, but the post-mortem examinations were omitted, as it was not practical feasible to process 56 pigs day within one day. The pastures should be vacated at once for a immediately introduction of new non-infected pigs (the day after) which was considered important for elucidating the possible transmission of salmonella from a contaminated environment.

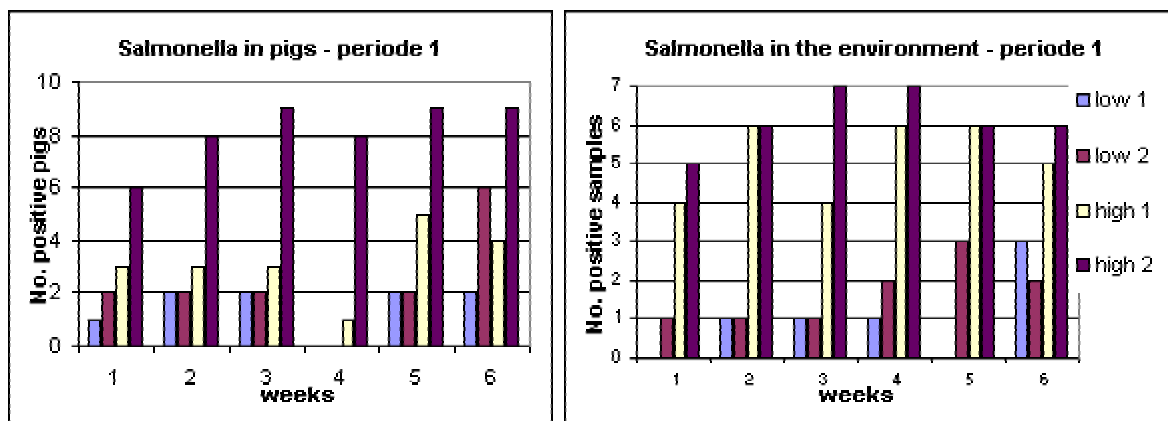


Figure 1: Number of salmonella excreting pigs and level of salmonella contamination in each pastures (1st. periode)

In the second period, salmonella infected pigs were merely found in one of the high dosis pastures (one pig were killed after 4 weeks due to clinical signs of salmonellosis), whereas only few pigs became infected in the other pastures. However, it was still possible to detect the inoculum strain in some of the environmental samples for all 6 weeks of the second period.

On basis of these results it was assumed that only one of the high dosis pastures could pose an infection risk to new non-infected pigs introduced in a possible third period. Thus, an evaluation of the pathogen reducing effect of ploughing was not possible. However, it was tested whether the reduced contamination level in high dosis pasture still could cause and infection in new non-infected pigs. In this pasture the *Salmonella* inoculum strain from the first period persisted in the pasture throughout the whole experiment and was in the third period transmitted to two pigs at different time.

In the other 3 pastures, the set-up from the first periode was repeated, but with the high dosis only. To differentiate between salmonella strains potentially surviving from the first two periods and the newly inoculated strain, the inoculum strain was further selected for nalidixic acid resis-

tance. Again, detection of salmonella was not possible in all the inoculated pigs at 3 days post infection but transmission to some of the non-infected pigs did occur.

In addition to the inoculum strain, *S. Typhimurium* DT 12 rifampicin/nalidixic resistant, an unexpected high number of different serotypes and *S. Typhimurium* phagetypes were found in pig faecal and soil samples, and occasionally these were also found in the control pastures. To try to elucidate the source of these types, rodents and birds was caught in the surrounding environment for a period of 2 weeks. This was performed in collaboration with the Danish Pest Infestation Laboratory. However, no *Salmonella* was found, whereas *Campylobacter* was found especially in the crow birds. During the experiment, cases of PMWS were experienced among the pigs and some pigs of poor condition were killed. This may have increased the susceptibility of the pigs and partly explain the unexpected high colonisation of different *Salmonella* bacteria besides the inoculum strain.

The *Salmonella* inoculum strain was frequently detected in the environment of the pasture and to examine the extra-intestinal survival over time, the bacteriological examination of soil and water samples has been continued after the determination of the pig experiment. After three weeks, salmonella was still detected in a few samples.

An in-house real-time PCR method for discrimination of *Campylobacter coli* and *C. jejuni* was further developed for screening of pig faecal samples. Two different broths, Preston and Bolton without blood were evaluated for pre-enrichment of *Campylobacter* in faecal samples and furthermore two sample preparation methods, boil lysate and chelex were tested. The Bolton without blood and the boil lysate were chosen for the experimental study.

A DIG-labelled *Campylobacter jejuni* species specific nucleotide probe based on the hippuricase gene of *C. jejuni* has been developed. This probe is used for colony blot hybridisation, with chromogenic detection of target hybrids on the nylon membrane. With this method it has been possible to detect and isolate *C. jejuni* present in minority among other campylobacter species on mCCD Agar plates. The obtained isolates were confirmed to be *C. jejuni* by the real-time PCR test and furthermore tested for the ability to hydrolyse hippurate, which distinguish *C.jejuni* from other campylobacter species. However, in a few cases the obtained isolated was not confirmed as *C. jejuni*, probably because the isolate were picked from a non-single colony with possible overgrowth by other types of campylobacter.

In all three periods the control pigs were examined for excretion level of campylobacter, which fluctuated throughout the period with no clear tendency. Five *Camp.* ssp. were isolated randomly from each pig and environmental samples every second week. In addition, the specific detection of *Campylobacter jejuni* with real-time PCR screening of pre-enriched faecal samples and colony hybridisation was performed to determine the campylobacter species distribution in pigs. A freeze collection of isolated strains has been established for further analysis.

In the first two periods, only a few pigs were found to host *C. jejuni*, whereas *C. jejuni* (in minority) was found one or more times in 12 of 15 pigs in the third period. Preliminary results will be presented at SAFE PORK 2003, The 5th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork, Crete.

A large amount of results has been collected during the summer and the data analysis has just been initiated and will continue in the coming months.

C.2 Fulfilment of deliverables and milestones

(To be completed for each work package)

WP1: Investigation of introduction and spread of <i>Salmonella</i> in outdoor pigs	Time schedule according to application	Deviations, if any*
Deliverables		
1. Determination of detection limit for bacteriological methods	Jan. 03	
2. Information on the risk for <i>Salmonella</i> transmission to uninfected piglets constituted by infected animals and contaminated pasture environment representing different infection pressures	Sept. 03	
3. Information on whether survival of <i>Salmonella</i> in the environment under naturally conditions is high enough to cause/transmit infection in uninfected animals	Sept. 03	
4. Information on the pathogen reducing effect of ploughing pastures will be available if the infection level established is sufficiently high for this evaluation	Nov. 03	Evaluation not possible.
5. Scientific and international publication of results	June 04	
6. Practical guidelines for minimising the risk of salmonella infection in outdoor pig production	June 04	
Milestones		
Determination of detection limit for the bacteriological method applied	After 1. quarter of 03	
First experimental period – spread of infection between experimentally infected animals and uninfected tracer animals	After 2. quarter of 03	
Second experimental period – spread of infection from pasture environment to uninfected tracer animals	After 3. quarter of 03	
Third experimental period – <i>either</i> spread of infection from pasture environment to uninfected tracer animals and effect of soil treatment <i>or</i> spread of infection between experimental highly infected animals and uninfected tracer animals	After 3. quarter of 03	
Scientific and international publication of results and formulation of practical guidelines for minimizing the risk of salmonella infection in outdoors pig production	After 2. quarter of 04	

* *Deviations are to be further discussed in D*

WP2: Investigation of the dynamics of natural <i>Campylobacter</i> infection in outdoor pigs	Time schedule according to application	Deviations, if any*
Deliverables		
7. Development and evaluation of a new approach for determining the species composition (<i>C. jejuni</i> and <i>C. coli</i>) in faecal samples and subsequent isolation of the species in minority	Jan. 03	
8. Publication of method in the form of a short paper (note/letter)	Apr. 03	Jan. 04

9. Elucidation of the infection dynamics of thermophilic campylobacters in out-door organic pigs, including time of colonisation, excretion level in faeces, number of different strains co-colonising one piglet during time, persistence of strains, and the ratio between <i>C. jejuni</i> and <i>C. coli</i> in individual animals	Oct. 03	
10. Information on the interaction between individual pigs and between the pigs and environment with respect to <i>Campylobacter</i> infection	Nov. 03	
11. Scientific and international publication of results	June 04	
Milestones		
Implementation of a PCR-based method for determination of the composition of <i>Campylobacter</i> species in faecal samples and the subsequent isolation of the species in minority. Determination of detection limit of the method.	After 2. quarter of 03	
Results are obtained on the infection dynamics of thermophilic campylobacters in outdoor piglets repeated during three seasons	After 1. quarter of 04	
Results are obtained on the interaction between outdoor piglets and the environment with respect to <i>Campylobacter</i> infections	After 1. quarter of 04	
Publication of results in international journal	After 2. quarter of 04	

D. Description of deviations and subsequent adjustments of plans

A Ph.D. studium has been established based on the project. This has caused adjustment of the budget (see 8. A & B). In addition, related activities will be performed just after this project period.

Publication of method in the form of a short paper (WP2-8) has been postponed to Jan. 04 due to limited time and an inferior priority to the planning and performance of the experimental set-up.

Evaluation of the ploughing effect (WP-4) was not possible due to a large difference in the contamination level in "identical" pastures. In one pasture the contamination level were still high at the end of the second period, and the infectivity was evaluated by introduction of new non-infected tracer animals. In the 3 others were the infectivity study of the first repeated but with high inoculum doses only

In relation to the interaction with the surrounding environment, samples from birds and rodents has been included in the study, which will add to the information about potential sources of the pathogens in ourdoor production of pigs.

E. Project publications and other products

1. Articles in international, scientific journals with review procedures

None

2. Papers presented at congresses, symposiums, etc.

Campylobacter species distribution in outdoor pigs. Presentation no. O44. SAFE PORK 1st-4th Oct. 2003, The 5th International Symposium on the Epidemiology and Control of Foodborne Pathogens in Pork, Crete, Greece

3. Reports, articles in agricultural journals, etc.

None

4. Oral presentations, public meetings, field days, etc.

None

F. Scientific education

A Ph.D. studym has been established based on the project. This has been possible due to an additional grant given by Research School for Organic Agriculture and Food Systems, The Royal Veterinary and Agricultural University and Danish Research Centre for Organic Farming.

The Ph.D. study lasts three years from 1st of October 2002 to 31st of September 2005. In addition to the activities described in this project the Ph.D. study will include model investigations on the surveillance of pathogens in the extra intestinal environment. The model investigations have the aim to validate hypothesis described through the activities carried out in this project.

Cand. Scient. Annette Nygaard Jensen has been employed as a ph.d.-student. Associate professor Anders Dalsgaard, DVM, Ph.D., Institute of veterinary microbiology is main supervisor whereas Dorte Lau Baggesen, Senior research officer, Ph.D. and Eva Møller Nielsen, Senior research officer, Ph.D. are co-supervisors.

G. National and international cooperation

The outdoor experiments will be carried out at the research animal facilities of The Danish Veterinary and Agricultural University.

The examinations of rodents in the surrounding environment was carried out in cooperation with Jens Lodal, Danish Pest Infestation Laboratory.

H. Critical reflection on the project

The amount and quality of expected achievements will increase because of the establishment of a Ph.D. study with additional activities. The contact to other researchers in the area of organic farming will be improved through the participation in the Research School for Organic Agriculture and Food Systems.

The pigs used in the experiment were delivered by an organic farmer. It was believed that the organic feed and the outdoor life would influence the health condition and intestinal microflora of organic bred animals. Thus, for the most realistic picture of the infection dynamics in organic pigs, conventionally pigs should probably be avoided. Only rather few farmers had the capacity to deliver 56 pigs of similar age at one time, but a farmer was recommended by an agricultural con-

sultant. The information about the herd was limited, besides a sampling of faecal samples from the herd to examine the salmonella status before the experiment. The pigs were delivered in connection to weaning at the age of 7 weeks, but there was a big variation in their weight, which may affect their resistance to infection, however, the results reflected apparently no clear correlation between weight and infection. In the second period, cases of PMWS were experienced among the pigs and some pigs of poor condition were killed. This may of course also influence the infection dynamics of salmonella, as the pigs may be more prone to infection, but such potential interaction could not be controlled. Although it may seem like a "worst case scenario" it may reflect a rather realistic situation among organic pig farmers.

The *Salmonella* inoculum strain was frequently detected in the environment of the pasture and to examine the extra-intestinal survival over time, the bacteriological examination of soil and water samples will continue in the Autumn 2003 after the determination of the pig experiment.

Furthermore, to examine the long-term infectivity of salmonella potentially persisting in the environment, it will be considered to introduce new tracer-animals in the spring 2004, to elucidate whether the contaminated pastures still pose an infection risk to pigs.

8. Budget

A. Account for any change in budgets

Within the time schedule and the budget of this project some adjustments have been necessary to fulfil the requirements of the Ph.D. study. The investigations carried out in this study will cover the main part of a ph.d. study (see pkt. F).

B. Budget for the whole project (1.000 DKK) – NB ! AJUSTED BUDGET

Total consumption of funds from DARCOF and expected consumption this year and coming years

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel	3,75	12	9		24,75
Technical personnel		19			19

Year:	Consumption before 2003*	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel	123	320	244		
Technical personnel		430			
Other operational costs	18	287	50		
Equipment		130			
Others (please specify)		45	45		
Direct costs	141	1.212	339		
Indirect costs (20% of direct costs)	23	242	68		
Total	164*	1.454	407		

* The FØJO-financed part of the consumption constitutes 138.000 DKr.

9. Signatures and stamps

Name	Institute	Date	Signature
Head of project Dorte Lau Baggesen	Danish Veterinary Institute	30. september 2003	

Appendix I. Detailed budget

A. Budget for each participating institute (1.000 DKr)

Name of Institute:

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel					
Technical personnel					

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel					
Technical personnel					
Other operational costs					
Equipment					
Others (please specify)					
Direct costs					
Indirect costs (20% of direct costs)					
Total					

Comments:

B. Budget for each participating department (1.000 DKK)

Name of Institute and department:

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel					
Technical personnel					

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel					
Technical personnel					
Other operational costs					
Equipment					
Others (please specify)					
Direct costs					
Indirect costs (20% of direct costs)					
Total					

Comments:

C. Budget for co-financing from each participating institute (1.000 DKK)

Name of Institute:

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Man-months					
Scientific personnel					
Technical personnel					

Year:	Consumption before 2003	Expected consumption 2003	2004	2005	Total
Salaries					
Scientific personnel					
Technical personnel					
Other operational costs					
Equipment					
Others (please specify)					
Direct costs					
Indirect costs (20% of direct costs)					
Total					

Comments: