PRO-LAB DIAGNOSTICS

MICROBANK™

WORLD WIDE PERFORMANCE PORTFOLIO

www.pro-lab.co.uk

Dear Customer,

We are pleased to provide that latest updated version of the Microbank™ World Wide Performance Portfolio for 2015.

Microbank™ now enjoys over 21 Years of successful storage and retrieval of bacterial and fungal cultures with over 20 million vials manufactured and supplied. We have included in the following pages a number of key reference trials that have contributed significantly to the success and reputation of the Microbank™ system over the years. There are of course many other papers and references for the Microbank™ and we are happy to include any of these in future publications of the Portfolio upon request.

We are extremely grateful to all who have contributed to the success and reputation of the Microbank™ system.

We are also grateful to our friends at Bestbion dx in Germany for supplying the contributions from the many German laboratories using the system.

Finally, our product mascot simply can't go with mention. This cute little guy has become synonymous with Microbank™ system and has his own calendar monitoring his travels.

If you have any pictures for inclusion in the calendar, or if you need a new penguin (we do see quite a few with missing heads!) please email:

uksupport@pro-lab.com

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MICROBANK™ INTRODUCTION

The Original Long Term Bacterial & Fungal Storage System

Microbank™ is a convenient, ready-to-use system designed to greatly simplify the storage and retrieval of bacterial cultures. It comprises of a unique cryovials system incorporating treated beads and a special cryopreservative solution.

Microbank™ has proven performance and is now the natural choice for Microbiologists worldwide including many specific reference culture collection centers.

Microbank™ is a more reliable method for maintaining important cultures than repetitive subculture, which can result in altered characteristics, lost organisms, or contaminated cultures.

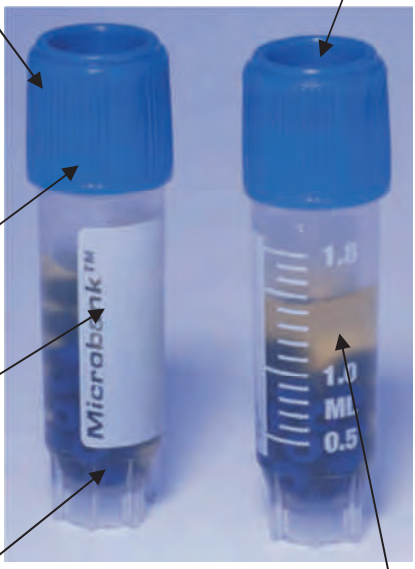
Microbank™ provides microbiologists with a much simpler option to traditional methods of lyophilization or use of glycerol broth.

MICROBANK™ KEY FEATURES

Industry standard
robust cryovial which can withstand snap freezing with liquid nitrogen.

Available in five colours
which provides laboratories a system to colour code different bacterial species.

Large 2 ml size vials
with triple depth external threaded cap. The larger cap reduces the possibility of contamination and the wider tube diameter provides more room for mixing to ensure beads are properly coated.



Larger writing area
allows for complete coding and reference data.

Chemically treated beads
provides improved bacterial adhesion.

Specially formulated preservative
ensures longer survival of fastidious bacteria and higher quantitative recoveries.

PRODUCT AVAILABILITY

MICROBANK™

Advanced presentation of 80 vials supplied in a plastic freezer box manufactured from durable plastic with "see through" lids, number locator printed screens, and tube collection device.

Products	Code	Size
Microbank™ - Blue	PL.170/B	80 Vials
Microbank™ - Green	PL.170/G	80 Vials
Microbank™ - Red	PL.170/R	80 Vials
Microbank™ - Yellow	PL.170/Y	80 Vials
Microbank™ - Light Blue	PL.170/LB	80 Vials
Microbank™ - Mixed (16 x each colour)	PL.170/M	80 Vials

MICROBANK™ SPECIAL PRESERVATION ONLY

Supplied in the same format as traditional Microbank™ but with specially formulated broth only.

Products	Code	Size
Microbank™ - Blue	PL.173/B	80 Vials
Microbank™ - Green	PL.173/G	80 Vials
Microbank™ - Red	PL.173/R	80 Vials
Microbank™ - Yellow	PL.173/Y	80 Vials
Microbank™ - Light Blue	PL.173/LB	80 Vials
Microbank™ - Mixed (16 x each colour)	PL.173/M	80 Vials

MICROBANK™ DRY

Supplied in the same format as traditional Microbank™ but without the specially formulated cryopreservation solution.

Products	Code	Size
Microbank™ - Blue	PL.172/B	80 Vials
Microbank™ - Green	PL.172/G	80 Vials
Microbank™ - Red	PL.172/R	80 Vials
Microbank™ - Yellow	PL.172/Y	80 Vials
Microbank™ - Light Blue	PL.172/LB	80 Vials
Microbank™ - Mixed (16 x each colour)	PL.172/M	80 Vials

MICROBANK™ ACCESSORIES

Freezer storage boxes are available which are suitable for collection and organization in low temperature freezers. Available in blue and red, each freezer box will hold 81 Microbank™ vials. Also available is the Cryoblock for maintaining low temperatures while working with Microbank™ vials on the laboratory bench.

Products	Code	Size
Freezer Storage Box - Blue	PL.169/B-1	Each
Freezer Storage Box - Red	PL.169/R-1	Each
Freezer Storage Box - Blue	PL.169/B-4	4 Pack
Freezer Storage Box - Red	PL.169/R-4	4 Pack
Cryoblock	PL.155-1	20 Well
Insulated Base & Lid	PL.156	2 Pack
Laminated Log Book	PL.165	12 Charts
Aluminium Cryocanes	PL.166	12 Canes

MICROBANK™ INSTRUCTIONS FOR USE

INTENDED USE

Microbank™ is a sterile vial containing porous beads which serve as carriers to support microorganisms.

SUMMARY AND EXPLANATION

Long term storage of microorganisms is a challenge in routine microbiology. Organisms should be stored at low temperatures utilizing a mechanical technique that offers the least possibility of disturbance, yet, permits ready access to stored material. Microbank™ offers a solution to this problem.

DESCRIPTION

Individual coloured beads are packaged approximately 25 beads in a cryovial containing cryopreservative. The beads are washed and are of a porous nature allowing microorganisms to readily adhere onto the bead surface. After inoculation the cryovials are kept at -70°C for extended storage. When a fresh culture is required, a single bead is easily removed from the vial and used to directly inoculate a suitable bacteriological medium.

PROCEDURE

A. PREPARATION

Using a permanent marker code the vial as desired, one organism per vial to be inoculated.

Under aseptic conditions open the screw cap cryovial.

Inoculate the cryopreservative fluid with young colonial growth (18-24 hours) picked from a pure culture to approximately a 3-4 McFarland standard.

Close vial tightly and invert 4-5 times to emulsify organism. **DO NOT VORTEX**!

At this point the microorganisms will be bound to the porous beads. The excess cryopreservative should be well aspirated leaving the inoculated beads as free of liquid as possible.

Close the vial finger tight.

Record the inoculation coding on the grid provided or on other permanent record as desired.

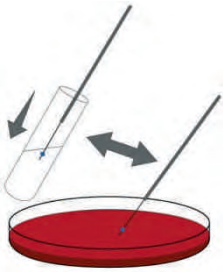
Store the inoculated cryovial at -70°C for best long term results.

B. RECOVERY

Under aseptic conditions, open the cryovial and using a sterile needle or forceps remove one coloured bead.

Close the vial finger tight and return as soon as possible to low temperature storage. Excessive changes in temperature reduce the viability of the organisms.

The inoculated bead may then be used to directly streak on to solid medium or may be dropped into an appropriate liquid medium.



When used as recommended, each cryovial will store approximately 25 identical potential cultures.

THERE IS A VIDEO DETAILING THIS PROCESS AVAILABLE TO VIEW ON YOUTUBE: https://www.youtube.com/watch?v=Rfh680H1jBM

LIMITATIONS

Microbank™ is offered solely as a means of providing extended storage possibilities for organisms. In use, aseptic technique should be practiced to ensure continued integrity of the stored microorganism.

Microbank™ should not be used if any of the following conditions are present before inoculation:

- The vial shows any evidence of leakage (loss of cryopreservative).
- Turbidity in cryopreservative suggesting contamination.
- The expiry date on the outer label has elapsed.

After removal, beads should not be returned to the cryovial for any reason.

Microbank™ is supplied in a variety of colours. These colours do not imply any change in the product function. They are provided only for colour coding convenience.

SAFETY PRECAUTIONS

A microbiological safety cabinet should be used when making and manipulating a heavy suspension of a culture.

Observe biohazard precautions when discarding used or partly used cryovials.

When storing Microbank™ in liquid nitrogen the following precautions should be taken:

- Ensure the cryovial screw cap is tightened finger tight: over-tightening may cause distortion of the silicone O-ring in the cap which may cause leakage.
- Ensure that the thread of the cryovial and screw cap is completely dry before closing: liquid drops will impair the seal in liquid nitrogen.

- All Microbank™ vials should always be stored in the gas phase, above liquid nitrogen. If immersed, they might develop leaks or even shatter when returned to room temperature.
- When removing vials from liquid nitrogen containers always use safety equipment such as gloves, hoods, face shields etc.

PRESENTATION

Microbank™ is packaged in shelf packs of 80 vials.

STORAGE

Before use, unused Microbank™ may be stored at 4°C or at room temperature but kept away from direct light. Stored under these conditions Microbank™ may be used up to the date of expiry shown on the product label.

REFERENCES

White and Sand, R.L. 1985. Medical Laboratory Sciences 42:289-290(U.K).
Feltham et al. 1978. Journal of Applied Bacteriology. 44:313-316.
Nagel, J.G. and Cunz, L.J. 1971. Applied Microbiology, 23(4):837-838

ANAEROBE REFERENCE UNIT MICROBANK™ STORAGE TRIAL 5 YEARS

Dr J Brazier, Dr V Hall.

Anaerobic Reference Centre, Cardiff UK.

Method and Materials

One bead from each vial will be inoculated onto Fastidious Anaerobe agar (Lab M, Bury) with 5% horse blood, and spread for single colonies. Plates will be incubated promptly in an anaerobic chamber (Concept 300 or Concept Plus) at 37°C for 48 hours. On removal from the chamber, cultures will be examined for growth consistent with the intended isolate. Cultures yielding no growth will be incubated for a further 3 days and re-examined.

Results

Growth will be recorded as + (intended isolate recovered) or - (isolate not recovered). Original inocula were not standardised, therefore, quantitation of growth would be fairly meaningless. Besides, for our purposes, density of growth is unimportant as long as the isolate is recoverable.

Bacterial strains (n = 100) frozen in 1993

ARU ref.	Organism	Growth	Comments
R5774	*A. gerencseriae*	+	
R5554	*A. israelii*	+	
R5557	*A. israelii*	+	
R5634	*A. naeslundii*	+	
R5568	*A. odontolyticus*	+	
R5639	*A. turicensis*	+	
R5552	*Actinomyces naeslundii*	+	
R5571	*Actinomyces* spp.	+	
R5619	*Actinomyces* spp.	+	
R5718	*Actinomyces viscosis*	+	

R5956	*B. fragilis*	+	
R5801	*B. ovatus*	+	
R5867	*B. splanchnicus*	+	
R5868	*B. splanchnicus*	+	
R5933	*B. thetaiotaomicron*	+	
R5791	*B. vulgaris*	+	
R5600	*B.distasonis*	+	
R5762	*B.distasonis*	+	
R5587	*B.fragilis*	+	
R5620	*B.fragilis*	+	
R5589	*B.fragilis* (metronidazole resist.)	+	
R5745	*B.ovatus*	+	
R5755	*B.thetaiotaomicron*	+	
R5631	*B.uniformis*	+	
R5570	*Bacteroides thetaiotaomicron*	+	
R5921	*Bif. Animalis* group	+	
R5824	*Bif. Longum*	+	
R5556	*Bifodobacterium* spp.	+	
R5588	*Bifodobacterium* spp.	+	
R6043	*Bilophilia wadsworthia*	+	
R5760	*C. glycolicum*	+	
R5601	*C.bifermentans*	+	
R5559	*C.butricum/beijerinckii*	+	
R5635	*C.cadaveris*	+	
R5584	*C.clostridioforme*	+	
R5573	*C.difficile*	+	
R5606	*C.novyi* type A	+	
R5558	*C.paraputrificum*	+	
R5586	*C.perfringens*	+	
R5628	*C.ramosum*	+	
R5560	*C.septicum*	+	
R5642	*C.sordellii*	+	
R5759	*C.sporogenes*	+	
R5572	*C.tetani*	+	
R6001	*Camp .ureolyticus*	+	
R5756	*Camp. ureolylicus*	+	
R5669	*Camp. gracillis*	+	
R5551	*Campylobacter recta*	+	

R5738	*Campylobacter* spp.	+	
R5555	*Clostridium perfringens*	+	
R5800	*E. aerofaciens*	+	
R5598	*E. lentum*	+	
R5670	*E. lentum*	+	
R5837	*Eu. Lentum*	+	
R5569	*Eubacterium aerofaciens*	+	
R5769	*F. naviforme*	+	
R5585	*F. necrophorum*	+	
R5778	*F. necrophorum*	+	
R5748	*F. nucleatum*	+	
R6000	*F. nucleatum*	+	
R6003	*F. nucleatum*	+	
R6066	*F. nucleatum*	+	Light growth after 3 days
R5716	*F. russii*	+	
R5641	*F. varium*	+	
R5565	*Fusobacterium necrophorum*	+	
R6097	*L. acidophilus*	+	
R6085	*Lactobacillus acidophilus*	+	
R5927	*Peptostrep .asaccharolyticus*	+	
R5562	*Peptostrep. Asaccharolyticus*	+	
R5840	*Peptostrept. anaerobius*	+	
R5767	*Peptostrept. Magnus*	+	
R5805	*Peptostrept. micros*	+	
R5997	*Peptostrept. micros*	+	
R5806	*Peptostrept.anaerobius*	+	
R5630	*Peptostreptococcus productus*	+	
R5720	*Peptostreptococcus magnus*	+	
R5761	*Peptostreptococcus productus*	+	
R5563	*Peptostreptococcus* spp.	+	
R5622	*Peptostreptococcus* spp.	+	
R5624	*Peptostreptococcus* spp.	+	
R5807	*Porph. Endodontalis*	+	
R5995	*Porph. Endodontalis*	+	
R6079	*Porph. Levii*	+	
R5550	*Porphyromonas asaccharolytica*	+	
R5838	*Porphyromonas* spp.	+	
R5954	*Prev. denticola*	+	

R5955	*Prev. melaninogenica*	+	light growth after 3 days
R5974	*Prev. melaninogenica*	+	light growth after 3 days
R6080	*Prev. oris*	+	
R5783	*Prevotella loescheii*	+	
R5836	*Prevotella* spp.	+	
R5839	*Prevotella* spp.	+	
R5764	*Prop. Acnes*	+	
R5671	*Prop. granulosum*	+	
R5567	*Prop. Propionicum*	+	
R5561	*Propionibacterium acnes*	+	
R5668	*Staphylococcus saccharolyticus*	+	
R5826	*Streptococcus mutans*	+	
R5848	*V.parvula*	+	
R5675	*Veillonella parvula*	+	

ANAEROBE REFERENCE UNIT MICROBANK™ STORAGE TRIAL 7 YEARS

Dr J Brazier, Dr V Hall.

Anaerobic Reference Centre, Cardiff UK.

Method and Materials

Microbank™ vials were inoculated with clinical isolates of obligately anaerobic bacteria referred to the Anaerobe Reference Unit for confirmation of identity and were frozen at -80°C for seven years. Isolates for study (n=100) were selected to represent the range of species commonly isolated from clinical material. One bead from each vial had been removed for culture after five years. Beads may have been removed on other occasions.

One bead from each vial was placed aseptically onto Fastidious Anaerobe agar (IDG, Bury) containing 5% horse blood. Plates were spread for single colonies and promptly incubated in an anaerobic chamber (Concept Plus) for 48 hours.

On removal from the chamber, plates were examined for growth of colonies characteristic of the intended isolates. If growth was not apparent at this stage, plates would be re-incubated for a further three days but, in the event, this was not necessary.

Results

All 100 isolates were cultured after anaerobic incubation for 48h.

Isolates examined were:
Actinomyces spp. (2)
Actinomyces gerencseriae
Actinomyces israelii (2)
Actinomyces naeslundii (2)

Actinomyces odontolyticus
Actinomyces turicensis
Actinomyces viscosis
Bacteroides distasonis (2)
Bacteroides fragilis (4)
Bacteroides opvatus (2)
Bacteroides splanchnicus (2)
Bacteroides thetaiotaomicron (3)
Bacteroides uniformis
Bacteroides vulgaris
Bifidobacterium spp. (2)
Bifidobacterium animalis group
Bifidobacterium longum
Clostridium bifermentans
Clostridium butyricum / beijerinckii
Clostridium cadaveris
Clostridium clostridioforme
Clostridium difficile
Clostridium glycolicum
Clostridium novyi type A
Clostridium paraputrificum
Clostridium perfringens (2)
Clostridium septicum
Clostridium sordellii
Clostridium sporogenes
Clostridium tetani
Clostridium ramosum
Eggerthella lenta (*Eubacterium lentum,* 3)
Eubacterium aerofaciens (2)
Peptostreptococcus spp. (3)
Peptostreptococcus anaerobius (2)
Peptostreptococcus asaccharolyticus (2)
Peptostreptococcus micros (2)
Peptostreptococcus magnus (2)
Peptostreptococcus productus (2)
Propionibacterium acnes (2)

Staphylococcus saccharolyticus
Streptococcus mutans

Results and Discussion

The sampling procedure chosen for the trial was deliberately stringent as it included a dilution step which would not normally be part of the recovery of a strain from cryogenic storage. Survival and recovery of fastidious anaerobes with this protocol, therefore, is a more rigorous test of the system, and makes the results more meaningful.

The overall performance of the Microbank™ preservation system for anaerobes was highly satisfactory. Although variations in recovery are apparent between samples, these are probably due to a combination of heterogeneity of inoculum and sampling error. There was no evidence of a gradual decline in recovery over time as compared to the control.

Three organisms failed to survive the trial period; these were *Actinomyces odontolyticus, Actinomyces israelii* and *Prevotella intermedia*. The latter two also failed in the control vial, however, and the former was contaminated with a P.acnes, presumably at the date of freezing.

In light of these results the Anaerobe Reference Unit has adopted the Microbank™ system for the preservation of strains in its culture collection.

ANAEROBE REFERENCE UNIT MICROBANK™ STORAGE TRIAL 10 YEARS

Dr J Brazier, Dr V Hall.

Anaerobic Reference Centre, Cardiff UK.

Method and Materials

Microbank™ vials were inoculated with clinical isolates of obligatory anaerobic bacteria referred to the PHLS Anaerobe Reference Unit (now the National Public Health Service for Wales Anaerobe Reference Laboratory, ARL) for confirmation of identity, and were frozen at -80°C for ten years. Isolates for study (n = 100) were selected to represent the range of species commonly isolated from clinical material. One bead from each vial had been removed to demonstrate viability after five years and seven years. Beads may have been removed on other occasions.

One bead from each vial was placed aseptically onto Anaerobe Basal Agar (Oxoid, Basingstoke) containing 5% horse blood. Plates were spread for single colonies and were incubated promptly at 37°C in an anaerobic chamber (Concept Plus) for 48 hours. On removal from the chamber, plates were examined for growth of colonies characteristic of the intended isolates. If growth was not apparent at this stage, plates were re-incubated for a further three days.

Results

All of 100 strains of anaerobic bacteria of clinical origin were viable after storage in Microbank™ vials at -80°C for ten years. Most strains yielded moderate to heavy growth from a single bead after 48 hrs incubation but three strains yielded only light growth after five days incubation. These comprised one of the two strains of *Prevotella denticola* examined, one of

four *Fusobacterium nucleatum* strains and one strain of *Fusobacterium varium.*

Discussion and Conclusions

One hundred anaerobic bacteria representing a wide range of genera isolated from clinical sources remained viable after ten years storage in Microbank™ vials at -80°C. The same 100 vials were previously sampled for viability after five and seven year's storage. However, 34 of those 100 isolates have changed names since their original identification at the ARL**.** These changes reflect advances in taxonomy and identification methods over the decade.

Some species have simply been placed in novel genera and, in some cases, have changed gender in the process e.g. *Eubacterium lentum* became *Eggerthella lenta*. The gram-positive anaerobic cocci have undergone major taxonomic review resulting in the removal of most former *Peptostreptococcus* species to novel genera e.g. *Finegoldia, Anaerococcus, Peptoniphilus*. Additionally, several novel species of anaerobic cocci have been described e.g. *Peptoniphilius harei*, *Peptoniphilus ivorii*. Several novel species have been described in other genera e.g. *Prevotella, Porphyromonas, Actinomyces*. The identification methods used at the ARL have been revised to accommodate such taxonomic changes.

The ARL has developed novel molecular methods, principally amplified 16S ribosomal DNA restriction analysis (ARDRA) for identification of *Bacteroides, prevotella* and *Porphyromonas* and for *Actinomyces* and other non-sporing gram-positive bacilli. ARDRA is more accurate and discriminatory than conventional phenotypic tests for identification of these groups; consequently some strains examined in the Microbank™ storage trial have been re-designated as a result of retrospective identification by ARDRA. Application of ARDRA to isolates stored at the ARL has resulted in the recognition of several novel *Actinomyces* species e.g. *Actinomyces*

cardiffensis and a novel genus and species *Varibaculum cambriense*. By chance, two strains previously included in the Microbank™ storage trial as *Actinomyces* species have subsequently been identified as members of these novel species.

Conclusion

Microbank™ vials are easy to use, compact, maintain viability and, therefore, are convenient for the long-term storage of anaerobic bacteria. The vials are particularly practical for repeated retrieval of strains as they ensure that the same strain is sub-cultured on each occasion with minimal effort. The ARL holds a collection of approximately 20,000 referred isolates dating back to the early 1980's. Isolates received in the past decade have been stored in Microbank™ vials. This collection is a valuable resource for retrospective research in fields such as evaluation of novel identification methods, monitoring of antimicrobial susceptibilities and development of molecular typing schemes.

ARL Ref	*Identification*	5Yrs	7Yrs	10Yrs
R5762	*B. distasonis*	+	+	+
R5956	*B. fragilis*	+	+	+
R5867	*B. splanchnicus*	+	+	+
R5868	*B. splanchnicus*	+	+	+
R5801	*B. thetaiotaomicron*	+	+	+
R5933	*B. thetaiotaomicron*	+	+	+
R5600	*B.distasonis*	+	+	+
R5587	*B.fragilis*	+	+	+
R5620	*B.fragilis*	+	+	+
R5589	*B.fragilis mer.res*	+	+	+
R5745	*B.ovatus*	+	+	+
R5570	*B.thetaiotaomicron*	+	+	+
R5755	*B.thetaiotaomicron*	+	+	+
R5631	*B.uniformis*	+	+	+
R5791	*Bacteroides* spp.	+	+	+
R5760	*C. glycolicum*	+	+	+

R5601	*C.bifermentans*	+	+	+
R5559	*C.butricum/beijerinckii*	+	+	+
R5635	*C.cadaveris*	+	+	+
R5584	*C.clostridioforme*	+	+	+
R5573	*C.difficile*	+	+	+
R5606	*C.novyi* type A	+	+	+
R5558	*C.paraputrificum*	+	+	+
R5555	*C.perfringens*	+	+	+
R5586	*C.perfringens*	+	+	+
R5628	*C.ramosum*	+	+	+
R5560	*C.septicum*	+	+	+
R5642	*C.sordellii*	+	+	+
R5759	*C.sporogenes*	+	+	+
R5572	*C.tetani*	+	+	+
R5585	*F. necrophorum*	+	+	+
R5716	*F. russii*	+	+	+
R5641	*F. varium*	+	+	+/-
R5565	*Fusobacterium necrophorum*	+	+	+
R5550	*Porph. asaccharolytica*	+	+	+
R5995	*Porph. endodontalis.*	+	+	+
R5838	*Porph. levii*	+	+	+
R5807	*Porph. uenonis*	+	+	+
R6079	*Porphyromonas* spp.	+	+	+
R5954	*Prev. denticola*	+	+	+
R5974	*Prev. denticola*	+	+/-	+/-
R5955	*Prev. melaninogenica*	+	+/-	+
R6080	*Prev. oris*	+	+	+
R5783	*Prevotella* spp.	+	+	+
R5836	*Prevotella* spp.	+	+	+
R5839	*Prevotella* spp.	+	+	+
R5573	*R5562*	*Peptoniphilus harei*	+	+
R5570	*R5562*	*Peptoniphilus harei*	+	+
R5584	*R5563*	*Peptoniphilus lacrimalis*	+	+
R5587	*R5563*	*Peptoniphilus lacrimalis*	+	+
R5586	*R5622*	*Peptostreptococcus* spp.	+	+
R5589	*R5622*	*Peptostreptococcus* spp.	+	+
R5601	*R5624*	*Anaerococcus octavius*	+	+
R5600	*R5624*	*Anaerococcus octavius*	+	+

R5606	*R5630*	*Ruminococcus productus*	+	+
R5620	*R5630*	*Ruminococcus productus*	+	+
R5555	*R5769*	*F. naviforme*	+	+
R5628	*R5769*	*F. naviforme*	+	+
R5631	*R5769*	*F. naviforme*	+	+
R5558	*R5778*	*F. necrophorum*	+	+
R5635	*R5778*	*F. necrophorum*	+	+
R5745	*R5778*	*F. necrophorum*	+	+
R5559	*R6000*	*F. nucleatum*	+	+
R5642	*R6000*	*F. nucleatum*	+	+
R5755	*R6000*	*F. nucleatum*	+	+
R5560	*R6003*	*F. nucleatum*	+	+
R5759	*R6003*	*F. nucleatum*	+	+
R5572	*R6066*	*F. nucleatum*	+	+/-
R5760	*R6066*	*F. nucleatum*	+	+/-

MICROBANK™ -20°C REFERENCE STUDY

Professor Valerie Edwards Jones.

Manchester Metropolitan University.

The Microbank™ bead system will be tested over a two year period for viability of protected bacteria and *Candida* spp. Organisms will be sub cultured and placed into the Microbank™ system and stored at -80 and -20°C and revived at set time periods for up to two years.

The details of sub culture are listed below:
Year 1 1 -12 months / revive monthly
Year 2 12-24 months / revive 3 monthly

The organisms will be revived from beads by dropping one bead into nutrient broth, vortexing and sub culturing onto the appropriate enriched medium, and incubating in the appropriate atmosphere for a 24/48hr period. Growth or No Growth will be recorded.

Organisms to be tested

Organism	Reference
Aeromonas hydrophila	NCTC 8049
Aspergillus niger	NCPF 2275
Bacillus cereus	NCTC 7464
Bacillus subtilis	NCTC10400
Bacteroides fragilis	NCTC 9343
Burkholderia cepacia	NCTC 10661
Campylobacter jejuni	NCTC 11322
Candida albicans	NCPF 3179
Candida albicans	NCPF 3255
Clostridium perfringens	NCTC 8237
Clostridium sporogenes	NCTC 532
Enterococcus faecalis	NCTC 775
Enterococcus faecalis	NCTC12697

Escherichia coli	NCTC 12241
Escherichia coli	NCTC 11954
Escherichia coli	NCTC 11560
Escherichia coli O157*	NCTC 12900
Haemophilus influenzae	NCTC 12699
Haemophilus influenzae	NCTC 11931
Klebsiella pneumoniae	NCTC 9633
*Klebsiella pneumoniae***	NCTC 13368
Listeria monocytogenes	NCTC 11994
Neisseria gonorrhoeae	NCTC 8375
Neisseria gonorrhoeae	NCTC 12700
Proteus mirabilis	NCTC 10975
Proteus vulgaris	NCTC 4175
Pseudomonas aeruginosa	NCTC 12903
Pseudomonas aeruginosa	NCTC 10662
Salmonella poona	NCTC 4840
Salmonella typhimurium	NCTC 12023
Serratia marcescens	NCTC 13382
Shigella sonnei	NCTC 8574
Staphylococcus aureus	NCTC 12981
Staphylococcus aureus	NCTC 12973
Staphylococcus aureus	NCTC 12493
Staphylococcus aureus	NCTC 6571
Staphylococcus epidermidis	NCTC 13360
Streptococcus agalactiae	NCTC 8181
Streptococcus pneumoniae	NCTC 12977
Streptococcus pyogenes	NCTC 12696
Yersinia enterocolitica	NCTC 12982

* Non Toxigenic Strain, *Escherichia coli* O157 NCTC 12900

** ESBL (Extended Spectrum Beta Lactamases) *Klebsiella pneumoniae* NCTC 13368

Results

All strains were viable after storage in Microbank™ vials at -20°C and -80°C at 12 months. All strains yielded moderate to heavy growth from a single bead after 24hrs incubation.

Conclusion

Microbank™ vials are easy to use, compact, maintain viability and therefore are convenient and effective for the long-term storage of bacteria. The vials are particularly practical for repeated retrieval of strains as they ensure that the same strain is sub-cultured on each occasion with minimal effort.

This trial will continue for a further 12 months following which a second report will be released.

Long term storage of multiple large research led culture collections of zoonotic enteric pathogens and commensal bacteria.

Williams N.J. and others.

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Many large research programmes have been undertaken at the University of Liverpool involving extensive field sampling of livestock, wildlife, their environment and companion animals. Considerable expense, time and effort are employed to obtain such samples and detect the presence of enteric zoonotic pathogens, namely *Campylobacter* species, *E. coli* and *Salmonella*, and explore antimicrobial resistance among these organisms, as well as commensal *E. coli* and *Staphylococcus* species. These collections are held on Microbank™ beads (Pro-Lab Diagnostics), which have been in use within our laboratories for the long term storage (at -70oC) of bacteria for the past 16 years. These culture collections serve as a valuable resource for further examination of the population structure, for example of different bacteria among different hosts in different environments, or to explore specific phenotypes relating to their survival in different environments, therefore it is vital they remain viable for future work.

Within our culture collection we house approximately 65,000 bacterial isolates comprising of isolates from previous studies. Some date back to 2000 with isolates of *E. coli*, *C. jejuni*, and *Staphylococcus* species still being resuscitated on a regular basis and used for further studies, both at the University of Liverpool as well as other institutions within the UK. In most cases isolates can be resuscitated easily on appropriate solid media, however for *Campylobacter* species specifically when kept for a significant number of years (>5 years), we have found from experience that a pre-enrichment step

in a non-selective broth incubated overnight under microaerobic conditions before plating onto a non-selective blood agar plate enhances recovery.

The published papers below relate to the most recent studies which we have conducted using Microbank™ vials for storage of isolates.

1. Wedley A, Dawson S, Maddox TW, Coyne KP, Pinchbeck GL, Clegg P, Jamrozy D, Fielder MD, Donovan D, Nuttall T, Williams NJ (2014). Carriage of *Staphylococcus* species in the veterinary visiting dog population in mainland UK: molecular characterisation of resistance and virulence. Vet.Microbiol. 170 (1-2): 81-8.
2. Humphrey S, Chaloner G, Kemmett K, Davidson N, Williams N, Kipar A, Humphrey T, Wigley P (2014). *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. MBio. 1:5(4):e01364-14.
3. Merga JY, Royden A, Pandey AK, Williams NJ (2014). *Arcobacter* spp. Isolated from untreated domestic effluent. Lett.Appl.Microbiol. 59(1):122-6.
4. Schmidt VM, Williams NJ, Pinchbeck G, Corless CE Shaw S, McEwan N, Dawson S, Nuttall T (2014). Antimicrobial resistance and characterisation of *staphylococci* isolated from healthy Labrador retrievers in the United Kingdom. BMC.Vet.Res. 10 (7). doi: 10.1186/1746-6148-10-17.
5. Kemmett K, Williams NJ, Chaloner G, Humphrey S, Wigley P, Humphrey T (2014). The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. Avian.Pathol. 43(1): 37-42.
6. Merga JY, Winstanley C, Williams NJ, Yee E, Miller WG. (2013). Complete genome sequence of the *Arcobacter butzleri* cattle isolate 7h1h. Genome.Announ.1(4): pii: e00655-13.
7. Merga JY, Williams NJ, Miller WG, Leatherbarrow AJ, Bennett M, Hall N, Ashelford KE, Winstanley C (2013). Exploring the diversity of *Arcobacter butzleri* from cattle in the UK using MLST and whole genome sequencing. PLoSOne. 8(2):e55240.

8. Kemmett K, Humphrey T, Rushton S, Close, Wigley P, Williams NJ. (2013) A Longitudinal Study Simultaneously Exploring The Carriage of APEC Virulence Associated Genes and The Molecular Epidemiology of Faecal and Systemic *E. coli* in Commercial Broiler Chickens.PLoSOne. 25; 8(6):e67749.
9. Williams, A, Christley RM, McKane SA, Roberts VLH, Clegg PD, Williams, NJ. (2013). Antibiotic resistance changes in equine enteric *E. coli* during hospitalisation. Vet.J.196(1):121-6.
10. Parsons BN, Porter CJ, Stavisky JH, Williams NJ, Birtles RJ, Miller WG, Hart CA, Gaskell RM, Dawson S (2012). Multilocus sequence typing of human and canine *Campylobacter upsaliensis* isolates. Vet.Microbiol. 153(3-4):391-7.
11. Ahmed MO, Williams NJ, Clegg PD, van Velkinburgh JC, Baptiste KE, Bennett M (2012). Analysis of risk factors associated with antibiotic resistant *Escherichia coli*. Microb.Drug.Resist. 18(2): 161-8.
12. Maddox T, Clegg PD, Wedley A, Dawson S, Pinchbeck G, Williams NJ. (2012) Cross-sectional Study of Antimicrobial Resistant Bacteria in Horses. Part 2: Risk Factors for Faecal Carriage of Antimicrobial Resistance *Escherichia coli* in Horses. Equine Vet J. 44(3):297-303
13. Maddox T, Clegg PD, Diggle PJ, Wedley A, Dawson S, Pinchbeck G, Williams NJ. (2012) Cross-sectional Study of Antimicrobial-resistant Bacteria in Horses. Part 1: Prevalence of antimicrobial-resistant *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* (MRSA). Equine Vet J. 44(3):289-296.
14. Wedley AL, Maddox TW, Westgarth C, Coyne KP, Pinchbeck GL, Williams NJ, Dawson S. (2011). Prevalence of antimicrobial-resistant *Escherichia coli* in dogs in a cross-sectional, community-based study. Vet.Rec.2;168(13):354.

LONG TERM STORAGE OF FASTIDIOUS CAMPYLOBACTER AND HELIOBACTER USING MICROBANK™

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Abstracts presented at the VIIth International Workshop on Campylobacter, Helicobacter and related organisms, Brussels, Belgium, Sept. 21-25 1993

Long term storage of fastidious Campylobacter and Helicobacter has proved to be difficult, as various workers have described low recovery rates on revitalizing freeze-dried cultures and other methods of preservation. We have used the Microbank™ (Pro-Lab Diagnostics, Texas, USA) system, in which porous beads act as carriers to support micro-organisms.

Campy*lobacter mucosalis, C. concisus, C. hyointestinalis, C. curvus, H. pylori and H. fennelliae* were tested in this system. Two or three day culture grown on tryptose blood agar plates (Oxoid CM233) under 11' enriched microaerophilic conditions were inoculated into the Microbank™ media and then stored at -70°C.

For revitalization, one bead was placed on the surface of a blood agar plate, allowed to thaw and gently rolled over the surface. Plates were incubated in an enriched microaerophilic environment at 37°C for up to 7 days.

Twenty-two clinical and reference cultures of *C. mucosalis* and *C. conscisus* were successfully revitalized from Microbank™ storage at 3 weeks, 4 months and 6 months. Five isolates of *C. hyointestinalis* were successfully revitalized after 7 months. The type strain of *C. curvus*, NCTC 11649 was revitalized at 3 weeks, 3 months and 6 months.

Thirty nine of 41 (95%) clinical and reference isolates of *H. pylori* were revitalized at 2 weeks, 2 months and 8 months. Concurrent revitalization of

freeze-dried cultures of these *H. pylori* isolates indicated that only 10 of 31 (32%) strains were viable.

Seventeen of 24 (71%) *H. fennelliae* clinical and reference cultures were viable. Fourteen isolates were revitalized after 6 months, and 3 isolates after 17 to 21 months. The initial inoculums must be heavy, and revitalization should be in a hydrogen enriched microareophilic atmosphere. The loss of viability in some of the *H. pylori* and *H. fennelliae* isolates is attributed to too sparse an inoculum.

The Microbank™ system provides a very simple solution to long term storage of fastidious *Campylobacter* and *Helicobacter* strains. As this commercial preservation system is freely available, it obliviates the need for specialized media and procedures. Additional testing is required for longer term storage in the Microbank™ system of these medically important micro-organisms.

LONG TERM STORAGE OF SALMONELLA ISOLATES SUBMITTED TO THE SALMONELLA REFERENCE TYPING LABORATORY USING MICROBANK™

The following collection of 312 reference Salmonella strains have been successfully stored on Microbank™ for a minimum of 14 Years at the Salmonella Reference Typing Laboratory, Veterinary Laboratory Agency, Weybridge, Surrey UK.

Vial	Species
1	*S. aarrhus*
2	*S. aberdeen*
3	*S. abortusovis*
4	*S. aderike*
5	*S. aesch*
6	*S. agona*
7	*S. agoueve*
8	*S. ajiobo*
9	*S. altendorf*
10	*S. altona*
11	*S. amager*
12	*S. amherstiana*
13	*S. anatum*
14	*S. anfo*
15	*S. angoda*
16	*S. ank*
17	*S. antarctica*
18	*S. apapa*
19	*S. arechavaleta*
20	*S. arizona*
21	*S. arkanass*
22	*S. artis*
23	*S. ashanti*
24	*S. askraall*
25	*S. babelsberg*

26	*S. bahrenfeld*
27	*S. bareilly*
28	*S. barranquilla*
29	*S. bergen*
30	*S. berkeley*
31	*S. berlin*
32	*S. berta*
33	*S. bilthoven*
34	*S. bispebjerg*
35	*S. blegdam*
36	*S. boecker*
37	*S. bonariensis*
38	*S. bonn*
39	*S. borreze*
40	*S. bradford*
41	*S. brancaster*
42	*S. bredeney*
43	*S. broughton*
44	*S. bruck*
45	*S. budapest*
46	*S. bulawayo*
47	*S. bulovka*
48	*S. c.suis v. kunzendorf*
49	*S. california*
50	*S. canastel*
51	*S. cannstatt*
52	*S. caracus*
53	*S. carrau*
54	*S. chailey*
55	*S. chameleon*
56	*S. chandans*
57	*S. chingola*
58	*S. chittagong*
59	*S. choleraesuis*
60	*S. chomedey*
61	*S. christiansborg*

62	*S. claibornei*
63	*S. clovelly*
64	*S. coein*
65	*S. congo*
66	*S. cotham*
67	*S. crossness*
68	*S. cubana*
69	*S. dabou*
70	*S. dahlem*
71	*S. dakar*
72	*S. dar es salaam*
73	*S. derby*
74	*S. deversoir*
75	*S. djakarta*
76	*S. dublin*
77	*S. duisburg*
78	*S. durban*
79	*S. durham*
80	*S. ealing*
81	*S. eimsbuettel*
82	*S. eingedi*
83	*S. elizabethville*
84	*S. enteritidis*
85	*S. essen*
86	*S. etterbeck*
87	*S. eves*
88	*S. falkensee*
89	*S. fischerkietz*
90	*S. fluntern*
91	*S. foulpointe*
92	*S. frankfurt*
93	*S. freemantle*
94	*S. freetown*
95	*S. freiburg*
96	*S. fresno*
97	*S. friedenau*

98	*S. gallinarum*
99	*S. georgia*
100	*S. gera*
101	*S. give*
102	*S. glostrup*
103	*S. gnesta*
104	*S. goerlitz*
105	*S. goldcoast*
106	*S. haduna*
107	*S. haelsingborn*
108	*S. haifa*
109	*S. halmstad*
110	*S. harburg*
111	*S. hartford*
112	*S. hato*
113	*S. havana*
114	*S. heidelberg*
115	*S. helsinki*
116	*S. hemingford*
117	*S. hindmarsh*
118	*S. hithergreen*
119	*S. hofit*
120	*S. houten*
121	*S. idikan*
122	*S. illinois*
123	*S. indiana*
124	*S. infantis*
125	*S. inganda*
126	*S. ipswich*
127	*S. isangi*
128	*S. ituri*
129	*S. jangwani*
130	*S. java*
131	*S. javiana*
132	*S. jerusalem*
133	*S. johannesburg*

134	*S. kalamu*
135	*S. kaolak*
136	*S. kapemba*
137	*S. karachi*
138	*S. karamoja*
139	*S. kegougou*
140	*S. kentucky*
141	*S. kiambu*
142	*S. kibi*
143	*S. kibusi*
144	*S. kidderminster*
145	*S. kiel*
146	*S. kimuenza*
147	*S. kirkee*
148	*S. kisarawe*
149	*S. koessen*
150	*S. kokomlemle*
151	*S. kottbus*
152	*S. krefeld*
153	*S. kuessel*
154	*S. landau*
155	*S. lanka*
156	*S. lansing*
157	*S. leiden*
158	*S. lexington*
159	*S. lille*
160	*S. litchfield*
161	*S. livingstone*
162	*S. llandoff*
163	*S. llobregat*
164	*S. london*
165	*S. luckenwalde*
166	*S. luke*
167	*S. luton*
168	*S. madjorio*
169	*S. makiso*

170	*S. malsatt*
171	*S. manchester*
172	*S. manhattan*
173	*S. manila*
174	*S. mara*
175	*S. marembe*
176	*S. marina*
177	*S. marshall*
178	*S. massenya*
179	*S. matopeni*
180	*S. mbandaka*
181	*S. meleagridis*
182	*S. mgulani*
183	*S. midway*
184	*S. mikawasima*
185	*S. millesi*
186	*S. milwaukee*
187	*S. minneapolis*
188	*S. minnesota*
189	*S. mississippi*
190	*S. mobeni*
191	*S. moero*
192	*S. molade*
193	*S. mondeor*
194	*S. mons*
195	*S. monschaui*
196	*S. montevideo*
197	*S. morehead*
198	*S. morningside*
199	*S. mountpleasant*
200	*S. mpouto*
201	*S. muenster*
202	*S. naestved*
203	*S. nagoya*
204	*S. napoli*
205	*S. nchanga*

206	*S. nessziona*
207	*S. newbrunswick*
208	*S. newport*
209	*S. newyork*
210	*S. nigeria*
211	*S. nima*
212	*S. nitra*
213	*S. nottingham*
214	*S. noya*
215	*S. nyanza*
216	*S. oakland*
217	*S. ochiogo*
218	*S. offa*
219	*S. ohio*
220	*S. okerara*
221	*S. omifisan*
222	*S. ona*
223	*S. onderstepoort*
224	*S. oranienburg*
225	*S. ordonez*
226	*S. orientalis*
227	*S. oslo*
228	*S. ouakam*
229	*S. panama*
230	*S. papuana*
231	*S. paratyphi A*
232	*S. paratyphi B*
233	*S. patience*
234	*S. pietersburg*
235	*S. plymouth*
236	*S. pomona*
237	*S. poona*
238	*S. portsmouth*
239	*S. pullorum*
240	*S. putten*
241	*S. rawash*

242	*S. rideau*
243	*S. riggil*
244	*S. riogrande*
245	*S. rosenthal*
246	*S. rubislaw*
247	*S. ruiru*
248	*S. rumford*
249	*S. saintpaul*
250	*S. salford*
251	*S. sarajone*
252	*S. schlessheim*
253	*S. selandia*
254	*S. sendai(miami)*
255	*S. senegal*
256	*S. senftenberg*
257	*S. seremban*
258	*S. shubra*
259	*S. simsbury*
260	*S. singapore*
261	*S. sinthia*
262	*S. soesterberg*
263	*S. solt*
264	*S. stanleyville*
265	*S. sternschanze*
266	*S. stockholm*
267	*S. stourbridge*
268	*S. strasbourg*
269	*S. sundsvall*
270	*S. tamale*
271	*S. tamberma*
272	*S. taunton*
273	*S. tees*
274	*S. telaviv*
275	*S. telelkebir*
276	*S. telhashomer*
277	*S. teltow*

278	*S. thielallee*
279	*S. thomasville*
280	*S. thompson*
281	*S. thompson*
282	*S. tione*
283	*S. toricada*
284	*S. toucra*
285	*S. typhi*
286	*S. typhimurium*
287	*S. uccle*
288	*S. uzaramo*
289	*S. vejle*
290	*S. vinohrady*
291	*S. virchow*
292	*S. vitkin*
293	*S. vleuten*
294	*S. vogan*
295	*S. wa*
296	*S. wandsbek*
297	*S. wandsworth*
298	*S. warral*
299	*S. wassenaar*
300	*S. wayne*
301	*S. weslaco*
302	*S. westerstede*
303	*S. westhampton*
304	*S. widemarsh*
305	*S. wildwood*
306	*S. windermere*
307	*S. worthington*
308	*S. wuppertal*
309	*S. wyldergreen*
310	*S. yeerongpilly*
311	*S. yerba*
312	*S. yoruba*

CRYOPRESERVATION OF FUNGAL SPORES USING MICROBANK™

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A simple method is described for the cryopreservation of *Conidia*, stored adhering to small porous beads in a robust polypropylene vial. For recovery, a single bead is removed from the vial and streaked onto a solid growth medium. Preparative work is minimised so the method is rapid. The storage life of an isolate can be increased greatly if a 'seed lot' system is employed.

The most reliable way to store fungi for extended periods is in liquid nitrogen or in the vapour of liquid nitrogen. Elliott (1976) used polypropylene drinking straws to store strains of *Agaricus bisporus* in liquid nitrogen as the straws were safer and less expensive than using glass ampoules. The small size of the straws also permitted greater storage capacity and sample replication in vivostats. However, there are problems with drinking straws as their small size makes them difficult to handle, they are hard to check for leaks after sealing and they can burst when removed from a vivostat. The preparative work is also time-consuming, so cultures tend to be handled in batches which increases the chances of cross contamination. Stalpers, De Hoog and Vlug (1987) recommended the use of special apparatus for large scale application of the straw technique but this apparatus is not available commercially.

This note describes cryopreservation of conidia of mitotic, entomopathogenic fungi on porous beads. The Microbank™ storage system (Pro-Lab Diagnostics) is a commercial variant of a system designed originally for storing bacteria (Feltham et al, 1978) but also appears suitable for storing certain fungi. In our laboratory at Wellesbourne, cultures are stored in the vapour above liquid nitrogen, but porous beads could be used for storing microorganisms in a deep freeze (ca -70°C). The Microbank™ system consists of sterile vials

containing beads (3 mm diam.) which act as carriers to support the microorganisms. Each vial has a volume of 2 ml and contains 25 beads in 1 ml of a cryopreservative (usually 10 or 15% glycerol). As the Microbank™'system is available commercially, preparative work is kept to a minimum. Vials and beads are available in a range of colours to aid identification and vials are robust and easy to handle.

In this laboratory, the preparation, inoculation and recovery of conidia are performed in a microbiological safety cabinet. A suspension of conidia, prepared in sterile 0.05% Tritron X-100 surfactant, is placed in a sterile Eppendorf tube, centrifuged, washed and centrifuged again. The pellet is then resuspended in cryopreservative taken from one of the vials and pipetted back into that vial, which is inverted 3 to 4 times to allow conidia to adhere to the beads. Most of the cryopreservative is then removed to prevent the beads sticking together during freezing, but a thin layer of free liquid can be left at the bottom of the vial to allow recovery of the fungus should the conidia fail to adhere to the beads. Vials are frozen overnight in a deep-freeze at -70° and transferred to a vivostat the following morning. The vials are stored in the vapour above liquid nitrogen as this avoids the problem of liquid nitrogen entering the vials through cracks or leaks.

For recovery, vials are removed from the vivostat and one bead is removed with sterilised forceps and streaked, using a loop, onto the surface of a suitable solid medium. Vials are prevented from thawing during this procedure by placing them into an insulated block of aluminium (10 x 8 x 4 cm) or 'Cryoblock', which has sample wells drilled into it (PL.155 available from Pro-Lab Diagnostics). The block is stored in the deep freeze and can be cooled further in liquid nitrogen immediately before use. The method is simple and rapid so that the vials are out of the vivostat for only two to three minutes. The temperature of a vial placed within the cryoblock was measured on the laboratory bench at room temperature (approximately 20°) using a thermocouple, after they had been frozen overnight at -70°.

Consistently, the cryoblock kept the vial at a temperature below -60°for five min. below -50° for 15 min and below -40° for 30 min.

The method has been used to store approximately 50 isolates of *Beauveria bassiana, Metarhizium Anisopliae, Verticillium lecanii* and *Paecilomyces* spp. Prima facie, the method appears to be suitable only for storing *Conidia*: it remains to be seen whether it is also suitable for preserving other life stages, e.g. hyphal bodies produced in liquid culture. To date, *Conidia* have been stored for 18 months, with recovery of a bead every 3 months, so that the suitability of the method for very long term preservation has not been assessed. However, all isolates stored in this way have been recovered without contamination and loss of pathogenicity to target insects has not been observed in our routine bioassays. A preliminary assessment of the germination of conidia was performed for two isolates each of *B. bassiana, M. anisopliae* and *V. lecanii*. Each isolate, obtained from cultures stored previously for a minimum of six years in polypropylene straws under liquid nitrogen, had been stored for 18 months using the bead system. *Conidia* were washed from beads in 1 ml 0.05% Triton X-100 and an aliquot (0.1 ml) pipetted onto Sabouraud dextrose agar in 5.5 cm Petri dishes. Dishes were incubated at 23° for 24 hr, after which *Conidia* were stained with lactophenol cotton blue and germinated/ungerminated *Conidia* were counted using a compound miscroscope. *Conidia* were considered to have germinated when the length of the germ tube exceeded the width of the conidium. Three replicates were used with a minimum of 300 *Conidia* counted each time. Germination of *Conidia* was greater than 95% in all cases. Each bead held between 10^5 and 10^6 *Conidia*.

An isolate can be stored in a 'seed lot' system to increase its storage life. Two vials are prepared from the isolate. One vial, the 'working' vial, is used for preparing cultures for experimental work. When all the beads from the working vial have been used, a bead from the other 'seed' vial is removed, a culture is grown from it and is used to prepare a second working vial. In this

laboratory, a further level of storage has been added. A slope culture from the working vial is kept in a refrigerator at 5° for 3 months and cultures for experiments are grown from this. As each working vial contains 25 beads, it should last for over 6 years. Theoretically, therefore, an isolate stored using this method of cryopreservation can be accessed for 156 years before it needs to be replaced.

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STORAGE OF NCTC REFERENCE SET OF CULTURES SELECTED FOR QUALITY CONTROL AND LABORATORY ACCREDITATION REQUIREMENT USING MICROBANK™

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Introduction

Ashford PHL carried out an investigation using cultures supplied by NCTC to select cultures suitable for use in QC procedures in Food, Water and Dairy products testing. These were initially directed to Public Health Laboratory's requirements but were extended to cover other Laboratories' requirements.

Following feedback from colleagues, both in the PHLS and other sources, a set of 44 cultures were selected.

Method

The freeze dried vials received from NCTC were checked for vacuum integrity using a spark tester. Vials were then opened following the NCTC instructions under a Class III safety cabinet. The hydrated cultures were then inoculated into appropriate media (in most cases Blood Agar). The incubation temperature, gaseous requirement and length of incubation were selected as appropriate to the culture eg 37°C aerobic 24 hours for most cultures.

The incubated cultures were examined for viability and purity. A subculture was prepared for identification check as appropriate to the individual culture.

Inoculation of the Vials and Beads

Using the primary culture obtained directly from the NCTC vial the vials were inoculated following the Pro- Lab Diagnostics Procedure A Preparation.

Storage

The vials were placed in a -70°C Cabinet (Kelvinator).

Check of Viability of Beads

All inoculated beads were checked for viability within 1 week of inoculation, all were viable.

Recovery Method

The Pro-Lab Diagnostics Procedure B for recovery was followed. In addition "cold blocks" were used to carry the culture from the -70°C Cabinet and during testing. Recovery was limited to 6 cultures at a time to reduce temperature loss. The beads were inoculated onto the appropriate media and appropriate culture conditions were used. Recovery was assessed by a semi-quantitative scoring method.

Recovery Results

The dates of bead inoculation and last date of viability (recovery) check are given on the enclosed list.

All cultures were viable at the recent dates shown. The majority have also been tested previously.

All cultures remained viable with no quantitative loss of viability detected. The majority of the cultures have been stored on beads at -70°C for over 2 years with no loss of viability.

Tests for storage at other temperatures e.g. -20°C were not performed.

VIAL	IDENTIFICATION	REFERENCE	MONTHS STORED
1	*Zygosaccharomyces rouxii*	NCPF 3879	26
2	*Aspergillus niger*	NCPF 2275	26
3	*Bacillus cereus*	NCTC 7464	26
4	*Bacillus subtilis*	NCTC 10400	26
5	*Campylobacter jejuni* (BT1)	NCTC 11322	26
6	*Saccharomyces cerevisiae*	NCTC 3178	26
7	*Citrobacter freundii*	NCTC 9750	27
8	*Clostridium difficile*	NCTC 11204	26
9	*Clostridium perfringens*	NCTC 8237	26
10	*Enterobacter aerogenes*	NCTC 10006	27
11	*Enterococcus faecalis*	NCTC 775	27
12	*Escherichia coli*	NCTC 10418	9
13	*Escherichia coli* 0157:H7	NCTC 12079	26
14	*Lactobacillus casei*	NCTC 10302	17
15	*Legionella pneumophila* SG 1	NCTC 12821	27
16	*Listeria monocytogenes* 4b	NCTC 11994	26
17	*Proteus mirabilis*	NCTC 10975	26
18	*Pseudomonas fluorescens*	NCTC 10038	24
19	*Pseudomonas aerugionosa*	NCTC 10662	26
20	*Pseudomonas cepacia*	NCTC 10661	26
21	*Salmonella typhimurium*	NCTC 12023	26
22	*Shigella sonnei*	NCTC 8574	26
23	*Staphylococcus aureus*	NCTC 6571	26
24	*Staphylococcus epidermidis*	NCTC 11047	20
25	*Lactobacillus lactis*	NCTC 662	26
26	*Listeria ivanovii*	NCTC 11846	24
27	*Campylobacter coli*	NCTC 11366	25
28	*Vibrio furnissii*	NCTC 11218	26
29	*Vibrio parahaemolyticus*	NCTC 10885	26
30	*Yersinia enterocoliticus* SG1	NCTC 10460	26
31	*Klebsiella aerogenes*	NCTC 9528	26
32	*Aeromonas hydrophila*	NCTC 8049	26
33	*Acinetobacter lwoffii*	NCTC 5866	26
34	*Serratia marcescens*	NCTC 11935	26
35	*Edwardsiella tarda*	NCTC 11934	26
36	*Protes rettgeri*	NCTC 7475	26
37	*Enterobacter cloacae*	NCTC 11936	26

38	*Vibrio cholerae* Non 0:1/0:24	NCTC 11348	26
39	*Salmonella Poona* 013, 22	NCTC 4840	26
40	*Rhodococcus equii*	NCTC 1621	26
41	*Staphylococcus aureus*	NCTC 1803	26
42	*Clostridium bifermentans*	NCTC 506	26
43	*Clostridium sporogenes*	NCTC 532	26
44	*Listeria innocua*	NCTC 11288	26

Conclusion

Storage of the NCTC Reference Set of Cultures has been demonstrated under normal working conditions in a Public Health Laboratory using the Microbank™ system, manufactured by Pro-Lab Diagnostics.

This method of storage of cultures is recommended for use in microbiological Laboratories requiring reference cultures for Laboratory Accreditation and QC procedures. The NCTC set of cultures is recommended for this purpose.

STORAGE AND REPEATED RECOVERY OF NEISSERIA GONORRHOEAE USING MICROBANK™

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Introduction

Due to the fastidious nature of *Neisseria gonorrhoeae* a simple, inexpensive and efficient system for the storage and recovery of clinical isolates and quality control strains is required for good clinical laboratory practice, in research and for epidemiological studies. Various methods such as use of cooked meat broth, lyophilisation or freezing in liquid nitrogen are available for the storage of bacteria but none is ideal, particularly for gonococci. The limitations associated with these methods are the variable recovery of bacteria, the time and inconvenience involved in the preparation and maintenance of cultures and the financial cost in the purchase and maintenance of expensive equipment.

The technique of storing organisms at -70°C described by Nagel and Lawrence in 1971 has given rise to simple and convenient commercial storage systems such as the Pro-Lab Microbank™ which uses coloured beads in a 'cryovial' containing cryopreservative fluid. After inoculation and storage a single bead can be removed to inoculate culture media.

We examined the Microbank™ system with a view to its overall convenience of use for storage and recovery. The recovery rate after medium-term storage and repeated sampling was analysed and an evaluation made of the efficacy of recovery in relation to the spectrum of antigenic types (serovars) of gonococci that occur in nature.

Materials and Methods

Bacterial strains

One hundred clinical isolates of *N. gonorrhoeae*, including 30 penicillinase-producing *N. gonorrhoeae* were included in the study. These strains represented the wide variety of antigenic types of gonococci encountered in natural infection and comprised 26 serogroup IA strains covering eight different serovars, 61 serogroup IB strains covering 14 different serovars and 13 serogroup IB strains which were non-typeable with the standard monoclonal antibody serotyping panel.

Preservation and storage of strains

Using a sterile cotton bud, gonococcal colonies were harvested from an 18-24 hour culture on modified New York City medium and a suspension made in the cryopreservative fluid of the cryovial approximately equivalent to a McFarland No. 4 standard. The inoculated vial was closed and the contents inverted 4-5 times to coat the beads with bacteria. Excess cryopreservative fluid was removed with a sterile pastette. The vial closed and immediately placed in a -70°C freezer.

Retrieval of bacteria

All 100 gonococcal isolates were sampled each month for 24 consecutive months. Twenty cryovials at a time were removed from the -70°C freezer, using an aluminium transfer block to retain a low temperature. Using sterile forceps a single bead was removed from the cryovial, placed onto the surface of a culture place containing modified New York City medium, and allowed to thaw. A sterile loop was used to streak out the area around the bead to obtain separate colonies and the culture plates incubated for 48 hours in a carbon dioxide-enriched atmosphere.

Serotyping

Sereotyping of the gonococcal strains was performed using the Genetic Systems[6] panel of monoclonal antibodies.

Statistical analysis
The chi-squared test was used for all statistical analysis.

Results

As shown in Table 1, the overall recovery rate from 2400 retrievals for the 24 months of the trial was 98.6% (2365/2400), and all strains were recovered in eight of the 24 months (100% recovery). The recovery rate for the remaining months ranged from 99% to 96%: 99% in five of the months, 98% in five of the months, 97% in four of the months and 96% in two of the months. Although the recovery rate was extremely good there were 0.8% (9/1191) failures in the first 12 months compared with 2.2% (26/1174) in the last 12 months – a significant difference ($x^2 = 8.4$; $P<0.01$).

Thirteen separate isolates accounted for the 35 (1.5%) failures and the distribution of these failures by serovar and month is shown in Table 2. Five isolates failed on only one occasion, four on two occasions, two isolates on five occasions and two isolates on six occasions.

The distribution of the 13 serovar failures in relation to the total number of isolates for each of the 23 sereovars tested is given in Table 3. Although there were only three serovar IB15 isolates, each one failed on at least one occasion and together they accounted for 37.1% (13/35) of the total failures. Sereovars IA16 and IB25 were also associated with multiple failures on five or more occasions (Table 2).

Thirty-five isolates comprising serovars IA02, IA06, IB01, IB02 and IB03, classified as major serovars from continuous prevalence studies in our geographical area, accounted for only 2.0% (1/35) of the failures. The remaining 65 isolates, classified as minor serovars, accounted for 18.5% (12/65) of the failures – a significant difference ($x^2 = 4.9$; $P<0.05$).

Table 1: Monthly recovery rate for 100 gonococcal isolates

Month	No. of Recovered Strains	No. of Failures
1	100	0
2	100	0
3	100	0
4	99	1
5	100	0
6	100	0
7	100	0
8	100	0
9	99	1
10	98	2
11	98	2
12	97	3
13	99	1
14	97	3
15	97	3
16	98	2
17	96	4
18	100	0
19	97	3
20	98	2
21	99	1
22	99	1
23	96	4
24	98	2
Total	**2365 (98.6%)**	**35 (1.5%)**

Table 2: Distribution of 35 failures by serovar and month

Serovar	Month
IA05	15,16
IA16	11,15,17,19,23
IA21	17
IA25	17,19
IB02	21
IB15	12,24
IB15	11,14,16,20,22
IB15	13,14,15,20,23,24
IB19	23
IB25	4,9,10,12,14,17
IB29	19
IB00	10,23
IB00	12

IB00 (Non-typeable strain)

Table 3: Distribution of 13 failures in relation to individual serovars

Serovar	No. of Isolates	No. of Failures
IA02	6	0
IA04	3	0
IA05	2	1
IA06	5	0
IA07	1	0
IA16	4	1
IA21	3	1
IA25	2	1
IB01	10	0
IB02	7	1
IB03	7	0
IB05	2	0
IB06	8	0
IB07	5	0
IB08	4	0

IB15	3	3
IB17	7	0
IB19	2	1
IB25	2	1
IB26	1	0
IB29	2	1
IB31	1	0
IB00	13	2

IB00 (Non-typeable strain)

Discussion

Lyophilisation has long been accepted as the 'gold standard' method for the preservation of microorganisms, but the high cost in equipment and processing time precludes its use in many routine laboratories. Modern technology has made -70°C facilities readily available and the small capacity required to store large numbers of isolates makes cryovial storage systems extremely convenient for the clinical laboratory which requires easy access to strains. Concerns over refrigeration failure and the subsequent loss of valuable strains can be alleviated by fitting carbon dioxide back-up systems, designed to activate at a pre-set temperature to the freezer.

Nagel and Lawrence[2] first described a method for the preservation of multiple replicate units of bacteria using sterile glass beads and a mixture of equal parts of broth culture and horse blood allowing storage of at least 200 beads in a plastic tube at -70°C. In a subsequent study Feltham et al used different concentrations of cryoprotectants in the storage media used to make the bacterial suspensions, and stored the beads at -70°C. They observed a reduction in the number of viable bacteria with nutrient broth containing 15% dimethyl sulphoxide. Nutrient broth supplemented with either: 10% dimethyl sulphoxide, 10% glycerol or 15% glycerol, showed no such reductions. In a further study White and Sand demonstrated the

viability of organisms after storage at -76°C for two years, using glass beads and brain-heart infusion broth containing 10% glycerol as the emulsifying fluid.

In this study we have shown that the Microbank™ system offers a simple commercially available system for medium-term storage and multiple recovery of *N. gonorrhoeae*. The overall recovery rate of 98.6% is extremely good and, together with the ability to sample up to 25 times, represents substantial cost benefits. The failure of four isolates to grow at least five times each may be associated with the strains, or may be a simple physical problem of insufficient primary inoculum in these vials. The overall recovery rate could possibly be improved with the use of special recovery medium. Morton and Smith advocated the use of a solution of 20% sucrose in phosphate-buffered saline for the recovery of fastidious organisms such as *Neisseria* spp, though clearly this is not essential for the vast majority of gonococcal isolates.

There was a small but significant decrease in recovery in the last 12 months of the trial, which may reflect a sampling problem rather than a temporal phenomenon and further long-term studies are underway to differentiate between these possibilities. The finding that failures were significantly associated with minor sereovars suggests that the transmission/viability of minor serovars may be lower than that of common serovars and could be a significant factor in the overall epidemiology of gonococcal infection. he selective loss of minor serovars on storage could also lead to a bias in epidemiological studies based on isolates that have been stored for some time.

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VALIDATION FOR CRYO STORAGE OF BRUCELLA SPP. USING MICROBANK ™

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The *Brucella* research section of the Veterinary Laboratories Agency (VLA) under the auspices of the Food and Agriculture Organisation (FAO)/World Health Organisation (WHO) Collaborating Centre for Reference and Research on Brucellosis and on behalf of the Office International des Epizooties (OIE) Brucellosis Reference Centre, has as one of our roles to advise other laboratories on the identification and preservation of *Brucella* strains. As finances for these laboratories can be a problem it is not always possible to freeze-dry samples. On a recent VLA workshop in Morocco the delegates from North African countries were presented with Microbank™ cryo vials as an alternative storage method. They thought that these were ideal for their needs. We would like to be in a position to recommend these vials to colleagues at other laboratories worldwide with confidence of their suitability for the long-term storage of *Brucella*.

Materials and Methods

A representative isolate of each sub-species and in addition the most fastidious strain of *Brucella abortus* biovar 2 (Advances in Brucellosis Research, Texas A & M University Press, Texas) henceforth referred to as a set, were sub-cultured onto serum dextrose agar (SDA) to check for purity. Each reference strain was freeze dried allowing one vial for each date of testing in the trial, they were also added to the Microbank™ cryo vials in accordance with the manufacturers instructions. In order to ascertain the effects of freeze/thawing an individual vial was set up for each of the storage conditions and each testing date. After one month of storage at +4°C the first set of freeze dried vials were reconstituted and subbed onto SDA plates to

give a circle of approximately 5cm in diameter. The Microbank™ cryo vials from the -20°C and -80°C freezers, a bead was removed from these and spread on SDA plates to give a circle approximately 5cm in diameter, all plates were then incubated at 37°C in a 10% CO_2 atmosphere for 4 days. Growth was examined for morphology and the quantity was compared allowing a deviation of approximately 25% growth between the cultures of different storage conditions. Although the inoculum was not standardised, it is important that sufficient quantity of the isolate remains viable in order to carry out further work on the isolates. The morphology was examined visually, aided using obliquely reflected light from under the culture.

After six months this was repeated, however this time beads were removed from the original Microbank™ cryo vials, opened at one month and an additional new bead from a fresh vial was removed for the sixth month stage in order to assess the effect of freeze/thawing. This process was also repeated after one year of storage.

Results

Freeze-dried vials stored at +4°C

Isolate	1 month	6 month	1 year
Brucella melitensis (biovar 1) 16M	+	+	+
Brucella abortus (biovar 1) 544	+	+	+
Brucella suis (biovar 1) 1330	+	+	+
Brucella canis RM6/66	+	+	+
Brucella neotomae 5K33	+	+	+
Brucella abortus (biovar 2) 86/8/59	+	+	+
Brucella ovis 63/290	+	+	+

Microbank™ cryo vials stored at -20°C

Isolate	1 month	6 month	1 year	Time in storage
Brucella melitensis (biovar 1) 16M	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella abortus (biovar 1) 544	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella suis (biovar 1) 1330	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella canis RM6/66	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella neotomae 5K33	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella abortus (biovar 2) 86/8/59	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella ovis 63/290	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year

Microbank™ cryo vials stored at -80°C

Isolate	1 month	6 month	1 year	Time in storage
Brucella melitensis (biovar 1) 16M	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella abortus (biovar 1) 544	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella suis (biovar 1) 1330	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella canis RM6/66	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella neotomae 5K33	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella abortus (biovar 2) 86/8/59	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year
Brucella ovis 63/290	++	++	++	1 month
	++	++	++	6 months
	++	++	++	1 year

Table Key

++	Growth comparable to freeze-dried vials
+-	Growth, but insufficient to be comparable to freeze drying
+	Freeze dried vials growth (used as the standard for comparison against)
-	No growth
?	Change in morphology of the culture
!	Contaminant present

Conclusion

The preliminary results have so far shown that the procedure could be a cost effective alternative to freeze-drying negating the problems associated with freeze storage of cultures, such as loss of viable organisms through lysis during freeze thawing owing to the formation of ice crystals. The yield and morphology of the Microbank™ cryo vials in this first year have proved to be comparable with that of freeze-drying. All the cultures were still viable after the first year of this trial and as of yet there has been no significant difference in the products stored at -20°C and those stored at -80°C.

This study has also shown that the freeze thawing of the Microbank™ cryo vials has limited effect so far, proving the efficacy of the cryopreservative.

LONG TERM PRESERVATION OF FUNGAL ISOLATES IN MICROBANK™ VIALS

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Since 1994, 198 yeasts and 391 moulds belonging to 25 and 37 species, respectively, were stored in Microbank™ cryogenic vials at ≥ -130°C in liquid nitrogen and at -70°C in a freezer. All of the isolates, with the exception of 45 yeasts and 15 dermatophytes, were recovered from both storage temperatures. Good reproducibility was demonstrated for amphotericin B, fluconazole and voriconazole MICs determined for random isolates.

Long-term preservation of fungal strains is essential for their in-depth study; however, both the viability and the stability of living cells should be ensured during the preservation period. Fungal isolates are usually preserved in water at room temperature, an easy and economical procedure introduced for fungi by Castellani in 1939. Because the stability of fungal cells was not ensured by this simple procedure, other methods have been suggested, such as preservation in soil or on oil or water-covered slants, cryopreservation either in liquid nitrogen or at low temperature (-20 and -70°C), and lyophilization (the freeze-drying procedure). Cryopreservation in liquid nitrogen and lyophilization are the methods recommended and used by the American Type Culture Collection. Although lyophilization of living cells provides a mechanism for stabilizing these cells for long periods of time, this procedure is cumbersome and lengthy and requires expensive equipment. On the other hand, storage in liquid nitrogen vapour (above the liquid at ≥ -130°C) is a more convenient and less expensive alternative for long-term

storage of living cells. Storage above the liquid nitrogen prevents leakage of the liquid nitrogen into the vials.

The Microbank™ system (Pro-Lab Diagnostics) consists of sterile vials that contain 25 porous, coloured beads and a cryopreservative fluid; this system was originally developed for storage of bacterial cells. The beads are acid washed, and their porous nature allows the cells to adhere to the bead surface; the beads serve as carriers for the cells being stored (Microbank™ package insert). When an isolate is stored in this way, 25 or more identical cultures can be preserved. The purpose of this study was to evaluate the preservation in Microbank™ sterile vials of yeast and mould clinical isolates that were received from 1994 to the end of 2002 at the VCU Medical Center (Richmond, Va.) and the Valme University Hospital (Seville, Spain). Two temperatures ($\leq$ -130°C {liquid nitrogen vapour} and -70°C {freezer}) were evaluated.

Fresh, pure cultures of 6,198 yeast and yeast-like organisms and 391 moulds (Table 1) were grown on either Sabouraud dextrose agar (for yeasts) or potato dextrose agar (for moulds) at 35°C; some isolates of dermatophytes, *Histoplasma capsulatum, Blastomyces dermatitidis* and *Alternaria* spp. were incubated at 30°C. Yeast and Yeast-like isolates were incubated for 48 to 72 hours and moulds were incubated for 7 to 15 days. Each isolate was stored in accordance with the directions of the manufacturer. For each isolate, the cryogenic fluid of two Microbank™ vials was inoculated with the fungal growth to a density approximately equivalent to a McFarland standard of 3 or 4. The inoculated fluid was mixed four or five times to emulsify the suspension and to bind the cells to the beads. The extraneous cryogenic fluid was then removed, leaving the inoculated beads as free of liquid as possible to prevent the beads from sticking together during freezing but allowing a thin layer of suspension to stay at the bottom of the vial. The vials were then held overnight at -70°C. After overnight freezing, one of the vials was stored in liquid nitrogen vapour ($\leq$ -130°C) and the other was left at -70°C.

The viability and purity of the strains were monitored immediately after storage, at 1 and 6 months after storage, and once a year subsequently as follows. One of the inoculated beads was removed under aseptic conditions with a sterile needle, and each vial was returned immediately to the corresponding low temperature; the bead was then inoculated onto either Sabouraud dextrose agar or potato dextrose agar for at least 20 days. Both viability and the morphological characteristics of each culture were observed.

Each mould isolate was considered viable if the rate of growth present was the same as that of the original culture and if the morphology and colour of the colony matched the fungal identification documented for each strain. All of the mould strains, with the exception of 15 (of 61) dermatophyte isolates, were recovered each time from both storage temperatures and showed the initial colony characteristics, growth rates, and morphologies (Table 1). Only isolates of *B. dermatitidis, H. capsulatum*, and *Alternaria* spp. required more than one bead for harvesting; they required two to four beads. These results are in agreement with those described by Chandler, who preserved 50 uncommon moulds for 18 months in Microbank™ vials and found that only one bead was necessary for the recovery of most isolates. Each yeast strain was considered viable if growth was present; the identification and purity of yeasts were also randomly validated on CHROMagar medium. A very small percentage of yeasts (0.7%) were not recovered; *Candida dubliniensis* had the lowest recovery rate (33%; 28 of the 42 isolates were not recovered). The stability was validated by determining the antifungal susceptibilities of random samples of yeasts (200 isolates) and moulds (50 isolates) stored at both temperatures. Amphotericin B, fluconazole and voriconazole MICs were determined by following NCCLS guidelines (documents M27-A2 {for yeasts} and M38-A {for moulds}) before storage and 6 months and 4 years after preservation. MICs for the isolates after storage were either the same as, or within three dilutions of the MICs before storage, which is the criterion used in NCCLS studies to obtain percentages of intra-and inter-laboratory reproducibilities as well as for establishing quality control MIC ranges.

In general, the effects of both storage temperatures on the stability and viability of stored isolates were similar, which is fortunate because most laboratories have a -70°C freezer.

The advantage of using the Microbank™ system over other cryogenic systems is its commercial availability. The time-consuming procedure of preparing other preservative devices such as drinking straws or cryogenic fluid is avoided; Microbank™ vials are stored at room temperature prior to use. The harvesting of individual isolates is easier than that described by Pasarell and McGinnis, in which a portion of the frozen culture is chipped and sub-cultured. Because vials should not be outside the low-temperature device for more than 3 minutes to avoid thawing, it is recommended that the frozen vials be placed in an insulated cryoblock during harvesting.

In conclusion, the Microbank™ system appears to be an easy, convenient, economical and effective tool for the preservation of fungal isolates other than dermatophyte and C.dubliniensis strains. Longer monitoring of isolates and storage of other species would further validate the reliability of this system for the cryogenic preservation of yeast and mould strains. Also, the stability of fungal cells should be further assessed by molecular parameters.

TABLE 1 – Fungal isolates reserved between 1994 and 2002

Species	No. of isolates stored	No. (%) of isolates not recovered
Yeasts and yeast-like organisms:	4,453	5
Candida albicans	5	0
Candida ciferri	42	28
Candida dubliniensis	28	0
Candida famata	359	0
Candida glabrata	33	0
Candida guilliermondii	6	0
Candida humicola	10	0
Candida kefyr	279	5
Candida krusei	20	0
Candida lambica	5	0
Candida lipolytica	43	0
Candida lusitaniae	352	3
Candida parapsilosis	3	0
Candida rugosa	401	2
Candida tropicalis	14	0
Candida zeylanoides	112	1
Cryptococcus neoformans	3	0
Cryptococcus albidus	9	0
Cryptococcus laurentii	4	0
Hansenula anomala	1	0
Sporobolomyces salmonicolor	2	0
Trichosporon beigelii	6	1
Rhodotorula rubra	1	0
Rhodotrula glutinis	7	0
Saccharomyces cerevisiae		
TOTAL YEAST AND YEAST-LIKE ORGANISMS:	**6,198**	**45 (0.7)**

Species	No. of isolates stored	No. (%) of isolates not recovered
Moniliaceous moulds:		
Aspergillus fischeri	1	0
Aspergillus flavus	24	0
Aspergillus fumigatus	117	0
Aspergillus niger	10	0
Aspergillus nidulans	9	0
Aspergillus terreus	27	0
Aspergillus sydowii	1	0
Aspergillus versicolor	1	0
Fusarium incarnatum	1	0
Fusarium moniliforme	5	0
Fusarium solani	10	0
Fusarium oxysporum	5	0
Paecilomyces lilacinus	9	0
Rhizopus arrhizus	7	0
Rhizomucor pusillus	1	0
Trichoderma longibrachiatum	4	0
TOTAL MONILIACEOUS MOULDS:	**232**	**0**
Dematiaceous moulds:		
Alternaria spp.	4	0
Bipolaris hawaiiensis	3	0
Bipolaris spicifera	3	0
Cladophialophora bantiana	8	0
Cladosporium cladosporioides	1	0
Curvularia spp.	4	0
Dactylaria gallopava	3	0
Exophiala jeanselmei	6	0
Exophiala spinifera	2	0
Phoma spp.	1	0
Scedosporium apiospermum	17	0
Scedosporium prolificans	10	0
Wangiella dermatitidis	9	0
TOTAL DEMATIACEOUS MOULDS:	**71**	**0**

Dimorphic moulds:		
Blastomyces dermatitidis	5	0
Histoplasma capsulatum	5	0
Penicillium marneffei	17	0
TOTAL DIMORPHIC MOULDS:	**27**	**0**
Dermatophytes:		
Epidermophyton floccosum	4	2
Microsporum canis	12	3
Microsporum gyseum	6	1
Trichophyton mentagrophytes	26	2
Trichophyton rubrum	13	7
TOTAL DERMATOPHYTES:	**61**	**15**
TOTAL MOULDS:	**391**	**15**

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EFFICIENCY OF MICROBANK™ FOR THE CONSERVATION OF MICROORGANISMS RELEVANT TO VETERINARY MEDICINE

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In this paper the conservation of different bacteria, yeasts and molds from diagnostic material was examined with a commercial deep freeze system. 137 bacteria isolated from the stomachs of dogs, cats and pigs and from duodenal juice of cats and 7 isolates of yeasts and fungi from diagnostic material were conserved with the deep freeze system Microbank™. Furthermore 62 *Helicobacter pylori*-isolates and 1 Helicobacter felis-isolate were conserved with this system. After a storage period of 24 hours up to 20 months the isolates were recultured. 96% of the conserved microorganisms could be grown. It was not possible to cultivate 1 fungus-isolate (*Fusarium* sp.). 2 *Helicobacter pylori*-isolates, spiral bacteria from the stomach of a pig and a *Clostridium tyrobutyricum*-isolate were only recultured after 24 hours, 1 isolate *Moraxella* sp. after 2 months and another 4 anaerobe isolates after 5 months. The Microbank™ system proved to be suitable for conservation and was also efficient for the conservation of microorganisms, which could not easily be cultivated.

USE OF COMMERCIALLY AVAILABLE CRYOGENIC VIALS FOR LONG-TERM PRESERVATION OF DERMATOPHYTE FUNGI

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The use of commercially available cryogenic vials (Microbank™ vials) stored at −70°C for the storage and preservation of dermatophyte fungi was investigated. None of the 200 strains of dermatophytes examined, representing 21 species, showed a loss of viability after they had been stored for periods ranging from 1 week to 2 years at −70°C. All strains showed typical colonial and microscopic morphologies following revival.

Long-term storage of fungal isolates is critical for preservation of the germplasm and maintenance of stock cultures with minimal effort over long periods. Conservation of morphological, physiological, genetic, and metabolic stability is crucial for many purposes and is vital for isolates used as medical reference strains, for chemotaxonomic studies, or in the commercial production of biochemicals. In a comparative study of the effects of five different storage methods, cryopreservation was the method that best provided for the stability of secondary metabolite production and is now considered the best method available for the long-term storage of microbial cultures . Dermatophytic fungi can present problems for storage, as the cultures often become pleomorphic, with various levels of sporulation or mycelial growth. McGinnis et al. used sterile distilled water to store hyphal and spore suspensions of 147 different species of fungi at 25°C for periods of 12 to 60 months. These included more than 25 species of dermatophytes. The degree of sporulation and the quality of the inoculum appeared to be

critical factors; and when the inocula were “adequate” in size, even some poorly sporulating species such as *Trichophyton violaceum*, *Trichophyton schoenleinii*, and *Microsporum ferrugineum* survived storage well. The long-term storage of a wide range of fungal species, including dermatophyte isolates, in commercially prepared cryogenic freezer beads (Microbank™) at −70°C or in liquid nitrogen has been tested. Although most fungi were preserved well by this method, dermatophytic fungi did not show good recovery rates. For example, more than 50% of isolates of T*richophyton rubrum* were not recovered. Consequently, this method was not recommended for use for the long-term storage of dermatophytic fungi. In contrast, we have found contradictory results and we report on a simple and successful technique for the long-term storage of dermatophytes.

Fresh isolates were collected from clinical specimens submitted to the Microbiology Department at the William Harvey Hospital, Ashford, United Kingdom, between March 2002 and August 2005. Reference strains were obtained from the National Collection of Pathogenic Fungi, Bristol, United Kingdom. Representative strains of dermatophytes were used to assess a commercially available freezer bead storage kit (Microbank™; Pro-Lab Diagnostics). Each 2-ml tube contains approximately 50 plastic beads (diameter, 3 mm) with a hole through the center (this hole retains approximately 1 µl of suspension), which allows repeated recovery of an isolate before the preparation of a new stored culture is needed. This is in contrast to traditional long-term storage methods, in which the isolates are stored in multiple single-use vials, and has the added advantage of taking up less space.

Mycelium and conidia were harvested from 7-day-old cultures incubated at 27°C on Sabouraud dextrose agar (SDA; Oxoid Ltd., United Kingdom) by using a sterile scalpel and inoculated into a freezer bead tube containing a suspension medium prepared according to the manufacturer's instructions to give a density approximately equal to or greater than that of a McFarland no.

4 standard. The suspension was shaken vigorously to evenly distribute the fungus and was left to stand for 5 min. Excess fluid was removed with a Pasteur pipette. Before the tubes were frozen and stored at −70°C, a single bead was removed with sterile forceps and was placed on a fresh SDA plate, and the resulting drop of fungal suspension was spread by using a 10-µl loop to obtain single colonies and to check for viability and purity. These plates were incubated at 27°C for 7 days to assess the amount of inoculum present on a single bead. At various time intervals over 24 months, the tubes were removed from the freezer and a bead was removed from the frozen clump, plated, and incubated as described above. The tubes were immediately returned to the −70°C freezer before the contents had thawed. The number of colonies recovered, their growth rate, and the macroscopic and microscopic morphologies of the isolates were noted.

A detailed time course study was conducted with four isolates (*Trichophyton interdigitale* WHH1268, *Trichophyton mentagrophytes* WHH692, *T. rubrum* WHH3229, and *Epidermophyton floccosum* WHH1471). Single beads were removed at 0, 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20, 24, 32, and 36 weeks and were cultured as described above. *Epidermophyton floccosum* was included, as it is known to die rapidly if it is kept at 4°C. In all cases, at all time intervals, successful reestablishment of the cultures ensued. At least 1,000 CFU was typically recovered from each bead. The growth rates and the hyphal densities were comparable to those of an initial control culture before it was frozen. Colonial and microscopic morphologies remained true to type throughout.

In addition to this time course study, a second trial was conducted with 58 stored isolates representing 15 species of dermatophytes. The isolates were selected to give a range of species but also to sample a range of isolates within some of these species (e.g., Arth*roderma benhamiae, Trichophyton interdigitale*, and *Trichophyton tonsurans*). For this trial, isolates stored for different time periods over the previous 24 months were recultured in

triplicate to assess the uniformity of the distribution of viable organisms in frozen tubes. Three freezer beads were taken from each tube of preserved isolates and cultured as described above. In all cases, successful reestablishment from all three replicates occurred for all isolates tested. No adverse effects on morphology or growth rates compared to those of cultures not subjected to cryopreservation were noted.

Following these initial trials, this method of preservation was adopted for the storage of all stock strains in the laboratory. To date, all cultures kept in this manner have been successfully revived as required at times ranging from 1 week to 2 years, and these cultures represent 200 isolates of 21 species of dermatophytes (Table 1). Espinel-Ingroff et al. have discussed the advantages of using the Microbank™ freezer bead system in terms of its availability and ease of use. Their results suggested that dermatophytes would not be well preserved by use of this method. Our results for a wider range of isolates suggest that the limited numbers of dermatophytes that they tested were not representative or that a different preservation technique might have given better recovery rates. For example, half the specimens prepared by Epinel-Ingroff et al. were preserved in liquid nitrogen and kept for up to 8 years, whereas all our specimens were kept at −70°C and tested within 2 years. The recovery of all the isolates used in our study was successful, with no apparent effect on culture phenotype. All four specimens of *T. rubrum* were recovered in our study, including the isolate used in the detailed time course study. This is in contrast to the 54% recovery rate of *T. rubrum* isolates in the earlier study. As a consequence, we can now recommend this method of preservation of dermatophytes for clinical laboratories worldwide.

Table 1. Isolates recovered after preservation for 1 week to 24 months

Name	Isolate code	No. of strains	Time of storage (m)
Arthroderma benhamiae	NCPF410	1	24
A. benhamiae	NCPF456	1	3
A. benhamiae	NCPF460	1	0.25
A. benhamiae	Clinical isolates	13	2-10
Arthroderma simii	NCPF494	1	24
A. simii	NCPF471	1	1
Arthroderma vanbreuseghemii	NCPF452	1	24
A. vanbreuseghemii	NCPF750	1	24
A. vanbreuseghemii	NCPF749	1	24
Epidermophyton floccosum[b]	Clinical isolate WHH1471	1	24
E. floccosum	Clinical isolates	7	8-24
Microsporum audouinii	Clinical isolates	4	1
Microsporum canis	Clinical isolates	7	9-24
Microsporum gypseum-Microsporum fulvum	Clinical isolates	5	1-9
Microsporum persicolor	NCPF502	1	24
M. persicolor	Clinical isolates	4	1-9
Trichophyton ajelloi	NCPF364	1	12
Trichophyton equinum	NCPF526	1	24
T. equinum	NCPF565	1	24
T. equinum	NCPF673	1	18
T. equinum var. *autotrophicum*	NCPF488	1	24
T. equinum	Clinical isolate WHH2660	1	24
Trichophyton erinacei	NCPF652	1	24
T. erinacei	Clinical isolates	10	1-10
Trichophyton interdigitale	NCPF780	1	24
T. interdigitale b	Clinical isolate WHH692	1	24
T. interdigitale	Clinical isolates	55	1-24
Trichophyton mentagrophytes	NCPF224	1	24
T. mentagrophytes[b]	Clinical isolate WHH1268	1	24
T. mentagrophytes	Clinical isolates	27	1-24
Trichophyton quinckeanum	NCPF310	1	24
T. quinckeanum	NCPF341	1	24
T. quinckeanum	Clinical isolate	1	0.25

Trichophyton rubrum	NCPF113	1	24
T. rubrum[b]	Clinical isolate WHH3229	1	24
T. rubrum	Clinical isolates	2	1-24
Trichophyton schoenleinii	NCPF691	1	12
T. schoenleinii	Clinical isolate Bristol ST5	1	1
Trichophyton soudanense	NCPF800	1	12
T. soudanense	Clinical isolates	4	2-14
Trichophyton tonsurans	NCPF690	1	24
T. tonsurans	Clinical isolates	25	1-22
Trichophyton verrucosum	Clinical isolates	2	1-22
Trichophyton violaceum	Clinical isolates	5	7-22
Total		**200**	

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TWO-YEAR STUDY EVALUATING THE POTENTIAL LOSS OF METHICILLIN RESISTANCE IN A MRSA CULTURE COLLECTION

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A reported loss of mecA prompted us to monitor 360 cryostocked methicillin-resistant Staphylococcus aureus strains for stability. Concurrently, 14 well-characterized strains were stored in a Microbank preservation system and subjected to multiple freeze-thaw events. There were no significant declines in the methicillin-resistant populations with either method over a two-year period.

Strain stability in a research culture collection is critical for valid and consistent experimental results. The University of South Florida Center for Biological Defense (CBD) stocks well-characterized methicillin-resistant Staphylococcus aureus (MRSA) strains for use in validating molecular typing methods such as multilocus sequence typing, staphylococcal protein A typing, multilocus variable-number tandem repeat analysis, pulsed-field gel electrophoresis (PFGE), and staphylococcal cassette chromosome mec (SCCmec) typing. The CBD collection largely consists of community-acquired strains received from hospitals and clinical laboratories in Florida and Washington State as well as strains obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) and from H. de Lencastre [(13)]. Based on the known association of pulsed-field type with SCCmec type [(15)], the majority of the strains typed in our collection were USA300 (159 presumed SCCmec type IV), followed by USA100 (103 presumed

SCCmec type II), and 84 sporadic pulsed-field types were SCCmec types II, IIA, III, IIIA, and IV and undetermined. The integrity and stability of mecA and SCCmec within our MRSA strains are critical to CBD research.

In 2005, van Griethuysen et al. reported the loss of mecA in a MRSA strain collection after two years of cryostorage at −80°C in a Microbank bead-based preservation system (MBPS) (Pro-Lab Diagnostics, Austin, TX) [(16)]. Prior to storage in the MBPS, the strains were previously held as long-term stocks at room temperature (RT). Two years later, approximately 14% of the collection stored in the MBPS no longer carried mecA.

This report prompted an evaluation of the stability of 360 MRSA strains from the CBD collection. After confirmation of methicillin resistance and phenotypic characterization from a single, representative colony, each MRSA strain was cryostocked using a standardized cryostorage protocol as follows. The progenitor colony was inoculated to 100 ml of tryptic soy broth (TSB) and incubated at 35°C for approximately 15 h in a shaking water bath at 125 rpm. Thirty milliliters of overnight culture was pelleted at 3,571 × g at RT for 10 min, resuspended in 25 ml of TSB with 10% glycerol, and aliquoted as 1 ml into cryovials. After 30 min of equilibration at RT with the cryoprotectant, cryovials were placed in Nunc controlled freezing units (Nalgene, Rochester, NY) following the manufacturer's instructions and held at −85°C for 75 min to foster cooling of approximately −1°C/min to near −30°C. Immediately thereafter, cryovials were removed from the units and stored at −85°C.

One week after cryostorage, during which most cell death occurs [(1)], a single cryovial of each CBD MRSA strain was quick-thawed at 35°C and plate counts were obtained from a 1:10 dilution series to review viability and purity. To confirm that methicillin resistance had been maintained in the population after cryopreservation, disk diffusion susceptibility (DDS) was performed using 30-µg cefoxitin and 1-µg oxacillin antibiotic disks (Oxoid, Hants, United Kingdom) on Mueller Hinton (MH) plates. Zones of inhibition were measured

after incubation at 35°C for 24 h, followed by incubation at 30°C for another 24 h. The Clinical and Laboratory Standards Institute (CLSI) standards for these antibiotics were used to interpret the resistance of the strains [3, 4]. Control strains for this method and all others used in this study were a methicillin-susceptible Staphylococcus aureus strain, ATCC 25923 (American Type Culture Collection, Manassas, VA) and a confirmed SCCmec type IV MRSA strain, CBD 804 (HDE288). CBD strains were also tested for the presumptive presence of PBP2', the mecA gene product, from cultures grown at 35°C for 24 h on blood agar following the manufacturers' instructions (Oxoid, Hants, United Kingdom; Denka Seiken, Ltd., Derbyshire, United Kingdom).

At one- and two-year intervals, plate counts were performed on the 360 CBD MRSA strains to ensure that a robust heteroresistant population of MRSA had persisted after prolonged cryostorage at −85°C. CBD MRSA stocks were quick-thawed, and a 1:10 dilution series was spread in 100-µl volumes on tryptic soy agar (TSA) and MH agar containing 4% NaCl and 6 µg/ml oxacillin (MH-OXA) (Remel, Lenexa, KS). By comparing the CFU/ml of the entire population on TSA to that of the MRSA subpopulation on MH-OXA at each yearly time point, we were able to track the stability of the heteroresistant population. All the strains tested produced methicillin-resistant colony counts on MH-OXA equal to or within a log of the heteroresistant CFU/ml on TSA at one- and two-year time points. The means of the counts from year 1 on TSA and MH-OXA were 1.66 × 109 ± 4.28 × 107 and 1.26 × 109 ± 3.14 × 107, respectively, and in year 2, 1.89 × 109 ± 5.98 × 107 and 1.46 × 109 ± 3.87 × 107 (data not shown). Thus, none of the 360 cryostocked MRSA strains tested from the CBD culture collection demonstrated a significant decline in stability of their heteroresistant populations of MRSA after the two-year review (nonparametric Kruskal-Wallis test; P = 0.7175) (Table 1).

During this evaluation of our CBD MRSA collection stability, we also sought to recreate the loss of mecA reported by van Griethuysen et al. for strains

stored in the MBPS [16]. We subjected six previously described [13] MRSA strains comprised of SCCmec types I, IA, II, III, IIIA, and IV and eight PFGE types (USA100 through -800) obtained from NARSA to two years of cryostorage in the MBPS with periodic freeze-thaw events (Table 1). In contrast to our CBD cryostorage protocol, the MBPS technical instructions directed the inoculation of a cell suspension of young growth for each strain into a cryostorage vial containing 25 porous beads and a proprietary cryoprotectant. The vials were inverted five times and then immediately well aspirated of most liquid. This action gave the cells that had adhered to the beads very little equilibration with the cryoprotectant. The vials were frozen in an uncontrolled manner to −85°C; the manufacturer recommends storage at −70°C.

To monitor the 14 strains for a stable methicillin-resistant subpopulation, a single MBPS bead for each strain was tested at intervals spanning 1 to 6 weeks during the two-year period. At each time point, the MBPS vials were removed from −85°C and held on ice for approximately 10 to 15 min while a bead for each strain was aseptically transferred to 1 ml TSB with 10% glycerol at RT. The MBPS vials were immediately returned to −85°C. Following 5 s of vigorous vortexing of each sacrificed bead per strain, plate counts comparing the heteroresistant population on TSA to the MRSA subpopulation on MH-OXA were performed as described above. The vials containing individual beads suspended in TSB with glycerol were placed directly in −85°C to freeze and were saved for future testing. Within 10 min on ice, the MBPS vials transitioned from approximately −85°C to near 0°C and rose to 5°C during handling to remove a bead, as determined by inserting a temperature probe among the beads. This procedure allowed us to replicate freeze-thaw events.

During cryostorage in the MBPS for 104 weeks, the 14 MRSA strains were subjected to 24 such freeze-thaw events. Thirteen strains did not have a significant decline in the methicillin-resistant population from initial storage in the MBPS to final evaluation at 104 weeks (nonparametric Kruskal-Wallis

test; P = 0.1528) (Table 1). The means of the counts from week 0 on TSA and MH-OXA were 5.17 × 106 ± 1.59 × 106 and 2.62 × 106 ± 6.39 × 105, respectively, and in week 104, 8.10 × 106 ± 2.56 × 106 and 4.73 × 106 ± 1.23 × 106 (Table 1). Two strains struggled to tolerate a relatively high concentration of oxacillin from the beginning of the study. CBD 801 (N315), a SCCmec type II strain, repeatedly failed to grow on MH-OXA, and therefore, the methicillin-resistant cells in its population were not enumerated at each time point. CBD 804 (HDE288), a SCCmec type IV strain, also had difficulty thriving on MH-OXA, as it required extended incubation time to produce countable colonies. DDS and PBP2' latex agglutination testing performed at each freeze-thaw event on both strains demonstrated that they were resistant to 1 µg oxacillin and that the mecA gene product was presumptively present.

To investigate whether a decrease in resistance to methicillin occurred among the 14 strains over two years, we examined the stored beads from the first freeze-thaw event, which were collected 1 week after initial storage in the MBPS, and from week 104, in which the beads had undergone the stress of 24 freeze-thaw events. Susceptibility tests using the microbroth dilution method were performed with a Sensititre system (TREK Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions and with CLSI standards [(3)]. A methicillin-susceptible S. aureus ATCC 29213 strain was the control in all MIC testing. The microtiter plates containing ampicillin, penicillin, oxacillin, and vancomycin were incubated at 35°C for 24 h. The susceptibility tests gave identical or near-identical MICs (a <2-fold dilution difference) on all the antimicrobials for the sacrificed beads stored at week 1 and week 104 for all 14 cryostocked strains in the MBPS.

The presence of mecA was also tested in sacrificed beads stored at week 1 and week 104. Genomic DNA was extracted from overnight bacterial growth in TSB using a MagNa Pure LC instrument (Roche Diagnostics Corp., Indianapolis, IN) with the supplied DNA isolation kit III following the

manufacturer's instructions. PCR for mecA was performed as described previously [13]. The results confirmed that the mecA gene was present in all 14 strains from their initial storage in the MBPS to the endpoint of our MBPS evaluation.

There are various insults to cells that occur during a freeze-thaw event [11, 12] that may explain the previously reported loss of mecA-positive cells [16]. However, a distinction should also be made between the deletion or disruption of mecA and the overgrowth and eventual replacement of MRSA by a coexisting mecA-negative population. MRSA strains are heterogeneous, consisting of two subpopulations wherein the resistant population grows slower than the more-robust susceptible population [2]. Stressful environments such as storage at RT and prolonged freezing with repeated freeze-thaw events can play a significant role in evolving lineages that are better able to survive these stresses [14]. Therefore, the CBD protocol began with selection and enrichment of a predominantly MRSA population prior to preservation. Confirmation of a robust MRSA population was also done shortly after cryostorage in TSB with 10% glycerol.

The cryostocking process itself could have decreased the mecA-positive subpopulation, since it is known to inflict detrimental changes to bacterial cells, such as ice crystal formation, dehydration, decreased and/or increased activity of enzymes, accumulation of metabolites, increased molecular contacts, disruption of weak hydrogen bonds, distortion of molecules, and breakdown of the cell membrane [12]. The CBD cryostocking protocol incorporates osmotic equilibration with a 10% glycerol cryoprotectant. Equilibration with a cryoprotectant minimizes damage during freezing by penetrating and stabilizing the cell membrane and delaying freezing. While we allow 30 min or more for cells to equilibrate with the glycerol cryoprotectant, the MBPS protocol allows <1 min for cells to equilibrate with the proprietary cryoprotectant. It is possible that the removal of the cryoprotectant leaves the mecA-positive cells in the MBPS more vulnerable

to injury during freeze-thaw events. Also, the MBPS technical instructions do not specify how to handle the vials while retrieving beads. The injurious complexities of a freeze-thaw event stem largely from uncontrolled rates of cooling and warming during which ice in the cells (re)crystallizes [8, 11]. The MBPS vials in our experiment were held on ice during bead retrieval, in keeping with a common practice that results in relatively rapid thawing and reduced cell injury [8, 11]. Our standardized protocol includes a program of controlled, slow freezing to minimize intracellular ice formation, followed by uncontrolled, rapid freezing to forestall further dehydration.

We did not detect a significant decline in 13 MRSA populations in the MBPS after 24 freeze-thaw cycles in two years; the MRSA population for CBD 801 (N315) could not be enumerated on MH-OXA after initial evaluation, but methicillin resistance was confirmed at each time point. However, it is possible that low-temperature storage or multiple uncontrolled freeze-thaw events give “freeze-hardy” methicillin-susceptible cells in the heterogeneous population a “cryopreservation selection” advantage over the methicillin-resistant subpopulation during thawing and reculturing [11]. To avoid these potential problems, we stock multiple single-use cryovials for every strain in our collection and perform quality control checks with each use.

A previous study reported that genetic background affects the stability of mecA in MRSA [10]. The distribution of SCCmec contributes to the efficiency and stability of PBP2’ expression, which propagates methicillin resistance. The mecA gene is well maintained on an engineered plasmid in methicillin-susceptible Staphylococcus aureus strains from major clonal complexes, including the complexes containing USA100 and USA300 [10]. Conversely, strains from other lineages are more likely defective in mecA production [10]. A further analysis of the lineages of the strains that lose mecA could address any inherited instability, since apparent genetic insertion and deletion events may serve as indicators for strain stability [16].

The reported loss of methicillin resistance in MRSA is rare, and a confirmed explanation as to why it occurs is still elusive [6, 7, 9, 16]. The suggestion of genetic instability of MRSA strains in cryostorage prompted us to review our MRSA strain collection for the potential loss of a robust heteroresistant population. We have seen no decline in the methicillin-resistant population among the 360 MRSA strains cryostocked using a standardized protocol for more than two years in our collection, possibly due to the major presence of inherently stable strains. The genetic background of MRSA strains as well as the complexities of the cryostocking process can play a significant role in long-term preservation of these strains. Therefore, it is important to enrich and confirm a robust mecA-positive subpopulation before, during, and after cryostorage of MRSA strains critical to research.

Table 1: Plate counts of heteroresistant and methicillin-resistant populations

CBD no.	Strain	Description	Evaluation of CBD cryostocks[a]			
			Year 1		Year 2	
			TSA	MH-OXA	TSA	MH-OXA
799[c]	COL	SCC*mec* type I	2.0×10^9	2.1×10^9	2.4×10^9	2.0×10^9
800	PER34	SCC*mec* type IA	1.5×10^9	1.3×10^9	1.7×10^9	1.4×10^9
801	N315	SCC*mec* type II	1.3×10^9	NG[f]	1.3×10^9	NG
802	ANS46	SCC*mec* type III	1.9×10^9	1.8×10^9	1.6×10^9	1.1×10^9
803	HU25	SCC*mec* type IIIA	1.6×10^9	1.8×10^9	2.0×10^9	2.1×10^9
804	HDE288	SCC*mec* type IV	6.4×10^8	3.0×10^8	1.4×10^5	7.8×10^8
1064[d]	NRS 382	USA100 (II)[e]	2.4×10^9	1.7×10^9	6.1×10^9	8.5×10^8
1065	NRS 383	USA200 (II)	1.3×10^9	1.3×10^9	2.6×10^9	2.2×10^9
1066	NRS 384	USA300 (IVa)	1.8×10^9	3.5×10^8	1.2×10^9	1.1×10^9
1067	NRS 123	USA400 (IVa)	4.3×10^9	1.1×10^9	2.2×10^9	2.0×10^9
1068	NRS 385	USA500 (IV)	1.4×10^9	9.9×10^8	1.3×10^9	1.2×10^9
1069	NRS 22	USA600 (II)	5.9×10^8	2.8×10^8	5.7×10^8	6.9×10^8
1070	NRS 386	USA700 (IVa)	1.3×10^9	9.3×10^8	2.6×10^9	3.0×10^9
1071	NRS 387	USA800 (IV)	3.3×10^9	1.1×10^9	1.2×10^9	1.0×10^9

CBD no.	Strain	Description	Evaluation of cryostocks in MBPSb			
			Week 0		Week 104	
			TSA	MH-OXA	TSA	MH-OXA
799c	COL	SCC*mec* type I	1.8×10^6	1.6×10^5	7.8×10^6	6.0×10^6
800	PER34	SCC*mec* type IA	3.4×10^6	2.1×10^6	4.6×10^6	4.5×10^6
801	N315	SCC*mec* type II	5.6×10^6	NG	6.0×10^6	NG
802	ANS46	SCC*mec* type III	1.8×10^7	1.5×10^7	1.5×10^6	1.7×10^7
803	HU25	SCC*mec* type IIIA	7.6×10^6	8.3×10^6	9.6×10^6	9.6×10^6
804	HDE288	SCC*mec* type IV	8.4×10^6	5.2×10^6	7.9×10^6	1.4×10^5
1064d	NRS 382	USA100 (II)e	2.5×10^6	2.1×10^6	1.7×10^6	2.0×10^7
1065	NRS 383	USA200 (II)	1.0×10^5	7.6×10^6	1.9×10^6	2.1×10^6
1066	NRS 384	USA300 (IVa)	1.9×10^6	4.3×10^6	1.6×10^7	1.4×10^7
1067	NRS 123	USA400 (IVa)	5.8×10^6	1.0×10^6	1.2×10^7	1.2×10^7
1068	NRS 385	USA500 (IV)	4.6×10^6	4.5×10^6	9.6×10^6	8.1×10^6
1069	NRS 22	USA600 (II)	1.0×10^6	1.4×10^6	3.2×10^6	1.9×10^6
1070	NRS 386	USA700 (IVa)	1.0×10^7	5.4×10^6	1.8×10^7	1.3×10^7
1071	NRS 387	USA800 (IV)	1.5×10^6	9.6×10^5	1.6×10^7	1.7×10^6

[a] Representative data in CFU/ml for 14 of 360 MRSA strains cryostocked with a standardized protocol in the CBD collection.
[b] Representative data in CFU/ml from 14 well-defined MRSA strains stored in the MBPS.
[c] CBD 799 to 804 were derived from strains previously described [(10)].
[d] CBD 1064 to 1071 were derived from NARSA strains.
[e] Pulsed-field type (SCCmec type). SCCmec type information obtained from NARSA.
[f] NG, no growth. Strain confirmed for methicillin resistance by DDS and PBP2' testing.

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AN INTERNAL QUALITY ASSURANCE SCHEME FOR CLINICAL BACTERIOLOGY USING MICROBANK™

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The development of an internal quality control scheme in clinical bacteriology has been hampered by a lack of suitable cultures. However, work undertaken using Mircrobank™ beads may provide a solution to this problem.

Internal Quality Assessment in Clinical Bacteriology

One definition of quality involves meeting the predetermined requirements of users of a product or service. An effective quality management system (QMS) determines the needs and expectations of users and evaluates the processes, responsibilities and resources required to meet quality objectives. In the laboratory, quality control (QC) procedures should be used in conjunction with external and internal quality assessment (QA), audit and equipment monitoring as an integral part of the QMS. Quality control permits the day to day monitoring of assay, operator and equipment performance. It should detect both random and systematic errors.

Criteria for Quality Control Material

In general, QC material should be independent of kit controls, be stable over a long period of time, be of sufficient volume to monitor within and between kit and reagent and batches, and give results within a clinically significant range (for bacteriological cultures, this means target organisms).

Unfortunately, availability of suitable QC material presents a problem in developing a suitable internal QA scheme in clinical bacteriology. One scheme established for QA in clinical bacteriology involves the submission of

anonymised original specimen for analysis[1]; however, problems associated with this type of QC material include:

- Failure to meet at least two of the criteria mentioned above.
- Repeat inoculation of a swab on a second set of plates may present a different picture (depending on the number of organisms originally present).
- A high percentage of bacteriology samples are negative and this does not challenge the ability to isolate 'target' organisms

Although this type of scheme assesses reproducibility, it does not detect systematic errors (because you don't know what you might be missing).

Microbank™ Beads

An alternative scheme using simulated specimens preserved on Microbank™ beads (Pro-Lab Diagnostics) was evaluated. Microbank™ offers a ready-to-use designed to simplify the storage and retrieval of bacterial and fungal cultures.

Comprising a special cryovial system that incorporates treated beads and a cryopreservative solution, Microbank™ provides a more reliable means of maintaining cultures than is possible with repetitive subculture, which can result in contaminated cultures, lost organisms or changed characteristics. The special formulated preservative ensures longer survival of fastidious organisms and higher quantitative recoveries. This makes the Microbank™ system ideal for QC applications where organism integrity, consistency and quality are of paramount importance.

Each 2 ml Microbank™ vial contains approximately 25 beads, providing the facility for repeat culture of the original organism using a simple procedure. Extensive, proven performance reference data, updated for 2005, is available on request from Pro-Lab Diagnostics.

To demonstrate the utility of the Microbank™ system, seven simulated specimens were prepared. Briefly, using freshly isolated colonies, a suspension of the target organism was prepared (equivalent to a McFarland 2 standard) in a Microbank™ vial. To simulate a clinical specimen, colonies of typical mixed normal flora were added to make the final suspension equivalent to a McFarland 5 standard (Table 1). The vial was mixed thoroughly and the contents were decanted into a Petri dish. Using sterile forceps, each bead was placed in an individual cryotube, which was then labelled and stored frozen at -80°C.

Table 1: Simulated Specimens

No	Specimen Type	Target Organism(s)	Other Organisms
1	Throat Swab	Group A *streptococcus*	Mixed oral flora
2	Throat Swab	*Corynebacterium diphtheriae*	Mixed oral flora
3	Sputum	*Haemophilus influenzae*	Mixed oral flora
4	Sputum	*Streptococcus pneumoniae*	Mixed oral flora
5	Wound Swab (burn)	*Staphylococcus aureus* *Pseudomonas aeruginosa*	Coagulase-negative staphylococcus
6	Faeces	Group D *salmonella*	Escherichia coli Proteus mirabilis
7	Faeces	*Escherichia* 0157	Escherichia coli (sorbitol-positive)

Day-to-Day Use

Each of the seven simulated specimens was processed once a week. Briefly, a vial was removed from the -80°C freezer and allowed to warm to room temperature. Nutrient broth (1ml) was added to the bead and mixed

thoroughly. A routine set of culture plates was inoculated from a swab dipped in the broth. Finally, specimen details were entered on the laboratory computer system, following an agreed format.

Culture Results

Over a six-month period (June to December 2003) all target organisms were isolated and correctly identified from six out of the seven simulated specimens. On two occasions, isolation of *Haemophilus influenzae* from specimen 3 failed.

Overview

Once prepared, simulated samples are simple to set up, record and score, and are inexpensive to prepare. In addition, they satisfy all the criteria for a QC material. Drawbacks include the fact that simulated samples are not 'real' specimens, and that some organisms may survive better than others at -80°C after 'pooling'. Fastidious organisms such as *Neisseria gonorrhoeae*, *Campylobacter* spp. and anaerobic organisms have yet to be tested using the Microbank™ system.

It may be argued that staff would soon get to know which organisms are present in the samples, but this argument also can be applied to most QC material used across pathology and is not relevant unless a 'blame culture' exists in the organisation. Used properly, however, a successful internal QC scheme will increase confidence in results and, in conjunction with external QA and audit, identify problems and assess the effectiveness of remedial measures.

Reference

[1]Constantine CE, Amphlet M, Farrington M, et al. Development of an Internal Quality Assessment Scheme in a Clinical Bacteriology Laboratory. J Clin Pathol 1993; 46: 1046-1050

GERMANY DATA - BESTBION DX

Following many years of successful application of the Microbank™ system for the long term storage and retrieval of bacterial and fungal cultures in many German microbiology laboratories and institutes, a request was made for inclusion of this data in the Microbank™ World Wide Performance Portfolio. Our exclusive distributor in Germany for the Microbank™ system (Bestbion dx. www.bestbion.com) conducted an extensive market survey inviting laboratories to submit data showing the successful use of the Microbank™. The following laboratories kindly contributed to the summary that follows below. We are grateful to all of our German customers and Bestbion dx for the continued support and confidence in the Microbank™ product.

Participating Laboratories:
Sanitätsakademie d. Bundeswehr München, Institut für Mikrobiologie
Diakonissenkrankenhaus Flensburg, Zentrallabor
MVZ Labor Schweinfurt, Bakteriologie
Hamburger Wasserwerke, Wasserlabor-Mikrobiologie
Eberhard-Karls-Universität Tübingen, Institut für Tropenmedizin
Klinikum Ansbach, Bakteriologie
Klinikum Bamberg, Institut für Labormedizin
Klinikum Kulmbach, Zentrallabor
Klinikum St. Marien Amberg, Zentrallabor
LaboKlin GmbH & Co. KG, Labor für klinische Diagnostik
Labor Bamberg
Labor L+S AG
LADR GmbH MVZ Dr. Kramer & Kollegen
Max Rubner-Institut Kulmbach
Labor an der Salzbrücke MVZ GmbH
Medizinisches Versorgungszentrum Dr. Stein u. Kollegen
Medizinisches Versorgungszentrum Dres. Cornely, Riebe & Berndt
Tierärztliche Gemeinschaftspraxis Dres. Windhaus & Hemme
Universitätsklinikum Magdeburg, Institut für Medizinische Mikrobiologie und Krankenhaushygiene
Universitätsklinikum Heidelberg, Medizinische Mikrobiologie und Hygiene

Universitätsklinikum Ulm, Institut für Medizinische Mikrobiologie und Hygiene
Universitätsklinikum Würzburg, Klinik u. Poliklinik f. Dermatologie, Mykologielabor
Westpfalz-Klinikum Kaiserslautern, Institut f. Laboratoriums- und Transfusionsmedizin

Results

Storage Temp -20°C

Organism / Species	≤12 m	≤24 m	≤36 m	≤48 m	≤60 m	> 5 y
Bacteroides spp.	2		1			1
Bordetella spp.					1	
Campylobacter jejuni	1		1			1
Candida spp.		1	1			1
Clostridium spp.	2		2			1
EHEC		1				
Escherichia coli	1		2		1	2
Haemophilus spp.	1		1			1
Klebsiella pneumoniae	1					
Listeria monocytogenes				1		
Myroides odoratus						1
Neisseria gonorrhoeae	1		1			
Ochrobactrum anthropi					1	
ORT			1			
Pasteurella spp.					1	
Pasteurella multocida						1
Pseudomonas aeruginosa	1					
Riemerella spp.		1				
Rhodococcus equi						1
Staph aureus / MRSA			1	1		3
Streptococcus Group B		1	1			1
Streptococcus pneumoniae	1		1			1
Taylorella equigenitalis					1	
Yersinia enterocolitica		1			1	

Storage Temp -40°C

Organism / Species	≤12 m	≤24 m	≤36 m	≤48 m	≤60 m	>5 y
Bacteroides spp.	1	1			1	
Campylobacter jejuni	2					1
Candida spp.	1	1			1	1
Clostridium spp.	2	1				1
Escherichia coli		1	1		1	1
Haemophilus spp.	3					1
Helicobacter pylori				1		
Mycobacterium fortuitum						1
Neisseria gonorrhoeae	3					
Staph aureus / MRSA		1	1		1	1
Streptococcus Group A						1
Streptococcus Group B	1		1		1	
Streptococcus pneumoniae	3					1

Storage Temp -70°C

Organism / Species	≤12 m	≤24 m	≤36 m	≤48 m	≤60 m	>5 y
Aspergillus spp.	1					
Bacteroides spp.	1		1			2
Campylobacter jejuni	1			1	1	1
Candida spp.	1	1			1	2
Clostridium spp.	1					2
Enterococcus faecalis	1					1
Escherichia coli	3	1				4
Haemophilus spp.		1		1	1	2
Helicobacter pylori				1		
Mycobacterium spp.				1		
Mycobacterium tuberculosis	1			1		
Neisseria gonorrhoeae		1			1	2
Propionibacterium acnes	1					
Pseudomonas spp.						1
Pseudomonas aeruginosa	1					
Pseudomonas fluorescens			1			
Staph aureus / MRSA	1	1		1		4
Streptococcus Group B		1			1	3
Streptococcus pneumoniae		1			1	3
Yersinia spp.			1			

Storage Temp -80°C

Organism / Species	≤12 m	≤24 m	≤36 m	≤48 m	≤60 m	>5 y
Aspergillus spp.						1
Bacteroides spp.						1
Blastomycetes						1
Campylobacter jejuni	1					1
Candida spp.			1			1
Closdridium spp.						1
Cryptococcus albidus						1
Dermatophytes						1
Escherichia coli				1		1
Enterococcus faecalis						1
Haemophilus spp.	1	1				
Helicobacter pylori	1	1				
Listeria monocytogenes						1
Moraxella catarrhalis						1
Mycobacterium tuberculosis			1			
Neisseria gonorrhoeae	2					
Nocardia spp.						1
Staph aureus / MRSA				1		1
Streptococcus Group B		1				1
Streptococcus pneumoniae	1					1

TEXT BOOK REFERENCES FOR MICROBANK™

The following text books reference the Microbank™ Storage system as a recommended method:

Bailey & Scott's Diagnostic Microbiology, by P. Tille.
ISBN:9780323083300.

Laboratory Methods in Food Microbiology by W. F. Harrigan.
ISBN: 9780123260437

Fungal Plant Pathogens - Principles and Protocols Series by C. Lane, P. Beales, K. Hughes.
ISBN: 9781845936686

Probiotics in Food Safety and Human Health by I. Goktepe, V. K. Juneja, M. Ahmedna.
ISBN: 9781574445145

Cryopreservation and Freeze-Drying Protocols by J. G. Day, M. R. McLellan.
ISBN: 9780896032965

Manual of Techniques in Invertebrate Pathology by L. A. Lacey
ISBN: 9780123868992

Bergey's Manual of Systematic Bacteriology by W. Whitman, A. Parte, M. Goodfellow, P. Kämpfer, H-J. Busse, M. E. Trujillo, W. Ludwig, K.I. Suzuki.
ISBN: 9780387950433

Manual of Clinical Microbiology by J. Versalovic
ISBN: 9781555814632

MICROBANK™

If you have data that you'd like to share with Pro-Lab Diagnostics on the successful recovery of organisms using Microbank™ please contact us.

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