Directoraat-Generaal Milieu
Directie Risicobeleid
Afdeling Straling, Nucleaire en Bioveiligheid

Rijnstraat 8
Postbus 30945
2500 GX Den Haag
Interne postcode 645

www.vrom.nl

ontwerp beschikking

DGM/RB IG 09-092/00

Gelezen de aanvraag van Intervet International B.V., te Boxmeer, van 30-09-2009, met de titel "Grootschalige produktie van Infectious Pancreatic Necrosis Virus (IPNV) VP2 antigeen", de aanvullende informatie van 29-10-2009 en van 02-11-2009,

De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

Gelet op artikel 17 van het Besluit genetisch gemodificeerde organismen milieubeheer en artikel 7 en bijlage 5, onder 5.7.2 van de Regeling genetisch gemodificeerde organismen,

Overwegende:
In de onderhavige aanvraag wordt ingevolge artikel 11 van het Besluit genetisch gemodificeerde organismen milieubeheer vergunning aangevraagd voor grootschalige productie van het VP2 antigeen afkomstig van het Infectious Pancreatic Necrosis Virus (IPNV) onder MI-II condities. Het gen coderend voor het antigeen bevindt zich in de vector pET-11d en wordt geproduceerd met behulp van een niet-koloniserende B stam van *Escherichia coli* . Deze productie vindt plaats in een fixed pipe fermentor met een maximaal werkvolume van 800 liter per batch.
De aanvrager voert de werkzaamheden momenteel uit onder MI-III condities. In 1995 is hiervoor vergunning verleend (IG 95-123), omdat zowel de gasheer als de vector nog geen erkende status verworven hadden. Op grond hiervan is destijds ingeschaald op het MI-III inperkingsniveau. Gezien het voortschrijdende inzicht en de erkenning van de gastheer en de vector wordt verzocht handelingen met het genetisch gemodificeerde organisme onder MI-II inperkingsniveau uit te mogen voeren.

Het genetisch gemodificeerde organisme is ingeschaald overeenkomstig de bijlage 5, en in het bijzonder onder 5.7.2 van de Regeling genetisch gemodificeerde organismen. Het gaat hier om een erkend gastheer/vectorsysteem, waarbij de gastheer erkend is voor handelingen van categorie IB en de vector erkend is voor handeling van categorie IA. Daarnaast is er geen reden om aan te nemen dat het VP-2 eiwit, een viraal eiwit, op enige wijze kan bijdragen aan de pathogeniteit van de bacterie *Escherichia coli* B.

Procedure:
De uniforme openbare voorbereidingsprocedure van Afdeling 3.4 van de Algemene wet bestuursrecht is van toepassing op de voorbereiding van deze ontwerpbeschikking.
De aanvraag en de onderhavige ontwerpbeschikking zullen voor advies worden voorgelegd aan de Commissie genetische modificatie (COGEM).

Besluit:
Intervet International B.V., te Boxmeer, vergunning te verlenen als bedoeld in paragraaf 2 van het Besluit genetisch gemodificeerde organismen milieubeheer. Aan de vergunning, waarvan de op 02-10-2009 ingediende vergunningaanvraag en de daarbij behorende stukken deel uitmaken, worden de hierna volgende voorschriften verbonden:

Artikel 1: toegestane werkzaamheden

1. Betreft: Productie van antigeen met behulp van genetisch gemodificeerde *Escherichia coli* met een maximaal volume van 800 liter;
   gastheer soort: *Escherichia coli;*
   stammen: B;
   vectoren: pET-11d;
   donorsequenties: VP2 major capsid protein (Infectious Pancreatic Necrosis Virus).

   De in dit lid bedoelde werkzaamheden moeten volgens de bepalingen van bijlage 4 van de Regeling genetisch gemodificeerde organismen, onder 4.2.2 (MI-II), worden uitgevoerd.

Artikel 2: plaatsen van uitvoering

Intervet International, Wim de Körverstraat 35, te Boxmeer.

Artikel 3: medewerkers

De bij de in artikel 1 bedoelde werkzaamheden betrokken medewerkers moeten van de bepalingen van deze vergunning in kennis zijn gesteld voordat die werkzaamheden aanvangen.

Artikel 4: nadere eisen

1. De vergunninghouder dient te voldoen aan de door de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (hierna: VROM) te stellen nadere eisen als bedoeld in artikel 9.2.2.3 Wet milieubeheer.
2. Voor het interne toezicht op de vergunning worden één of meerdere biologischeveiligheids-functionarissen aangesteld die door De Minister van VROM zijn toegelaten. Deze functionarissen zijn deskundig op het gebied van de inperkingsniveaus waarvoor vergunning wordt verleend.
3. Wijziging van de verantwoordelijk medewerker of biologischeveiligheidsfunctionaris en wijziging (in de naamgeving) van de rechtspersoon zoals vermeld in de aanvraag, moet binnen een week na wijziging schriftelijk aan De Minister van VROM gemeld worden p/a RIVM/SEC/Bureau GGO, Postbus 1, 3720 BA, Bilthoven, of via faxnummer 030-2744401.
4. Onvoorziene omstandigheden waarbij mogelijk ernstig risico voor mens en milieu is ontstaan moeten onverwijld aan De Minister van VROM gemeld worden. Hiervoor kunt u 24 uur per dag contact opnemen met het Ministerie van VROM, telefoonnummer 070-3832425. Tijdens kantooruren kunt u ook contact opnemen met het RIVM/SEC/Bureau GGO, telefoonnummer 030-2742793.

Den Haag, datum<<ONDERTEKENING_DATUM>>
de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,
voor deze:
de directeur-generaal van het Rijksinstituut voor Volksgezondheid en Milieu,
op last:
het hoofd van het Bureau Genetisch gemodificeerde organismen,
<<ONDERTEKENING_OMSCHRIJVING>>,

hier komt de handtekening

<<ONDERTEKENING_HANDTEKENING>>

en de naam van de ondertekenaar<<ONDERTEKENING_OFFICIELE_NAAM>>

Kennisgeving Besluit genetisch gemodificeerde organismen milieubeheer

Ontwerpbesluit op de vergunningaanvraag van Intervet International te Boxmeer voor ingeperkt gebruik van genetisch gemodificeerde organismen

Vergunningsaanvraag Intervet International

Op 30-09-2009 is van Intervet International te Boxmeer een vergunningsaanvraag op grond van het Besluit genetisch gemodificeerde organismen milieubeheer (hierna: Besluit ggo) ontvangen voor ingeperkt gebruik van genetisch gemodificeerde organismen. De aanvraag is ingeschreven bij DGM/RB onder nr. IG 09-092. De aanvraag betreft het uitvoeren van een grootschalige productie in fermentoren van het antigeen VP-2 van het infectious pancreatic necrosis virus door de genetisch gemodificeerde bacterie *Escherichia coli*. De productie zal binnen een MI-II ruimte plaatsvinden en betreft een werkvolume van maximaal 800 liter. De werkzaamheden zijn voorgenomen plaats te vinden in de gemeente Boxmeer.
Op deze aanvraag dient op grond van het Besluit ggo de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer (hierna: VROM) te beslissen.

Procedure

Voor de behandeling van bovengenoemde aanvragen zal de uniforme openbare voorbereidingsprocedure worden doorlopen, conform afdeling 3.4 van de Algemene wet bestuursrecht.
Deze kennisgeving geldt tevens als een mededeling als bedoeld in artikel 14 van het Besluit ggo.

Ontwerpbesluit

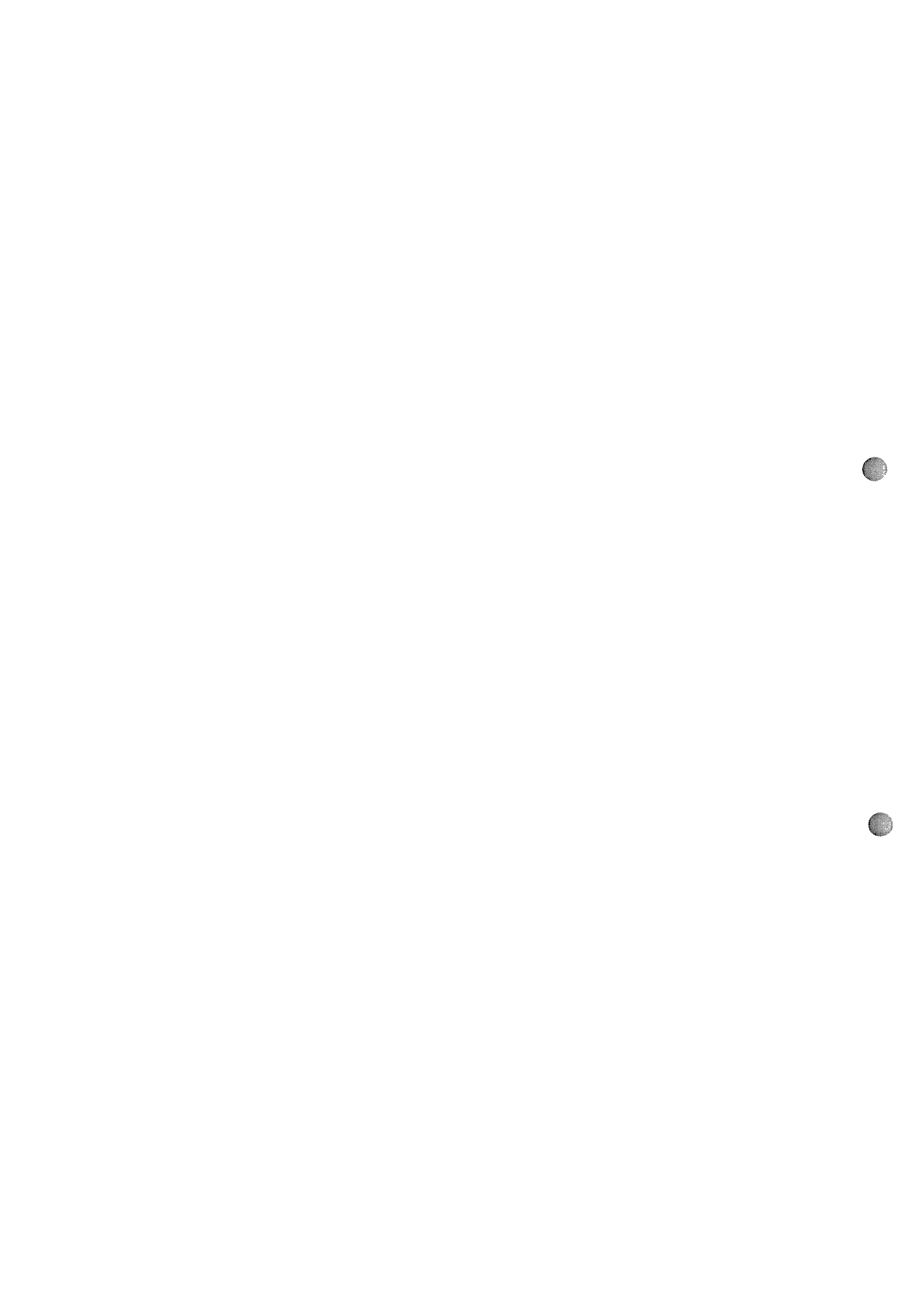
Naar aanleiding van de aanvraag is een ontwerpbeschikking opgesteld waarbij met de aanvraag wordt ingestemd.

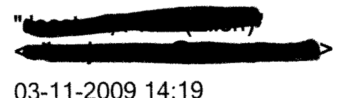
Inzage aanvraag en ontwerpbeschikking

De aanvraag, het ontwerpbesluit en de overige relevante stukken liggen vanaf 03-12-2009 op werkdagen ter inzage bij het Ministerie van VROM, afdeling Documentaire Informatie (C01 70), Rijnstraat 8 te Den Haag. De stukken kunnen daar ingezien worden van maandag t/m vrijdag van 8:30 uur tot 17.00 uur na afspraak via telefoon of mail (tel. 070-3393156, email: secretariaat.risicobeleid@minvrom.nl). De bezoeker dient zich te melden bij de receptie.
Deze kennisgeving, de ontwerpbeschikking en de bijbehorende stukken zijn ook beschikbaar op de internetpagina *www.vrom.nl/ggo-vergunningverlening*.

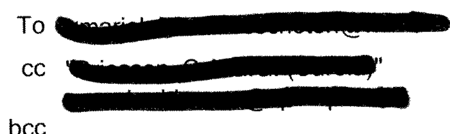
Inspraak

Tot en met 13-01-2010 kan een ieder zijn of haar zienswijzen schriftelijk of mondeling naar voren brengen met betrekking tot het ontwerpbesluit. Voor mondelinge zienswijzen kan contact opgenomen worden met het Ministerie van VROM / Bureau Genetisch Gemodificeerde Organismen, telefoon 030-2742793.
Schriftelijke zienswijzen dienen te worden gezonden aan:
De Minister van VROM
T.a.v.RIVM/SEC/Bureau GGO
postbus 1
3720 BA Bilthoven.
De zienswijze moet zijn ondertekend en van een datum, naam en adres voorzien zijn. Zienswijzen die per email worden ingestuurd, worden niet geaccepteerd.
Voor verdere vragen over het indienen van zienswijzen zie het veel gestelde vragen gedeelte op de internetpagina *www.vrom.nl/ggo-vergunningverlening*.





03-11-2009 14:19



To
cc
bcc
Subject RE: IG09-092

History: This message has been replied to.

Inderdaad gaat het ook bij 800 liter om een fermentorsysteem met fixed piping.
Groet,

SHE specialist/Biosafety officer
Safety, Health, Environment
Intervet / Schering-Plough Animal Health

From:)
Sent: 2 november 2009 10:07
To
Cc:
Subject: IG09-092

Beste
Zoals zojuist telefonisch aangegeven zouden we in de wijziging voor IPN (IG09-092) het kweekvolume willen vergroten naar 800 liter.
Groeten,

SHE specialist/Biosafety officer
Safety, Health, Environment
Intervet / Schering-Plough Animal Health

Intervet International bv
Wim de Körverstraat 35
Postbus 31, 5830 AA Boxmeer
The Netherlands
www.intervet.com

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-- please immediately and permanently delete

INGEKOMEN 0 2 OKT. 2009

Intervet
Schering-Plough Animal Health
Wim de Körverstraat 35
P.O. Box 31, 5830 AA Boxmeer
The Netherlands
www.schering-plough.com

RIVM/SEC/Bureau GGO
Postbus 1
3720 BA Bilthoven

16-09-092

Boxmeer, 30 september 2009
Betreft: kleine wijziging IG 95-123

Geachte Heer/Mevrouw,

Hierbij doet Intervet International bv een verzoek tot een kleine wijziging met betrekking tot beschikking IG 95-123.

In artikel 2 van deze vergunning is grootschalige productie toegestaan onder GS-I (= MI-III) inperking.

Ten tijde van de aanvraag van de beschikking (mei 1995) stond de gastheer, E. coli stam B, nog niet in appendix C, hoewel het niet koloniserende karakter reeds was aangetoond. Ook de vector, pET11d stond toen niet in appendix D. Daarom is het GGO toen beschouwd als een groep III GGO, waaruit een GS-I inperking volgde.

E. coli stam B wordt inmiddels beschouwd als een micro-organisme van klasse 1. De betreffende vector, pET11d, staat vermeld in Bijlage 2.1.1. Ons inziens kan hiermee grootschalige productie plaatsvinden onder MI-II inperking. Wij verzoeken u dan ook om de inperking in artikel 2 van IG 95-123 te verlagen van MI-III naar MI-II.

Hoogachtend,

Intervet / Schering-Plough Animal Health

cc: (Site Director)
(VM)

- 4 MEI 1995
ONTVANGEN 0 8 MEI 1995
Intervet

Ministerie van VROM
p/a directie SVS/655
Postbus 30945
2500 GX Den Haag

Onze ref.:JBO/DJ/B.300 — Boxmeer, 3 mei 1995

Betreft: kennisgeving ingeperkt gebruik, getiteld "Grootschalige produktie van Infectious Pancreatic Necrosis Virus (IPNV) VP2 antigeen".

Hierbij doet Intervet International kennisgeving van voorgenomen ingeperkt gebruik van genetisch gemodificeerde organismen als bedoeld in paragraaf 2 van het Besluit genetisch gemodificeerde organismen Wet milieu-gevaarlijke stoffen.

Aangezien het hier handelingen van categorie B betreft met een groep II organisme, zijn tevens aanvullende gegevens verstrekt volgens artikel 11 van het Besluit.
Bij deze aanvullende gegevens zijn 4 Addenda toegevoegd, te weten

I Bacteriologische produktie; een onderdeel van de aanvraag Hinderwet vergunning d.d. 26 November 1993.
II Veiligheidsvoorschrift voor het werken met GGO's in de Afdeling Bacteriologische Produktie; SOP no. 0212-5519-002.
III Ongevalsprogramma Bacteriologische Produktie; SOP no. 0212-5519-003.
IV Ontruimingsprocedures; Uit: Veiligheidsvoorschriften Intervet lokatie Boxmeer, VGWM-dienst, mei 1991.

De kennisgeving bevat 1 als zodanig aangemerkte vertrouwelijke Bijlage.

Hoogachtend,

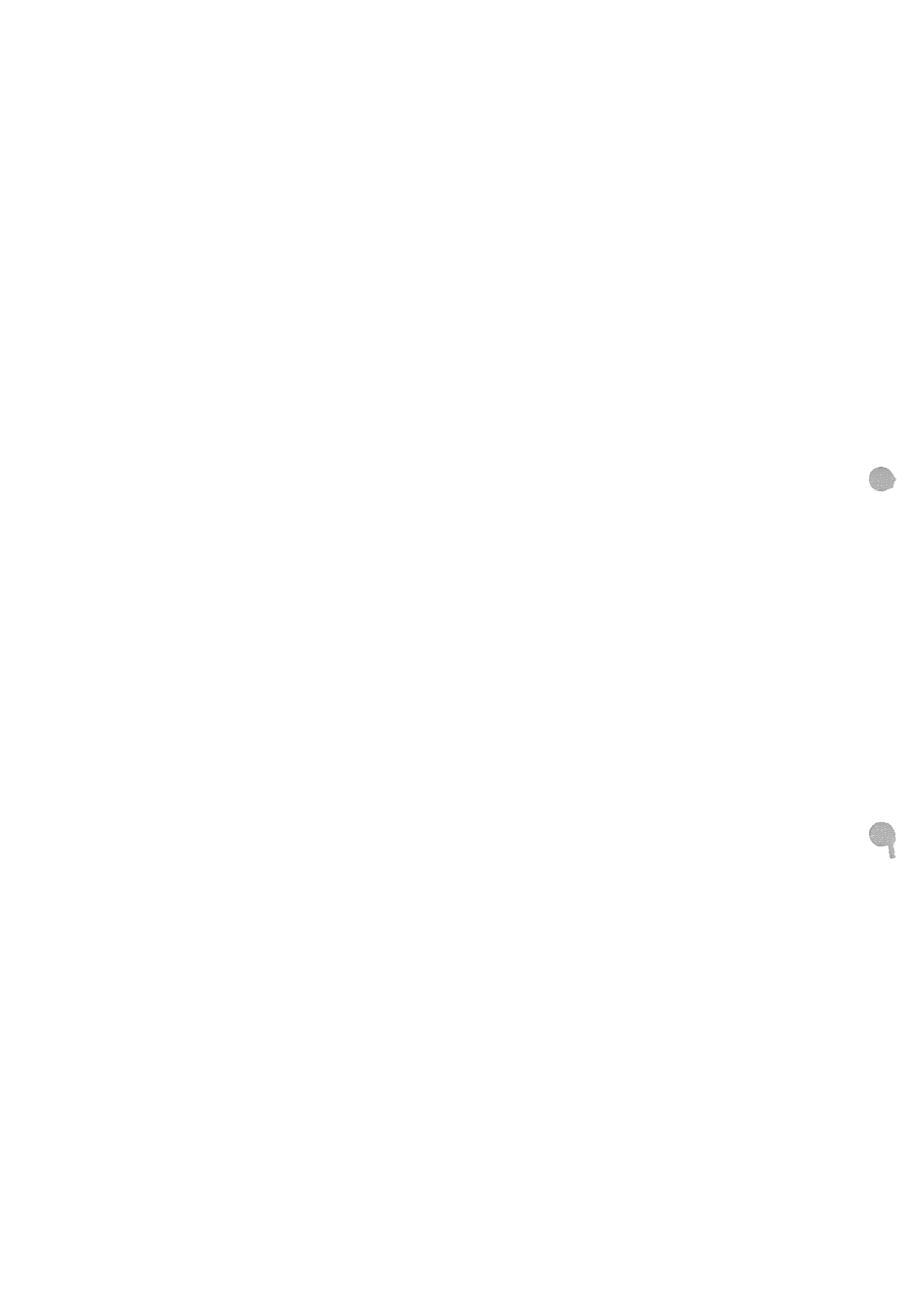
Dr. J.F. van den Bosch
Intervet International BV

cc: Secretariaat COGEM
Drs. P. van Gelder (Intervet)
Dr. Ir. V.F.M. Rijnierse (Intervet)
F. van der Zande (Intervet)
Dr. P.K. Storm (Intervet)
Dr. D. Lütticken (Intervet)

Bijlagen

Intervet

Intervet International bv
Wim de Körverstraat 35
P.O. Box 31
5830 AA Boxmeer
The Netherlands
Tel. (08855) 87600
Telex 37306
Telefax (08855) 77333



KENNISGEVING VAN VERVAARDIGING VAN EN HANDELINGEN MET GGO's IN LABORATORIA, PLANTEKWEEKCELLEN, KASSEN EN DIERVERBLIJVEN

In te vullen door VROM / VCOGEM
KGnr:

Deze kennisgeving bestaat uit:

Algemeen deel			met ..... Bijlagen
Tabel van GGO's			met ..... Bijlagen (waarvan ...... vertrouwelijk)
Deel 1		J~~a~~/Nee	met ..... Bijlagen (waarvan ...... vertrouwelijk)
Deel 2		J~~a~~/Nee	met ..... Bijlagen (waarvan ...... vertrouwelijk)
Deel 3	deel 3A	Ja/~~Nee~~	met ..1.. Bijlagen (waarvan ..1... vertrouwelijk)
	deel 3B	J~~a~~/Nee	met ..... Bijlagen (waarvan ...... vertrouwelijk)
	deel 3C	J~~a~~/Nee	met ..... Bijlagen (waarvan ...... vertrouwelijk)

ALGEMEEN DEEL *(Lees voordat u begint bij iedere vraag eerst de Toelichting !)*

1. KENNISGEVER

1.1 Naam - Intervet International bv

1.2 Postadres - Postbus 31

POSTCODE: 5830 AA PLAATS: Boxmeer

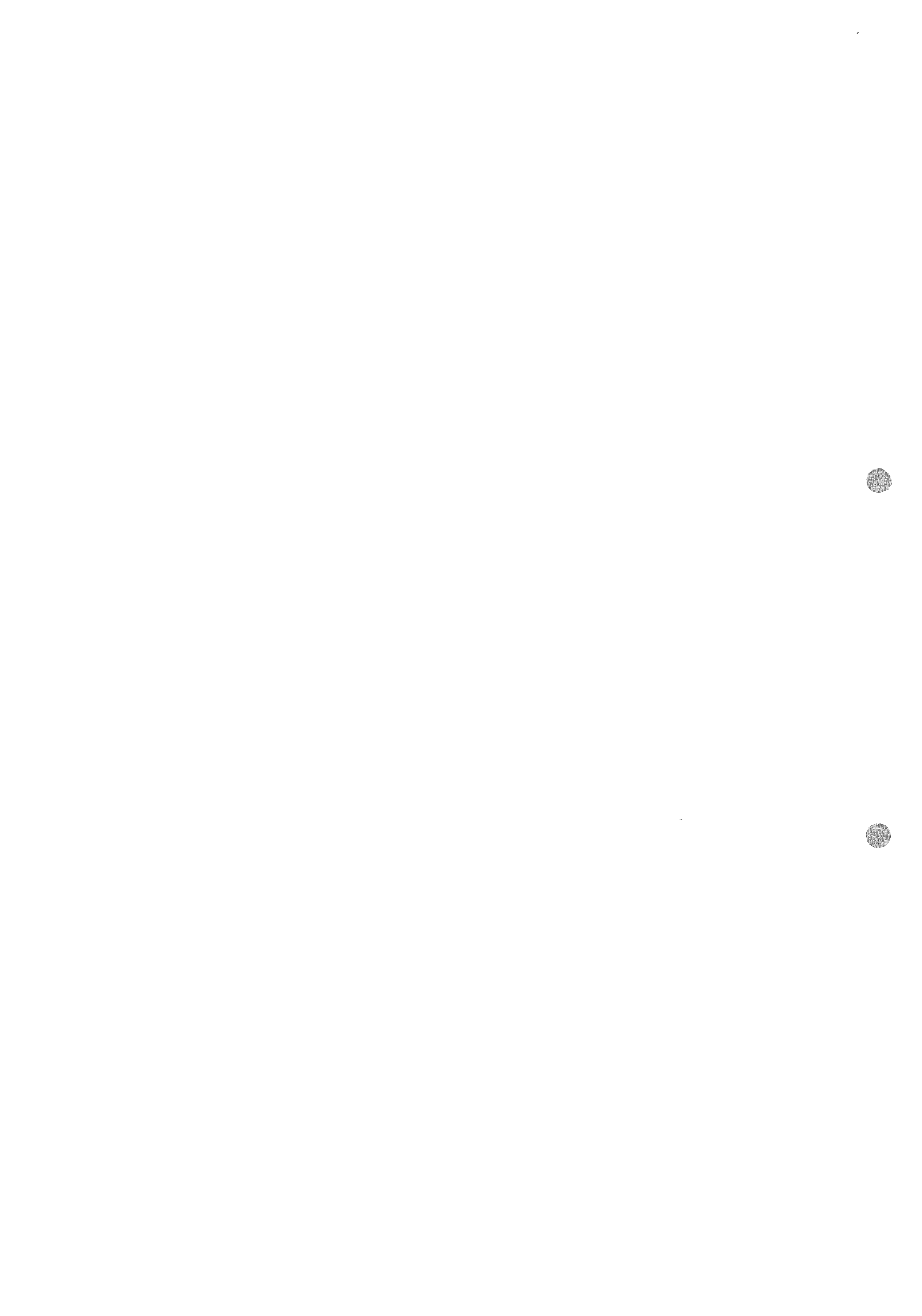
1.3 Contactpersoon voor deze kennisgeving - Dr. J.F. van den Bosch

1.4 Telefoon-/telefaxnummer - TEL: 08855-87322 FAX: 08855-87490

2. TITEL VAN HET PROJECT EN DOEL VAN DE HANDELINGEN

2.1 Beschrijvende titel - Grootschalige produktie van Infectious Pancreatic Necrosis Virus (IPNV) VP2 antigeen.

2.2. Doel van de handelingen - Vaccin productie



3. PLAATS VAN UITVOERING

3.1 Bezoekadres van de inrichting – Afd. Bacteriologische Productie
Intervet International bv, Wim de Körverstr. 35
POSTCODE: 5831 AN PLAATS: Boxmeer
Naam van de inrichting:

3.2 Contactpersoon – Dr. J.F. van den Bosch

3.3 Telefoon-/telefaxnummer – TEL: 08855-87322 FAX: 08855-87490

3.4 Inperkingsniveaus van de ruimten binnen de inrichting waarvoor een Hinderwetvergunning/vergunning Wet milieubeheer voor activiteiten met GGO's is afgegeven. – GILSP/GS-1
aangevraagd (concept raamvergunning dd 26-11-1993)

3.5 Vergunningverlenende instantie, afgiftedatum(-data) en nummer(s) van de onder 3.4 bedoelde vergunningen – INSTANTIE: Gemeente Boxmeer
NR: in aanvraag DATUM:

4. DUUR VAN DE ACTIVITEITEN.

4.1 Geplande begindatum – Medio 1995 (na verkregen toestemming)

4.2 Verwachte einddatum – n.v.t.

5. MEDEWERKERS.

5.1 Eerstverantwoordelijke medewerker – Dr. Ir. V.F.M. Rijnierse
Telefoon-/telefaxnummer – TEL: 08855-87700 FAX: 08855-87707

5.2 Overige medewerkers –

NAAM	BEOORDEELD
M.H. Brummans	ja/~~nee~~
J.M.G. van Loon	ja/~~nee~~
W.J.M. de Kleijnen	ja/~~nee~~
R.J.B. Lemmens	ja/~~nee~~
M.J.W. Litjens	ja

6. BIOLOGISCHE-VEILIGHEIDSFUNCTIONARIS

6.1 Naam – Drs. P.T.J.A. van Gelder

6.2 Correspondentieadres – Intervet International bv
Wim de Körverstraat 35
POSTCODE: 5831 AN PLAATS: Boxmeer

6.3 Telefoon-/telefaxnummer – TEL: 08855-87352 FAX: 08855-87339

6.4 Oordeel BVF over medewerkers –
☒ Allen hebben voldoende ervaring.
☐ Voorstel aanvullende opleiding voor:

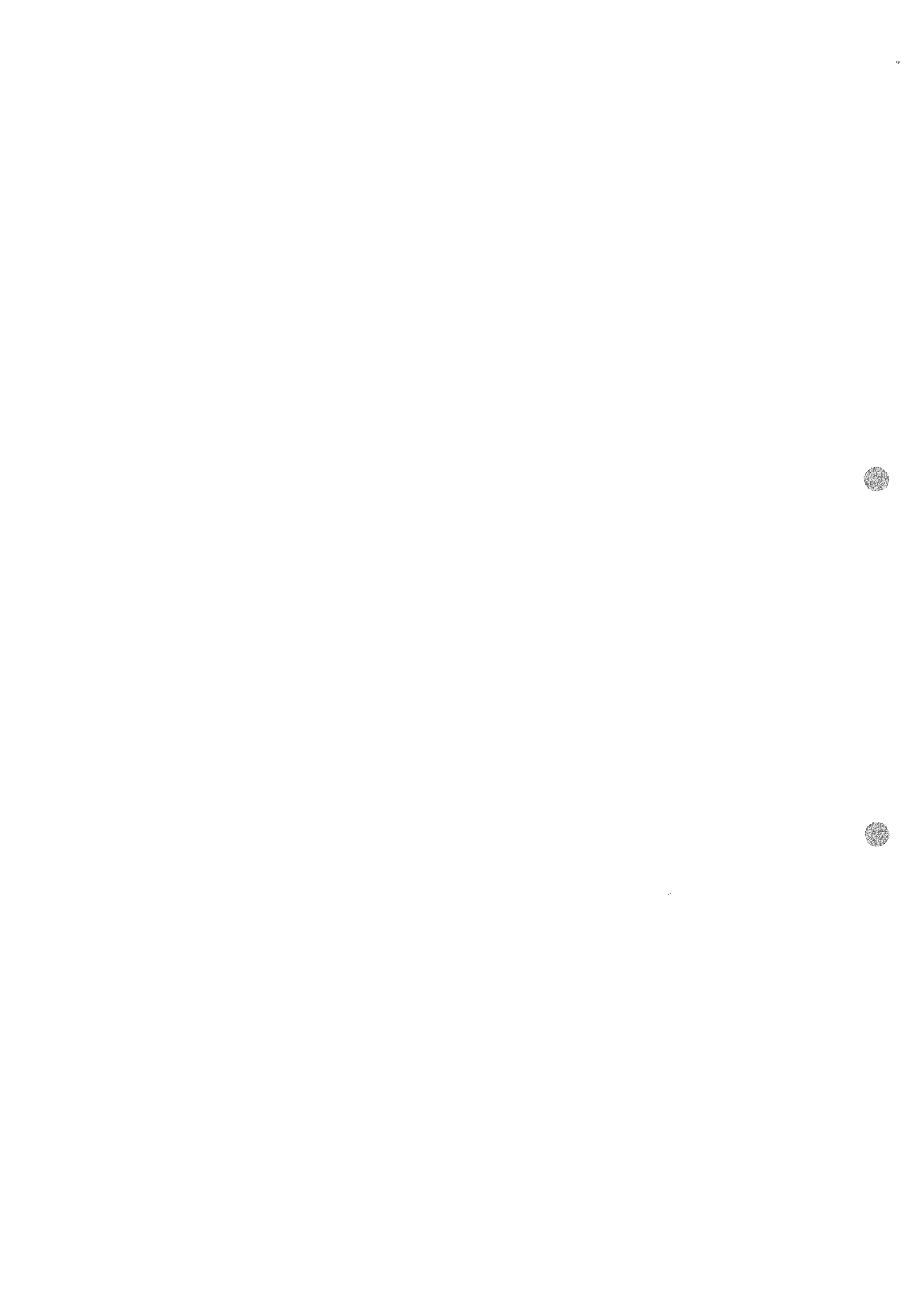
NAAM	TRAINING
....	....
....	....
....	....
....	....

De BVF verklaart zich akkoord met de inhoud van deze kennisgeving.

Plaats: Boxmeer

Datum: 14-April-1995

Handtekening: [signature] 27-04-95

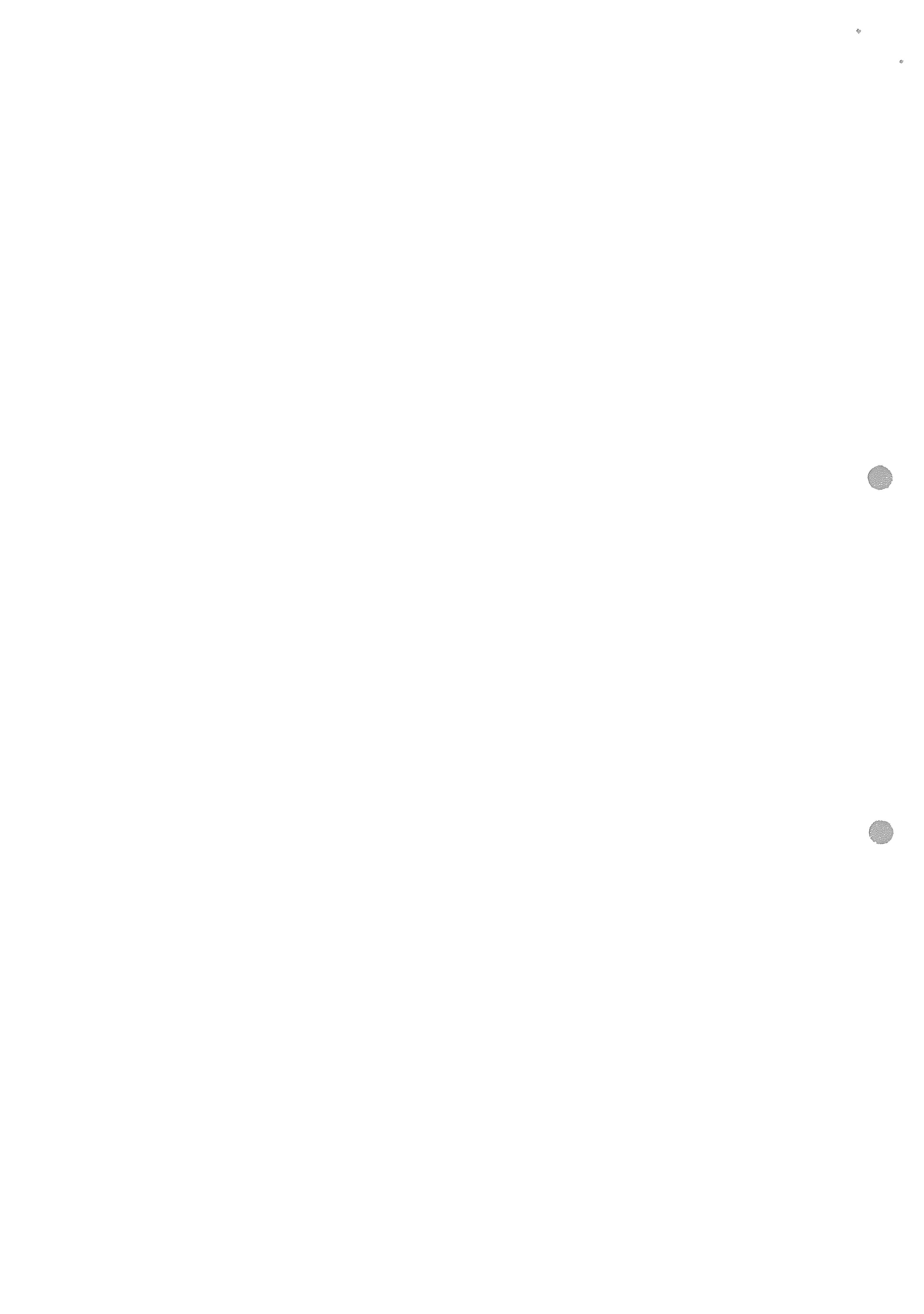


TABEL VAN GGO's die onder deze kennisgeving worden vervaardigd of toegepast *(Zie toelichting!)*

Nr.	GASTHEER -soort -stammen	Indeling; PG-/T-kl.	VECTOREN	Indeling	TE CLONEREN GENEN/SEQUENTIES; INSERTIES Naam	Functie	DONORORGANISMEN (Soort)	Indeling; PG-/T-kl.
1	E. coli BL21 (DE3) /pLysS (*)	g1 c6	pET11d	V8 (**)	VP2-major capsid protein		Infectious Pancreatic Necrosis Virus (IPNV)	d8 PG-1

(*) Gastheerstam is een E. coli B, nog niet officieel in Appendix C opgenomen, maar het niet-koloniserend karakter is aangetoond door Prof. W. Hoekstra

(**) Vector pET11d is nog niet opgenomen in Appendix C, maar volgens schrijven GGO 940216.03 van Min. VROM wel erkend.



<u>DEEL 3</u> Handelingen met GGO's.
Deel 3A: Micro-organismen
(Lees eerst de toelichting bij de vragen!)

Geef voor de in de linkerkolom van de Tabel van GGO's opgegeven nummers aan (voor zover het gaat om handelingen met die GGO's):

1. Volgnummer van dit deelformulier - Deel 3A-.1

2. Herkomst van de GGO's; wijze van vaststelling van de identiteit -

Het GGO BL21-pET11-VP2 (IPNV) is geconstrueerd door Intervet Norbio, Bergen, Noorwegen. De constructie en wijze van vaststelling van de identiteit staat beschreven in Bijlage 1.

3. Voorgenomen handelingen met de GGO's (specificaties in bijlagen).

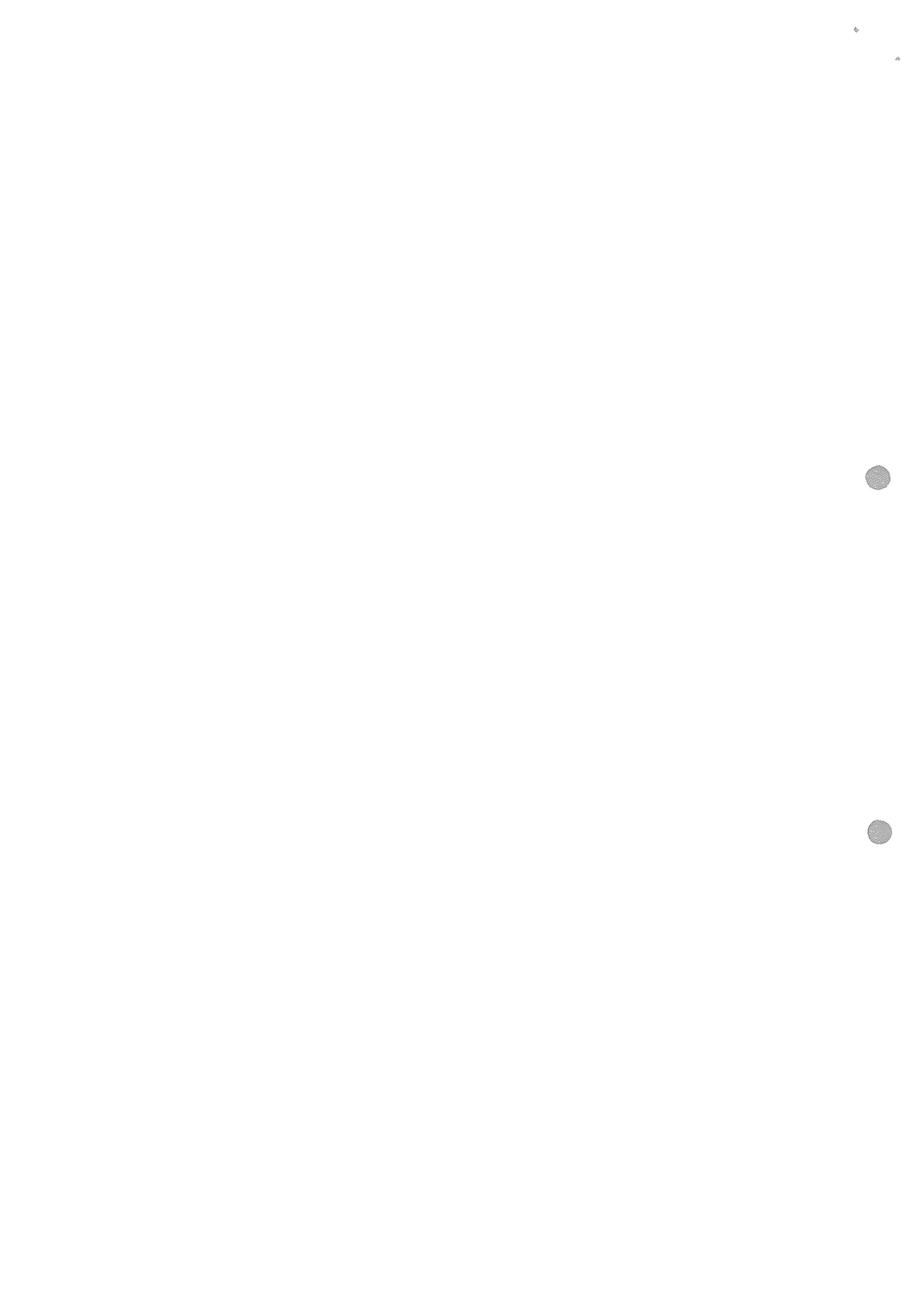
- ☐ Handelingen in laboratoria met volumina van minder dan 10 l.
- ☐ Handelingen in laboratoria met volumina groter dan 10 l. doch die op andere gronden als kleinschalig kunnen worden aangemerkt.
- ☐ Handelingen in associatie met planten in plantekweekcellen.
- ☐ Handelingen in associatie met planten in kassen.
- ☐ Handelingen in associatie met dieren in dierverblijven.
- ☒ Anders. Grootschalige productie

4. Risico-analyse.

4.1 Relevante eigenschappen van de GGO's -

Er wordt een gekarakteriseerde sequentie gebruikt van een niet toxicogeen, vis-specifiek IPNV virus van klasse PG-1, coderend voor het "major capsid protein" VP2. Het gastheer-vector systeem staat niet vermeld in Appendix C van de Richtlijnen. Echter, de gebruikte vector pET11d zal volgens een schrijven van het Ministerie (GGO 940216.03) worden toegevoegd aan de lijst van erkende vectoren, als reactie op een eerder ingediend verzoek van onze kant. De gebruikte gastheer *E. coli* BL21 (DE3) pLysS behoort tot de *E. coli* B stammen, waarvan recent door Prof. W. Hoekstra is aangetoond dat ze een niet-koloniserend karakter hebben, alsgevolg waarvan B stammen waarschijnlijk in de lijst van erkende gastheren zullen worden opgenomen. Al met al kan met grote zekerheid gesteld worden dat van het GGO BL21-pET11-VP2 (IPNV) geen toxische effekten of pathogene eigenschappen verwacht kunnen worden. Desalnietemin voldoet het GGO niet aan de criteria voor Groep I volgens artikel 6.1 van de Richtlijnen, en dient derhalve als een Groep II GGO aangemerkt te worden.

4.2 Geef aan onder welke inperkingcategorie de handelingen volgens u kunnen worden verricht en waarop uw inschaling is gebaseerd. - GS-1 volgens artikel 10.2.3.b en 10.4.3.a van de Richtlijnen



BIJLAGE 4

behorende bij de artikelen 7, tweede lid, 9, tweede lid, 10, tweede lid, en 11, tweede lid, van het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen

Gegevens voor kennisgevingen

1. Vereiste gegevens voor de in artikel 7, eerste lid, bedoelde kennisgeving:

a. de naam van degene die verantwoordelijk is of de namen van degenen die verantwoordelijk zijn voor het ingeperkte gebruik, alsook van degenen die verantwoordelijk zijn voor het toezicht, de controle en de veiligheid, en gegevens over hun opleiding en kwalificaties;

b. het adres en de exacte ligging van de inrichting, en de beschrijving van de relevante delen van de inrichting;

c. een beschrijving van de aard van de uit te voeren werkzaamheden, in ieder geval inhoudende de vermoedelijke schaal van de activiteiten;

d. de in artikel 5, eerste lid, bedoelde samenvatting van de analyse van de risico's voor mens of milieu.

2. Vereiste gegevens voor de in artikel 9, eerste lid, bedoelde kennisgeving:

a. indien van toepassing, de datum van ontvangst van de in artikel 7, tweede lid, bedoelde kennisgeving en de naam van degene die de kennisgeving heeft gedaan;

b. het gebruikte ouderorganisme dan wel de gebruikte ouderorganismen of, indien van toepassing, het gebruikte gastheer-vectorsysteem of de -systemen;

c. de herkomst en beoogde functie of functies van het bij de genetische modificatie gebruikte genetische materiaal;

d. de identiteit en de kenmerken van het genetisch gemodificeerde organisme;

e. het doel van het ingeperkte gebruik;

f. de in artikel 5, eerste lid, bedoelde samenvatting van de analyse van de risico's voor mens of milieu;

g. de gebruikte kweekvolumes.

3. Vereiste gegevens voor de in artikel 10, eerste lid, bedoelde kennisgeving:

a. de onder 2, onder a tot en met f, bedoelde gegevens;

b. de methoden voor het hanteren van de genetisch gemodificeerde organismen;

c. een beschrijving van de voor de duur van het ingeperkte gebruik te nemen maatregelen voor bescherming en toezicht;

d. de van toepassing zijnde inperkingscategorie met vermelding van de voorzieningen voor afvalstoffenbehandeling en de te nemen veiligheidsmaatregelen.

4. Vereiste gegevens voor de in artikel 11, eerste lid, bedoelde kennisgeving:

a. de onder 3 bedoelde gegevens;

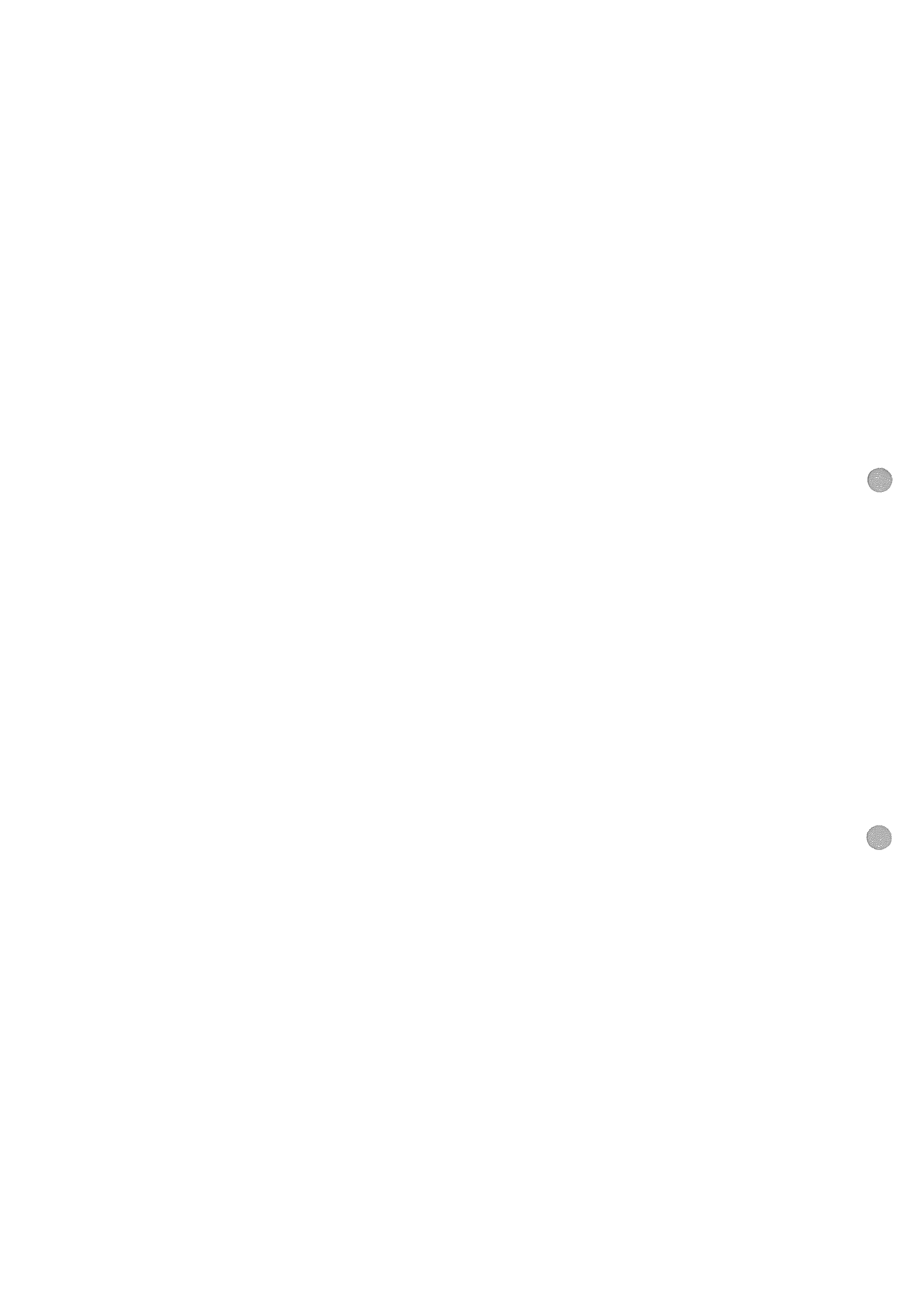
b. gegevens over het personeel;

d. gegevens over de installatie en de te gebruiken kweekvolumes;

e. gegevens over het afvalstoffenbeheer;

f. gegevens over de ongevalpreventie en de rampenplannen;

g. een analyse als bedoeld in artikel 5, eerste lid, van de risico's voor mens of milieu van het voorgestelde ingeperkte gebruik.



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Mapping of neutralization epitopes on infectious pancreatic necrosis viruses

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We have characterized and mapped variable and conserved neutralization epitopes of serogroup A strains of aquatic birnaviruses. Epitope mapping using monoclonal antibodies (MAbs) and *Escherichia coli*-expressed deletion fragments of VP2 of the N1 strain of infectious pancreatic necrosis virus (IPNV) demonstrated that two variable epitopes, H8 and B9, depend on the variable region between amino acid 204–330. A conserved neutralization epitope, F2, was shown to depend on the same region as epitopes H8 and B9 but was additionally dependent on amino acids between 153–203. The neutralization epitopes H8, B9 and F2 were also shown to overlap by a competitive binding assay. One conserved neutralization epitope, AS-1, was not exposed on any of the recombinant VP2 deletion fragments and was therefore not possible to map. However, the MAbs AS-1 and F2 were partly competitive indicating that these epitopes are overlapping. All neutralization epitopes were independent of a conserved non-neutralization epitope, E4. Our results demonstrate that the central third of VP2 contains several partly overlapping neutralization epitopes, both variable and conserved among serogroup A strains of IPNV.

Introduction

Infectious pancreatic necrosis viruses (IPNV) are aquatic birnaviruses responsible for infectious pancreatic necrosis (IPN) in various fish species. Since 1986, acute IPN with considerable losses has frequently been diagnosed among farmed Atlantic salmon (*Salmo salar*) in Norway (Christie *et al.*, 1990; Melby *et al.*, 1994). Mortality in salmonids is believed to be highest in fry at first feeding and to become negligible by approximately 6 months of age (Frantsi & Savan, 1971). However, recently an increasing number of IPN outbreaks with high mortalities have been reported among post-smolt of Atlantic salmon following transfer to seawater (Smail *et al.*, 1992; Melby *et al.*, 1994). Developing an effective IPN vaccine is a major priority.

The birnavirus genus, family *Birnaviridae*, contains animal viruses with double-stranded bisegmented (segments A and B) RNA genomes contained within a non-enveloped icosahedral capsid. Segment A encodes three known gene products within one large open reading frame (Duncan & Dobos, 1986; Håvarstein *et al.*, 1990), and segment B encodes a RNA-dependent RNA polymerase (VP1) (Duncan *et al.*, 1991). The segment A-encoded polyprotein, pVP2-NS-VP3, is autocatalytically cleaved by the endoprotease (NS) to produce the structural proteins VP2 and VP3 (Duncan *et al.*, 1987). Variations in the polypeptide patterns of different virus strains have been reported (Macdonald *et al.*, 1982; Christie *et al.*, 1988).

The classification of aquatic birnaviruses is disputed and varies depending on the method used (Caswell-Reno *et al.*, 1986, 1989; Christie *et al.*, 1988; Heppell *et al.*, 1993). Based on serological analyses, the aquatic birnaviruses have been divided into two serogroups (A and B) (Hill & Way, 1988). The predominant serogroup A is found globally and includes all serotypes known to be pathogenic to fish.

The major neutralization epitopes of aquatic birnaviruses are localized within VP2 (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Tarrab *et al.*, 1993), but neutralization epitopes have also been suggested for VP3 (Tarrab *et al.*, 1993).

An internal variable region located in the central part

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of VP2 has been identified (Håvarstein *et al.*, 1990) and was suggested to contain serotype-specific epitopes of birnaviruses. A VP2-specific monoclonal antibody (MAb), AS-1, neutralizing all serogroup A strains of IPNV has been reported (Caswell-Reno *et al.*, 1989; Lipipun *et al.*, 1991), indicating the presence of a conserved neutralization epitope on VP2. Due to the antigenic variability of aquatic birnaviruses a subunit vaccine against IPN should be based on neutralization epitopes known to be conserved among the relevant virus strains.

In order to develop an effective subunit vaccine the epitopes responsible for protective immunity have to be identified. In this communication we report the characterization and mapping of variable and conserved neutralization epitopes on serogroup A strains of aquatic birnaviruses using MAbs and *Escherichia coli*-expressed deletion fragments of VP2.

Methods

Cells and viruses. The VR-299 (A1) and West Buxton (A1) virus strains were obtained from Dr J. C. Leong (Oregon State University, USA), the Sp (A2) and Ab (A3) virus strains were obtained from Dr P. E. V. Jørgensen (The State Veterinary Serum Laboratory, Århus, Denmark), the Hecht (A4) virus strain from Dr W. Ahne (University of Munich, Germany), the Tellina (A5) virus strain from Dr D. A. Smail (Agriculture and Fisheries Department Marine Laboratory, Aberdeen, Scotland), the Canada-1, -2 and -3 (A6, A7 and A8) virus strains from Dr B. L. Nicholson (University of Maine, USA) and the Jasper-Dobos virus strain (A9) from Dr P. Dobos (University of Guelph, Canada). Isolation of the N1 virus strain (A10) from Norwegian Atlantic salmon (*Salmo salar*) has been described elsewhere (Christie *et al.*, 1988). All viruses were propagated in Chinook salmon embryo (CHSE-214) cells grown by standard methods. During virus propagation no serum was added to the cell growth medium. Virus used in sandwich ELISA or neutralization assay was either taken directly from the master seed or propagated once in CHSE-214 cells. Virus for SDS–PAGE and Western blotting was purified according to Christie *et al.* (1988).

Monoclonal antibodies. The VP2-specific MAbs F2 and E4 were produced against the N1 (A10) strain of IPNV as described elsewhere for the VP2-specific MAbs H8, B9 and for the VP3-specific MAb C12 (Christie *et al.*, 1990). MAb AS-1 (Caswell-Reno *et al.*, 1989), was kindly provided by Dr B. L. Nicholson (University of Maine, USA). MAbs H8, B9 and F2 were purified by the MAbTrap G procedure (Pharmacia) and labelled with biotin by standard methods using 100 µg *N*-hydroxysuccinimide biotin/mg immunoglobulin.

Isolation and subcloning of cDNA encoding VP2. A cDNA encoding VP2 was isolated from the lambda gt10 cDNA library previously used for sequencing segment A of the N1 virus strain Håvarstein *et al.* (1990). A VP2 cDNA of approximately 1·4 kbp was subcloned into the

Table 1. *Plasmid constructs for expression of VP2 fragments of the N1 strain of IPNV in Escherichia coli*

Name of plasmid	Origin*	VP2 codons encoded
pET11-VP2	Cleavage of pGEM-VP2-2x*Bam*HI with *Bam*HI and insertion of the VP2 fragment (*Bam*HI VP2) into *Bam*HI-linearized pET11d	3–453
pET11-VP2 Δ*Xmn*I	Complete digestion of the *Bam*HI VP2 fragment with *Xmn*I. Ligation of *Nco*I linker 5′ CAGCCATGGCTG 3′ to the blunt end, complete digestion with *Nco*I and *Bam*HI, and insertion into *Nco*I/*Bam*HI-linearized pET11d	92–453
pET11-VP2 Δp*Hinc*II	Partial digestion of the *Bam*HI VP2 fragment with *Hinc*II. Ligation of *Nco*I linker 5′ AGCCATGGCT 3′ to the blunt end, complete digestion with *Nco*I and *Bam*HI, and insertion into *Nco*I/*Bam*HI-linearized pET11d	153–453
pET11-VP2 Δ*Hinc*II	Complete digestion of the *Bam*HI VP2 fragment with *Hinc*II. Ligation of *Nco*I linker 5′ AGCCATGGCT 3′ to the blunt end, complete digestion with *Nco*I and *Bam*HI, and insertion into *Nco*I/*Bam*HI-linearized pET11d	234–453
pET11-VP2 Δ*Msc*I	Complete digestion of the *Bam*HI VP2 fragment with *Msc*I. Ligation of *Nco*I linker 5′ CAGCCATGGCTG 3′ to the blunt end, complete digestion with *Nco*I and *Bam*HI, and insertion into *Nco*I/*Bam*HI-linearized pET11d	280–453
pET11-VP2 Δp*Aat*II	Partial digestion of pET11-VP2 with *Aat*II and religation	3–269
pET11-VP2 Δ*Sac*IIΔ*Sma*I	Complete digestion of pET11-VP2 with *Sac*II and blunt ended with Klenow 3′–5′ exonuclease. Complete digestion with *Sma*I and religation	3–201
pET11-VP2 Δ*Bst*BI	Complete digestion of pET11-VP2 with *Bst*BI and religation	3–127
pET11-VP2 Δ*Aat*II	Complete digestion of pET11-VP2 with *Aat*II and religation	3–86
pET11-VP2 Δp*Hinc*IIΔ*Ava*II	Complete digestion of pET11-VP2 Δp*Hinc*II with *Xba*I and *Bam*HI. Complete digestion of the *Xba*I/*Bam*HI fragment with *Ava*II, blunt end repair with T4 DNA polymerase. Complete digestion with *Nco*I and insertion into *Nco*I/*Sma*I-linearized pET11-VP2	153–330
pET11-VP2 Δp*Hinc*IIΔp*Ava*II	Complete digestion of pET11-VP2 Δp*Hinc*II with *Xba*I and *Bam*HI. Partial digestion of the *Xba*I/*Bam*HI fragment with *Ava*II and blunt end repair with T4 DNA polymerase. Complete digestion with *Nco*I and insertion into *Nco*I/*Sma*I-linearized pET11-VP2	153–424
pET11-VP2 Δp*Hinc*IIΔ*Sac*IIΔp*Aat*II	Complete digestion of pET11-VP2 Δp*Hinc*II with *Sac*II followed by partial digest with *Aat*II, blunt end repair with T4 DNA polymerase and religation	153–201 & 270–453
pVABB-dVP2	Complete digestion of pRIT28-dVP2 with *Eco*RI and *Hin*dIII, and insertion of the dVP2 fragment into *Eco*RI/*Hin*dIII-linearized pVABBmp8†	204–331

* The location of the restriction sites used to construct the pET11-VP2 derived deletion mutants are shown in Fig. 1.
† Expression vector pVABBmp8 encodes a 25 kDa serum albumin binding domain (BB) of protein G of *Streptococcus* G148, 5′ to the polylinker.

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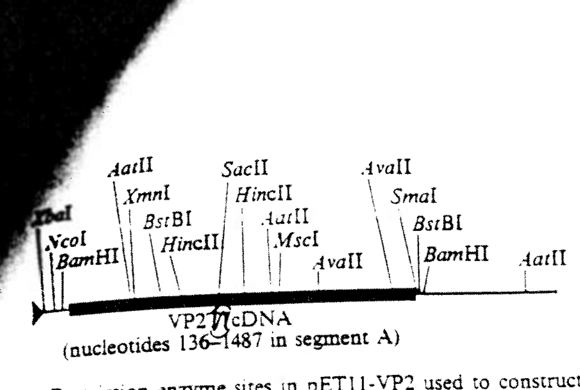

Fig. 1. Restriction enzyme sites in pET11-VP2 used to construct the pET11-VP2-derived deletion mutants described in Table 1.

*Eco*RI site of the pGEM-7Zf(+) vector (Promega) generating the plasmid pGEM-VP2. The coding potential of the subcloned VP2 cDNA (nucleotides 136–1487 of segment A) was determined by chain termination nucleotide sequencing of the 5′ and 3′ ends as described by Håvarstein *et al.* (1990), using primers (Promega) complementary to the T7 and SP6 promoter regions in pGEM-7Zf(+). A PCR fragment complementary to nt 739–1123 in segment A of the IPNV N1 strain was produced using the pGEM-VP2 as template. The upstream primer (26-mer) introduced a *Bam*HI site, while the downstream primer (33-mer) introduced a TAA translation stop codon and a *Hin*dIII site. The PCR fragment was digested with *Bam*HI and *Hin*dIII, ligated into similarly digested pRIT28 vector, generating pRIT28-dVP2, and sequenced by the direct solid-phase procedure (Hultman *et al.*, 1988).

Construction of expression plasmids encoding VP2 fragments. A *Bam*HI site was generated in pGEM-VP2 5′ to the VP2 cDNA by digestion with *Xho*I, blunt-ended with Klenow fragment and insertion of the *Bam*HI linker 5′-CGGATCCG-3′ (Pharmacia), generating the plasmid pGEM-VP2 2x*Bam*HI. The reading frame of the VP2 cDNA was determined by standard chain termination DNA sequencing using the T7 primer. Further DNA constructions for expression of VP2 fragments in the pET11d (Novagen) and the pVABBmp8 expression plasmids are described in Table 1. Plasmid pVABBmp8 is constructed by insertion of a *Xba*I–*Eco*RI fragment, encoding the serum albumin binding domain (BB) from streptococcal protein G, from plasmid pTrpBB (Öberg *et al.*, 1994), into a similarly digested pRIT44 (Köhler *et al.*, 1991).

The restriction sites in pET11-VP2 used to construct the VP2 deletion fragments as described in Table 1 are illustrated in Fig. 1. All constructions were verified by restriction fragment length analysis. The enzymes were obtained from Pharmacia (*Ava*II, *Aat*II, *Hin*dIII, *Nco*I, *Hin*cII, *Sac*II, *Sma*I, *Xba*I, T4 DNA Polymerase, T4 DNA Ligase and Klenow fragment), Promega (*Bam*HI, *Eco*RI and *Xho*I) and New England Biolabs (*Bst*BI, *Msc*I and *Xmn*I).

Gene expression and protein purification. E. coli strain BL21(DE3) pLysS (Novagen) was used for expression of VP2 fragments subcloned in the pET11d expression vector. *E. coli* containing pET11d constructions was grown at 30 °C in LB-medium containing ampicillin (Astra; 200 μg/ml) and chloramphenicol (20 μg/ml). Gene expression was induced when the cultures reached an OD_{550} of 0·6 to 1·0 by adding IPTG (1 mM; Sigma) and additional ampicillin (200 μg/ml). Following induction for 2 h, the cells were harvested by centrifugation (3000 *g* for 20 min) and concentrated 10 times by resuspension in ice-cold 1 × TNE-T buffer (0·1 M-Tris–HCl pH 8·0, 0·3 M-NaCl, 1 mM-EDTA, 0·1% Triton X-100) containing 1 mg/ml chicken egg-white lysozyme (Sigma). The cells were incubated on ice and constantly agitated for 2 h, at which point DNaseI (3 U/ml; Sigma) and $MgCl_2$ (1 mM) were added. Incubation and agitation continued overnight, after which the suspensions were heated to 70 °C for 15 min, cooled on ice and harvested by centrifugation (4300 *g*, 20 min). The pellets were washed for 10 min, twice in ice-cold 2 × TNE-T buffer and twice in ice-cold sterile distilled water prior to SDS–PAGE analysis and lyophilization. For gel filtration, lyophilized samples were solubilized in ice-cold 6 M guanidine.HCl, 16 mM-Tris–HCl pH 7·4, to a final concentration of 10–20 mg/ml. Undissolved material was removed by 0·22 μm filtration or by centrifugation (12000 *g* for 15 min) and samples of 200 μl applied on a Superose 12 HR 10/30 gel filtration column (Pharmacia) using the liquid chromatography (FPLC) system (Pharmacia) according to the manufacturer's instructions. Eluted fractions were monitored at 280 nm and analysed by SDS–PAGE. Relevant fractions were pooled and dialysed overnight against ice-cold 2 M-guanidine.HCl, 16 mM-Tris–HCl pH 7·4. The protein concentrations were determined according to the A_{280}, and all samples were diluted in additional 2 M-guanidine.HCl to a final protein concentration of approximately 100 μg/ml.

E. coli strain RR1ΔM15 (Rüther, 1982) was used for expression of the pVABBmp8 constructions. *E. coli* cells harbouring the pVABB-dVP2 or the pVABBmp8 vector were grown at 30 °C in tryptic soy broth medium (Difco; 30 mg/ml) with addition of yeast extract (Difco; 5 mg/ml) and ampicillin (Astra; 100 μg/ml). Induction of gene expression with indole acrylic acid was performed as described by Köhler *et al.* (1991) at OD_{550} of 1·5. Cells were harvested by centrifugation (3000 *g* for 20 min) at OD_{550} of 6 and resuspended in ice-cold TN-Tween buffer (25 mM-Tris–HCl pH 7·4, 150 mM-NaCl, 0·05% Tween-80). The intracellular proteins were released by pulsed sonication for 6 min and the BB-dVP2 and BB proteins purified on a HSA–Sepharose affinity chromatography column as described by Nygren *et al.* (1988). Eluted fractions were collected according to A_{280} *and analysed by SDS–PAGE. Relevant fractions were pooled, the protein concentration determined by* A_{280} analysis, lyophilized and resuspended in 2 M-guanidine.HCl to a final protein concentration of approximately 100 μg/ml.

SDS–PAGE and Western blotting. SDS–PAGE was performed by standard methods using 4% stacking gels and 12% resolving gels. Purified virus (10 μg/cm gel) or *E. coli* containing recombinant VP2 fragments was solubilized by boiling for 2 min in sample buffer (62·5 mM-Tris–HCl, pH 6·8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0·001% bromophenol blue). Samples containing guanidine.HCl (6 M or 2 M) were diluted 10-fold in pre-heated (98 °C) sample buffer, boiled for 2 min and loaded onto a pre-heated polyacrylamide gel (100 V for 10 min at room temperature). The gels were stained by standard methods using Coomassie brilliant blue or silver nitrate, or soaked for 30 min in electroblotting buffer (0·25 M-Tris–HCl pH 8·3, 0·192 M-glycine, 20% methanol) and electroblotted onto nitrocellulose (NC) sheets (0·45 μm; Schleicher & Schuell). Following post-coating of the NC sheets with 3% (w/v) dry milk (Nestlé non-fat instant milk) in TBS-Tween (25 mM-Tris–HCl, pH 7·4, 2·5 mM-KCl, 0·135 M-NaCl and 0·05% Tween-20) for 60 min at room temperature, the NC sheets were incubated overnight at 4 °C with hybridoma cell culture supernatant. For comparison of virus strains, biotin-labelled goat anti-mouse secondary antibodies (Amersham), diluted 1:200 were incubated at room temperature for 60 min before addition of peroxidase-conjugated streptavidin (Amersham) diluted 1:3000, and a further 30 min incubation. The NC sheets were developed for 10 min with peroxidase substrate (0·1% diaminobenzidine in 0·5 M-Tris–HCl pH 7·5 and 0·1% H_2O_2), and the reaction stopped by washing with distilled water. For analysis of recombinant VP2 fragments alkaline phosphatase (AP) conjugated goat anti-mouse antibodies (Bio-Rad) diluted 1:2000 were incubated at room temperature for 60 min. Colour development was performed using the AP conjugate substrate kit (Bio-Rad) according to the supplier's instructions.

All antibody dilutions were made in TBS-Tween containing 1% (w/v) dry milk and all antibody incubations were followed by washing four times with TBS-Tween for 10 min at room temperature.

Sandwich ELISA and competitive binding assay. Sandwich ELISA experiments with MAb AS-1 were performed as described by Melby &

Table 2. Analysis of epitopes on aquatic birnaviruses with VP2 specific monoclonal antibodies by Western blotting (WB), virus neutralization assay* (Neut) and ELISA†

Virus strain‡	Monoclonal antibodies														
	H8			B9			F2			AS-1			E4		
	ELISA	WB	Neut	ELISA	WB	Neut	ELISA	WB	Neut	ELISA	WB	Neut	ELISA	WB	Neut
VR-299 (A1)	−	+	−	−	−	−	+++	+	+	+	ND	+	++	+	ND
West Buxton (A1)	++	+	+++	−	+	−	+++	+	+	++	ND	+	++	+	ND
Sp (A2)	+++	+	+	+	+	+	+++	+	+	+	ND	++	+++	−	ND
Ab (A3)	−	+	−	−	+	−	++	+	+	++	ND	+	++	+	ND
Hecht (A4)	−	+	−	−	−	−	+	+	+	−	ND	−	+	+	ND
Tellina (A5)	−	−	−	−	−	−	+	−	−	−	ND	+	+	−	ND
Canada-1 (A6)	−	+	−	−	+	−	++	+	+	+++	ND	+	++	+	ND
Canada-2 (A7)	−	+	−	−	+	−	−	+	+	++	ND	+	+	−	ND
Canada-3 (A8)	−	+	−	−	+	−	−	+	+	++	ND	+	+	−	ND
Jasper-Dobos (A9)	++	+	+++	−	+	−	++	+	+	++	ND	++	+	+	ND
N1 (A10)	+++	+	+++	+++	+	++	+++	+	+	+++	−	+	+++	+	−

* The maximum dilution giving 50% reduction in plaque forming units were > 1:1000 (+ + +), between 1:100 and 1:1000 (+ +) or < 1:100 (+).
† The ELISA results except for MAb AS-1 are based on Melby & Christie (1994). The relative recognition of virus were quantified to be more than eight times (+ + +), between four and eight times (+ +), between two and four times (+) or less than two times the control value.
‡ The numbers in the parentheses correspond to the proposed serotyping of aquatic birnaviruses.
ND, Not done.

Christie (1994) using the N1 strain of IPNV as antigen. For competitive binding analysis of the MAbs excess of an unlabelled MAb (hybridoma medium) was incubated overnight at 4 °C, followed by a 3 h incubation at room temperature with a sub-saturating concentration (approximately 3 μg/ml) of a purified biotin-labelled MAb, and a 30 min incubation with peroxidase-conjugated streptavidin (Amersham) diluted 1:3000. The positive controls were incubated with 100 μl PBS-Tween (10 mM-Na_2HPO_4, 1·75 mM-KH_2PO_4, 150 mM-NaCl, 0·05% Tween-20) containing 1% dry milk instead of excess of unlabelled competitive MAb.

Neutralization plaque reduction assay. The dilution of the individual MAbs giving 50% plaque reduction after 1 h incubation with virus was determined. The concentration of infectious virus (p.f.u.) was estimated by a plaque assay using agarose with a gelling temperature less than 28 °C (SeaPlaque; FMC-BioProducts). Virus suspension (100 μl) diluted in Eagle's MEM (Flow) was placed centrally on a monolayer of CHSE-214 cells in a 26 × 33 mm tissue culture plate well (Nunc) and incubated at 20 °C with 0·5% CO_2. After 1 h, 5 ml growth medium with reduced fetal bovine serum concentration (2% v/v) containing 0·5% (w/v) solubilized agarose and equilibrated to 22 °C, was added. Following a further 36 h incubation, the cells were stained with 2 ml 0·001% neutral red in 0·15 M-NaCl for 2 h, and the plaques were counted.

Dot blot analysis with MAbs and deletion fragments of VP2. Purified *E. coli*-expressed VP2 fragments 1 μl (approx. 100 ng) and BB control antigen were dotted onto NC strips, air dried for 30 min and washed with TBS for 10 min at room temperature. CHSE-214 culture medium (1 μl) with propagated N1 virus strain diluted 1:3 in 6 M-guanidine-HCl (denatured) or in 16 mM-Tris–HCl pH 7·4 (native), were dotted onto the NC as positive control antigens. Protein immobilization was demonstrated using Colloidal Gold Total Protein Stain (Bio-Rad) according to the supplier's instructions. Post-coating, incubation with hybridoma cell culture supernatant, AP conjugate incubation and colour development were performed as described for Western blotting.

Sequence analysis. Jameson–Wolf antigenicity index analysis was performed using GCG package software (Madison, WI, USA).

Results

Characterization of epitopes on aquatic birnavirus strains

Table 2 shows the results of Western blotting and virus neutralization analyses of selected virus strains representing the ten proposed serogroup A serotypes of aquatic birnaviruses, with MAbs. Table 2 also shows the ELISA results with MAb AS-1 and for comparison the ELISA results with MAb's H8, B9, F2 and E4 previously reported elsewhere (Melby & Christie, 1994).

The neutralizing MAb AS-1 reported to react with all tested serogroup A virus strains by dot blot (Caswell-Reno *et al.*, 1989) recognized all tested virus strains except Hecht (A4) and Tellina (A5) by ELISA, and neutralized all virus strains except Hecht (A4). MAb F2 both neutralized and reacted by Western blotting with all virus strains except Tellina (A5), although it did give a weak reaction with this virus strain by ELISA. On the other hand, although MAb F2 neutralized Canada-2 and -3 (A7 and A8), these virus strains were not reactive by ELISA.

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Table 3. *Percentage binding inhibition of labelled VP2 specific MAbs to the N1 strain of IPNV by unlabelled VP2-specific MAbs in competitive ELISA**

Competitive MAb	Labelled MAb		
	H8	B9	F2
H8	95	16	17
B9	32	96	48
F2	38	62	76
AS-1	13	10	37
E4	4	8	6

* Based on three experiments.

MAbs H8 and B9 neutralized all virus strains recognized by ELISA. MAb H8 neutralized the virus strains West Buxton (A1), Sp (A2), Jasper-Dobos (A9) and N1 (A10), while MAb B9 only neutralized the Sp (A2) and the N1 (A10) virus strains. However, in Western blotting MAb H8 recognized all virus strains except Tellina (A5) and MAb B9 recognized all virus strains except Hecht (A4) and Tellina (A5). The non-neutralizing VP2-specific MAb E4, which recognized all tested serogroup A strains by ELISA, did not recognize the Sp (A2), Tellina (A5), Canada-2 (A7) and Canada-3 (A8) virus strains by Western blotting.

Competitive binding of virus neutralizing MAbs

Table 3 shows the results of competitive binding ELISA performed to determine whether the virus neutralizing MAbs were independent of each other and of the VP2-specific non-neutralizing MAb E4. Pre-incubation with MAb H8 showed a very limited effect on the binding of the labelled MAbs B9 (16%) and F2 (17%) to the N1 (A10) virus strain. However, MAb B9 partly blocked the binding of both MAb H8 (32%) and F2 (48%), and MAb F2 blocked the binding of MAb B9 (62%) very effectively relative to the blocking effect of MAb F2 on itself (76%). Furthermore, pre-incubation with MAb F2 partly blocked the binding of H8 (38%) to the virus. MAb AS-1 partly blocked the binding of MAb F2 (37%), but had little effect on MAb B9 (10%) and MAb H8 (13%). Pre-incubation with the non-neutralizing

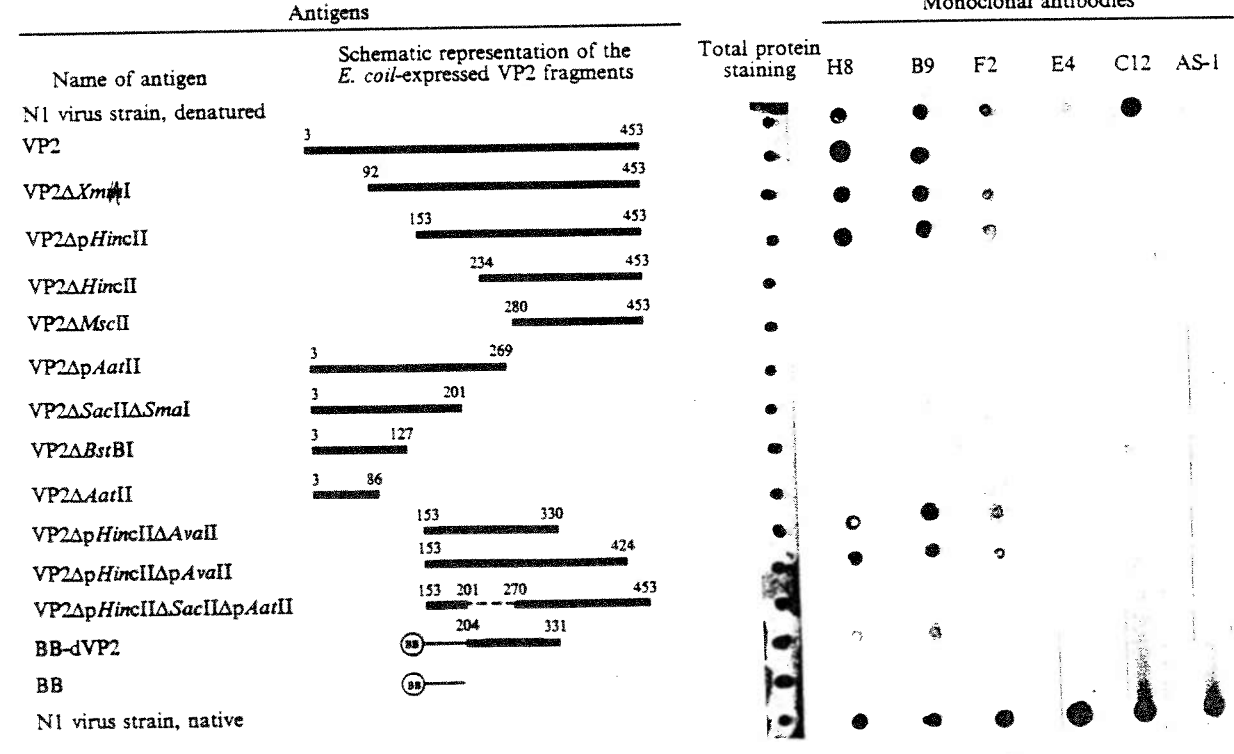

Fig. 2. Dot blot analysis of IPNV and purified recombinant deletion fragments of VP2 with MAbs against the N1 strain of IPNV. The VP3-specific MAb C12 was used as a negative control antibody and *E. coli*-expressed BB domain of streptococcal protein G as negative control antigen. All recombinant polypeptides were solubilized in 2 M-guanidine.HCl. N1 virus-infected CHSE-214 medium in 2 M-guanidine.HCl (top) or in buffer (bottom) were used as positive control antigens. Immobilization of the individual antigens on nitrocellulose was demonstrated by total protein staining. The numbers indicate the N- and C-terminal VP2 amino acids in the *E. coli*-expressed VP2 fragments (black bars), and correspond to the amino acid numbers of VP2 of the N1 virus strain. The dotted line in antigen VP2ΔpHincIIΔSacIIΔpAatII indicates the internal VP2 deletion made in this VP2 fragment.

MAb E4 showed no competitive binding for any of the virus-neutralizing MAbs tested.

Expression of VP2 fragments in Escherichia coli

The VP2-encoding cDNAs of the expression plasmid constructs described in Table 1 were successfully over-expressed in *E. coli*. With exception of the deletion fragment VP2ΔpAatII, which showed the lowest level of expression in *E. coli*, protein purification resulted in approximately 70–95 % pure deletion fragments of VP2, determined by SDS–PAGE and silver staining (data not shown).

Mapping of MAb specificity by reaction with deletion fragments of VP2

The recognition of recombinant deletion fragments of VP2 by MAbs was analysed by Western blotting and dot blot analysis. There were no differences in the qualitative reaction pattern of the individual virus-neutralizing MAbs with the VP2 fragments in the Western blotting (data not shown) compared to the dot blot analysis (Fig. 2). Following deletion of amino acids (aa) 153–234 at the N-terminal part (antigen VP2Δ*Hinc*II) or aa 270–453 at the C-terminal part (antigen VP2Δp*Aat*II) of VP2, the virus-neutralizing MAbs H8, B9 and F2 were all unable to bind these deletion fragments. Internal deletion of aa 201–270 (antigen VP2Δp*Hinc*IIΔ*Sac*IIΔp*Aat*II) also abolished the binding of the virus neutralizing MAbs H8, B9 and F2.

The smallest VP2 fragment recognized by the MAb's H8 and B9 was antigen BB-dVP2 (aa 204–331), and the smallest VP2 fragment recognized by the MAb F2 was antigen VP2Δp*Hinc*IIΔ*Ava*lI (aa 153–330). The weaker binding of MAb F2 in the dot blot analysis compared to MAbs H8 and B9 could not be detected by Western blotting (data not shown). The binding of the MAbs F2 and E4 to denatured virus was very weak compared to binding to native virus (Fig. 2). MAb E4 was unable to bind any recombinant VP2 fragments in the dot blot analysis but recognized antigen VP2 (aa 3–453) in Western blotting (data not shown). The conserved virus-neutralizing MAb AS-1 was only able to bind to native virus in the dot blot analysis. No binding of any MAb to the control antigen (BB) or binding of the VP3-specific control MAb C12 to any VP2 deletion fragments were detected.

Amino acid sequence analysis

Eleven regions with a theoretical Jameson–Wolf antigenicity index of 1·5 or more were identified within the VP2 amino acid sequence (aa 1–500) of the N1 virus strain (Fig. 3). All 11 regions were located between aa 149 and 390.

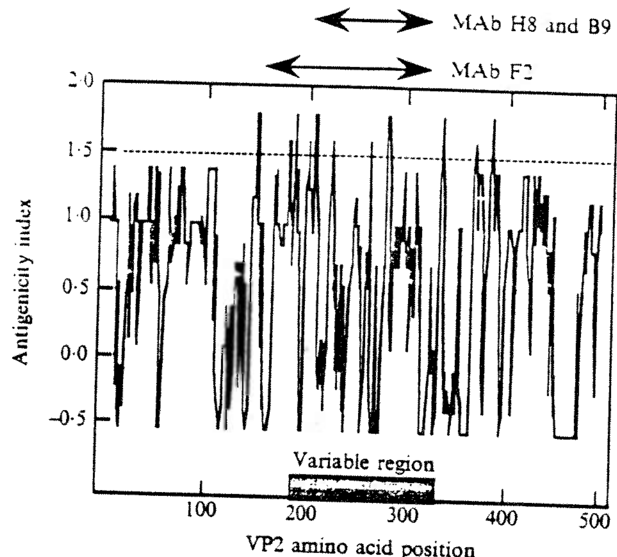

Fig. 3. Jameson–Wolf antigenicity index of the VP2 amino acid sequence of the N1 strain of IPNV compared to the minimum region of VP2 recognized by MAbs against variable (H8 and B9) and conserved (F2) neutralization epitopes and to the variable region of VP2 (hatched box) identified by Håvarstein *et al.* (1990).

Discussion

Our results demonstrate that the central third of VP2 of the serogroup A strains of IPNV contains at least three partly overlapping neutralization epitopes, two variable and one conserved. The variable epitopes B9 and H8 depend on amino acids between 204 and 330 which correspond to almost the entire variable region of VP2 identified by Håvarstein *et al.* (1990) (Fig. 3). The conserved neutralization epitope F2 depends not only on the region between amino acid 204 and 330 but also on some amino acids between 153 and 203. Deletion of amino acids 153 to 234 or 270 to 330 hindered binding of the MAbs H8, B9 and F2 to the deletion fragments of VP2 (Fig. 2), demonstrating the conformation-dependent nature of these epitopes. However, since all these MAbs recognized denatured VP2, the epitopes must easily renature to a form recognizable by the MAbs. The conserved and conformation-dependent epitopes AS-1 and E4 were not possible to map since no binding to VP2 deletion fragments could be detected. However, MAb AS-1 showed some competitive binding with the F2 MAb indicating that the F2 and the AS-1 epitope or parts of them merge into each other. The competitive binding assay further indicated that the neutralization epitopes H8, B9 and F2 are overlapping and that they are independent of the conserved non-neutralization epitope E4.

The results of the Jameson–Wolf antigenicity index sequence analysis also indicate that the central third of VP2 contains the dominant epitopes of VP2. Of the 11 regions within VP2 with a theoretical Jameson–Wolf antigenicity index of 1·5 or more, seven were located within the F2 epitope region which consists of approximately 35% of the VP2 sequence.

As far as we know regions of VP2 outside the variable region have not yet been shown to be part of any neutralization epitopes of birnaviruses. For infectious bursal disease virus (IBDV), a birnavirus pathogenic to chickens, only variable epitopes have been mapped (Azad *et al.*, 1987; Schnitzler *et al.*, 1993). Conserved epitopes of IBDV, like the AS-1 epitope of IPNV, appear to be highly conformation-dependent (Snyder *et al.*, 1988, 1992) and therefore difficult to map. Recently Vakharia *et al.* (1994) reported major amino acid variation within the variable region of VP2 between neutralizing MAb escape strains of IBDV. In spite of major sequence variation within the variable region of these virus strains, one conserved neutralization epitope was demonstrated for all virus strains, indicating that regions outside the variable region may be part of this epitope.

Previously we reported that the IPNV-neutralizing MAbs H8 and B9 were unable to recognize the Sp (A2) virus strain (Christie *et al.*, 1990). Later other clones of the same passage of the Sp (A2) type strain have shown positive results with MAb H8 and variable results with MAb B9 (Melby & Christie, 1994). This further demonstrates the high variability of epitopes H8 and B9. Although the Sp (A2) and the N1 (A10) virus strains are serologically related, the results from the characterization of epitopes on aquatic birnavirus strains presented in Table 2 demonstrate that they are not identical. Of the European virus strains only the two related virus strains Sp (A2) and N1 (A10) contain the variable H8 epitope. It was therefore surprising that the H8 epitope is present on the Canadian Jasper-Dobos (A9) and the American West Buxton (A1) virus strains. The amino acid sequences of the N1 (A10) and the Jasper-Dobos (A9) virus strains show a high degree of variation within the H8 epitope region (Håvarstein *et al.*, 1990) while the Jasper-Dobos (A9) and the West Buxton (A1) are believed to belong to the same genogroup (Heppell *et al.*, 1993). Furthermore, both MAb H8 and B9 recognized a greater number of virus strains by Western blotting when compared with ELISA and neutralization assays. This indicates that denaturation induces exposure of internal epitopes or change in conformation. Consequently, although located within a highly variable region, the heterogeneity of some epitopes of aquatic birnaviruses appears to depend on conformation variation, which can be induced by minor changes in the amino acid sequence. Major changes in the amino acid sequence are usually a consequence of evolution and would therefore not be as effective a mechanism for viruses to escape neutralizing antibodies (antigenic drift) as conformation variation following minor changes in the amino acid sequence. Recently, Pryde *et al.* (1993) identified only two amino acid variations within the variable region of a Scottish Sp (A2) virus strain compared to the N1 (A10) virus strain, both located at the periphery of the H8–B9 epitope region. For IBDV, sequence analysis of neutralization escape variants of IBDV has proven that minor amino acid variations may induce neutralization escape variants (Lana *et al.*, 1992; Vakharia *et al.*, 1994).

Virus-neutralizing MAbs that bind VP2 of aquatic birnaviruses by Western blotting have also been reported by others and the epitopes suggested to be of linear nature (Tarrab *et al.*, 1993). However, epitopes which are recognized by MAb following denaturation by, for instance, SDS–PAGE and Western blotting are not necessarily linear. In fact, specific procedures to increase binding of MAb's to conformation-dependent epitopes following Western blotting have been developed (Dunn, 1986). The neutralization epitope defined by MAb AS-1 and common to all serogroup A virus strains except Hecht (A4) appears unable to spontaneously renature, indicating that the conformation of epitope AS-1 on the virus capsid is of a more complex nature than that of epitopes H8, B9 and F2.

With regard to a candidate subunit vaccine against IPN in fish, the F2 epitope should be an important component since it is common to all serogroup A strains of IPNV. The Tellina strain (A5), which is not neutralized by MAb F2, is an aquatic birnavirus isolated from molluscs and is not known to cause IPN in fish. Furthermore, a structure resembling the epitope F2 is present on recombinant VP2.

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11 November 1994

Dear Dr Frost,

MANUSCRIPT 12953/94 ENTITLED : MAPPING OF NEUTRALIZATION EPITOPES ON INFECTIOUS PANCREATIC NECROSIS VIRUSES (IPNV) (Resubmission of manuscript 12743/94)

Your manuscript has now been evaluated by two independent referees.

I am happy to say that both referees found this manuscript to be a significant improvement of the previous submission and have requested only minor modifications, referee 1 by annotations of the manuscript (enclosed), referee 2 in his comments.

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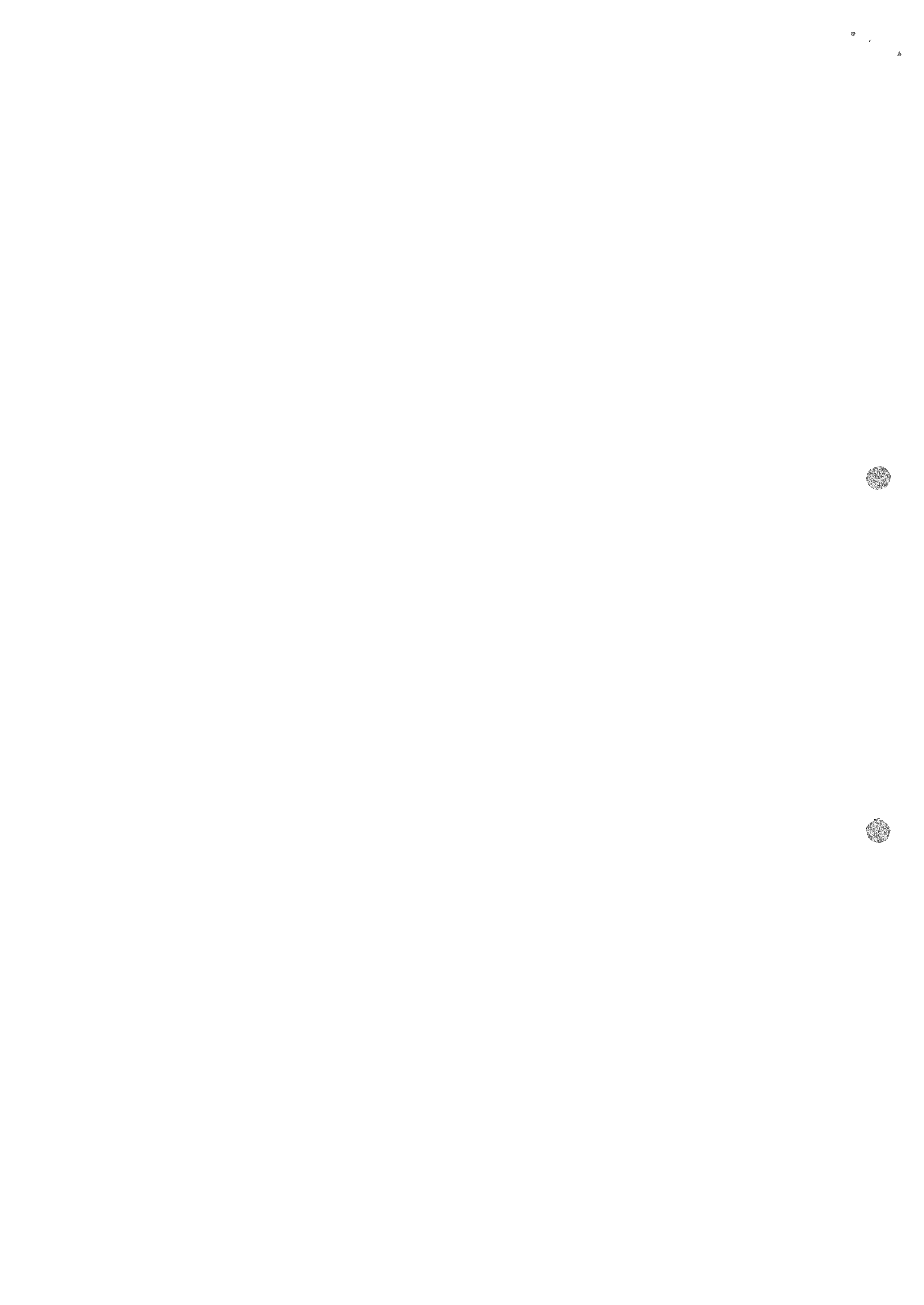

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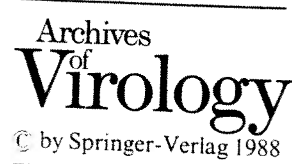

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Characterization of a new serotype of infectious pancreatic necrosis virus isolated from Atlantic salmon

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Summary. Virus particles isolated from hatchery reared fish with infectious pancreatic necrosis (IPN) were neutralized by homologous immune sera but not by immune sera raised against IPN virus serotype 1, 2, and 3. This virus isolate, called the N 1 strain, was detected in one year old Atlantic salmon (*Salmo salar*) during an outbreak with histopathological lesions of IPN and slightly increased mortality. The polypeptide pattern of N 1 virus differed markedly from that of the three classical IPN virus serotypes.

Double stranded RNA isolated from the N 1 virus particles, co-migrated during agarose gel electrophoresis with nucleic acid isolated from the IPN virus Jasper and Ab strains. Nucleic acid hybridizations using low stringency washing conditions and a synthetic DNA oligonucleotide probe (representing the 3′ end of the A segment of the Jasper strain) gave positive results with the IPN virus Jasper, Ab, Sp, and N 1 strains.

The results presented in this paper show that the N 1 isolate differs immunologically and biochemically from the IPN virus serotypes 1, 2, and 3 and may represent a new serotype of IPNV.

Introduction

Infectious pancreatic necrosis (IPN) is a well characterized acute disease of young hatchery reared brook trout (*Salvelinus fontinalis*) [28, 31] and rainbow trout (*Salmo gairdneri*) [27] first described in 1940 in Canada [26]. The viral etiology of IPN was confirmed by Wolf in 1960 [29], who isolated the IPN virus (IPNV) strain VR 299. The virus may also infect other kinds of salmonid fish [13] and now has a worldwide distribution. IPNV has been isolated from Atlantic salmon (*Salmo salar*), but the pathogenetic significance of these infections is not clear. The last two years several epizootics of IPN and subsequent mortality in the range of 10–20 per cent have been observed in Norwegian fish farms during the period of smoltification [H. O. Djupvik, pers. comm.].

IPNV is a member of the virus family *Birnaviridae* [2], which also includes viruses infecting other fish species, eel, molluscs as well as domestic fowl and drosophila [8]. The nomenclature of these viruses has not been made clear yet. In the first IPNV report from Europe [1] a strain serologically different from the VR 299 strain was described [30]. In 1971 two IPNV strains (Ab and Sp), both serologically different from the VR 299 strain, were isolated from rainbow trout in Denmark [18]. Since then, several isolates of IPNV which differ immunologically or biochemically from VR 299, Ab, and Sp, have been reported [3, 11, 14, 17, 25]. Some cross-reaction between all the IPNV strains is observed using cross neutralization assay. At present the most accepted serotyping of IPNV in fish includes three serotypes with the reference strains: ATCC VR 299 (West Buxton) (serotype 1), Ab (serotype 2), and Sp (serotype 3) [24].

In Norway serotype 2 and 3 have been isolated from farmed rainbow trout [16] and Atlantic salmon [J. Krogsrud, pers. comm.]. IPNV serotype 1 has not been detected in Norway.

The molecular biology of IPNV has been studied in detail for serotype 1 (VR 299, Jasper) [4–7, 9, 10, 23]. The IPNV genome contains two dsRNA segments [5, 12, 23]. The A segment encodes the structural proteins (preVP 2 and VP 3) and a non-structural protein (NS). The B segment encodes the RNA-polymerase (VP 1). Molecular cloning and sequencing of the Jasper strain genome have been performed and the cDNA sequence of the A segment was reported lately [10].

SDS-PAGE analysis of purified IPNV shows three major virus polypeptides, VP 1 with molecular weight (MW) 90–105 kD (VP 105), VP 2 MW 50–57 kD (VP 54), and VP 3 MW 29–31 kD (VP 31). In addition, one minor protein VP 4 MW 28–29 (VP 29), probably a degradation product of VP 3, is detected in virus of serotype 1 [24]. This polypeptide is especially abundant in purified Jasper virus. VP 4 may be demonstrated in purified Sp virus [12, 15] but has not been detected in Ab virus.

Polypeptide analysis of virus infected cells reveals seven virus specific polypeptides ICP 105, ICP 62, ICP 60, ICP 54, ICP 31, ICP 29, and ICP 25. Peptide map comparisons of the polypeptides [6] show that ICP 105 represents VP 1, the putative virion-associated RNA polymerase. ICP 65 and ICP 60 are precursor proteins of VP 2, the major capsid protein. Small amounts of the precursor proteins are also detected in purified virus preparations probably representing immature virus particles. ICP 31 is identical to VP 3 and VP 4 is a degradation product of VP 3. ICP 29 is identical to the NS protein and ICP 25 is a degradation product of NS.

The purpose of the present investigation was to carry out an immunological and biochemical characterization of the N 1 isolate.

Materials and methods

Fish samples

Atlantic salmon (*Salmo salar*) were obtained from hatcheries located on the west coast of Norway. The fish were transported on ice, and the kidneys were removed within 24 h post mortem and used directly for virus isolation.

Cells

Chinook salmon embryo cells (CHSE-214) (Flow) were grown at 20 °C in EMEM with 0.85 g/l $NaHCO_3$ supplemented with 10 per cent (vol/vol) fetal bovine serum (FBS), 1 per cent non-essential amino acids (Flow, 100 × concentrated), 1 per cent glutamine (Flow, 2 mM pr. ml), 1 per cent gentamycin (Schering, 10 mg pr. ml), and 0.5 per cent fungizone (Novo industri A/S, 0.25 µg pr. ml).

Virus

Two samples of the Ab reference strain were obtained from B. J. Hill (U.K.) and P.E.V. Jørgensen (Denmark). The Sp reference strain was obtained from Jørgensen. The Jasper strain, originally isolated from rainbow trout of the Jasper river in Alberta, Canada, was obtained from P. Dobos (Canada). The VR 299 and West Buxton strains were obtained from J. C. Leong (U.S.A.). IPNV strain N 1 was isolated from Norwegian Atlantic salmon collected during an outbreak of IPN.

Immune sera

Rabbits and mice were injected sub-cutaneously with purified virus (about 50 µg) in PBS containing 50 per cent Freund's complete adjuvants (Difco, England). The injection was repeated two weeks later and a booster injection without adjuvants was given intravenously another month later. The animals were bled weekly starting two weeks after the first injection.

Virus isolation and purification

The N 1 virus was isolated from kidney tissue suspention in PBS, homogenized, sterile filtered (0.22 µm), and diluted with EMEM containing 1 per cent gentamycin and 0.5 per cent fungizone. The concentration of virus was about 10^9 PFU/g of kidney tissue. Stock virus solutions were made from cells infected with 1 PFU/cell. The virus was stored at −70 °C in 50 per cent glycerol.

The viruses were propagated in CHSE-214 cells using 0.1 PFU/cell without addition of serum. Purification of virus was performed by a procedure based on a method described by Dobos [4]. Infected cells and medium were centrifuged at 4,000 × **g** at 4 °C for 15 min and the pellet was resuspended in 0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.3 (TNE buffer). The virus particles were first precipitated with 5 per cent polyethylene glycol (PEG) 20,000 in 2.2 per cent NaCl by stirring overnight at +4 °C and then centrifuged at 6,000 × **g** at +4 °C for 90 min. The pellet was solubilized in TNE buffer, sonicated 10 sec at 50 µA, and clarified by low speed centrifugation at 1,000 × **g** for 5 min. The PEG-concentrated virus was layered over a discontinuous gradient consisting of 1.5 ml of 40 per cent CsCl, 1.0 ml of 30 per cent CsCl and 0.5 ml of 20 per cent CsCl and centrifuged for 18 h at 35,000 rpm in a SW 50.1 rotor in a Beckman ultracentrifuge. The virus band was visualized by illumination with a Halogene lamp (Intralux 5000, Volpi AG) and removed by puncturing the tube.

Plaque-assay

Titration of virus was performed by plaque assay using agarose with a ultralow gelling temperature (<28 °C). A sample of 100 µl virus-suspension was placed centrally on 25 cm^2 cell monolayer previously washed with EMEM. Following adsorption for 1 h at 20 °C, 5 ml cell-culture medium containing 0.5 percent Agarose (SeaPlaque, FMC-Co. Rockland, Maine 04841) at 22 °C were added. After incubation for 48–72 h at 20 °C, the cells were stained with 2 ml 0.001% neutral red in 0.9 per cent saline. Cross neutralization plaque-reduction assay was performed by the method of Jørgensen [21].

Radiolabelling

Radiolabelled purified virus preparation was made from cells infected with 1 PFU/cell. The medium was exchanged 18 h post infection (p.i.) with methionine-free medium containing 16 µC ^{35}S-methionine/ml (Amersham) and virus particles were purified from the infected cell culture medium harvested two days p.i.

Radiolabelled virus infected cell preparation was made from cells infected with 5 PFU/cell. The medium was exchanged 22 h p.i. with methionine-free medium containing 20 µC ^{35}S-methionine/ml. Infected cells were washed with PBS and harvested 28 h p.i.

Electrophoresis

SDS-PAGE was performed by the method originally described by Laemmli [23]. The molecular weights of the polypeptides were calculated from their electrophoretic mobilities relative to ^{14}C-labelled standard proteins (Amersham) run in parallel.

Agarose gel electrophoresis was performed with naked dsRNA prepared by digesting purified virus (4–12 h) with proteinase K (0.2 mg/ml in TNE buffer containing 1 per cent SDS). The dsRNA was separated from the viral proteins by phenol-chloroform extraction, ethanol precipitated and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). The samples were run on a horizontal submarine 1.5 per cent agarose gel in TBE buffer (89 mM Tris, 89 mM Boric acid and 2 mM EDTA) and stained with ethidium bromide.

Preparation of the probe

Two oligonucleotides with the sequences given in Fig. 3 were synthetized. The oligonucleotide sequences, A (plus-strand) and B (minus-strand) are derived from the IPNV Jasper strain VP 3 sequence (bases 2679 to 2777) [10]. Oligonucleotide A and B were annealed and labelled with ^{35}P by primer extension, using Klenow's fragment of DNA polymerase I. The specific activity of the probes was approximately 5×10^8 dpm/µg.

Nucleic acid hybridization

The IPNV strains Jasper, Ab, Sp, and N 1 were used to infect a 75 cm^2 cell monolayer. Two or three days later when CPE was evident, the culture medium containing the virus was collected and centrifuged at 6,000 × g for 10 min. The virus was pelleted from the supernatant in a Beckman SW-27 rotor at 25,000 rpm for 150 min and suspended in a small volume of TNE buffer. Naked dsRNA was extracted in H_2O and denaturated by boiling for 5–10 min, and an equal volume of a solution consisting of 24 per cent formaldehyde, 50 mM EDTA and 0.5 M phosphate buffer pH 6.5 was added. The denaturation mixture was then incubated at 60 °C for 10 min. Three volumes of 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) were added and the samples were blotted onto a nitrocellulose membrane using a Minifold II slot-blot apparatus (Schleicher and Schuell). Nitrocellulose membranes were baked at 80 °C for 2 h to immobilize the RNA.

The hybridization was carried out overnight at 42 °C in sealed plastic bags. The hybridization solution contained 40 per cent formamide, 5 × SSC, 25 mM phosphate buffer pH 6.5, 0.1 (SDS), 1 × Denhardt's solution (0.02 per cent each of bovine serum albumine, polyvinylpyrrolidone and Ficoll-Pharmacia, Sweden) and 100 µg/ml of sheared salmon sperm DNA. The labelled oligonucleotide probe (10^6 dpm per ml hybridization solution) was added to the bag after boiling for 5 min and quenching on ice. Once the hybridization had been completed, the membranes were washed sequentially in 2 × SSC for 3 × 5 min at room temperature and 2 × 20 min at 48°. The membranes were exposed to Kodak XAR X-ray film at −80 °C for two days.

Results

By cross neutralization assay, 50% per cent PFU-reduction titre of rabbit anti-N 1 sera was 192,000 with homologous virus compared to 2000 or less with heterologous virus (Table 1). The neutralization titre of heterologous immune sera with N 1 virus was 12,000 for anti-Ab, 6,000 for anti-Sp, and 24,000 for anti-Jasper immune sera. The results indicate low cross reaction between the N 1 strain and the other IPNV strains. Very low neutralization titre of heterologous immune sera with Jasper virus was obtained. In contrast, high cross-reaction between the Ab and Sp strains was detected. Similar results were obtained by cross neutralization assay using immune sera raised in rabbit and mice.

Purified N 1 viral dsRNA contained two segments which co-migrated during agarose gel electrophoresis with the segments of the Jasper and Ab strains

Table 1. 50 per cent plaque-reduction titre of rabbit immune sera against the IPNV strains Ab, Sp, Jasper, and N 1

IPN virus strain	Anti-Ab	Anti-Sp	Anti-Ja	Anti-N 1
Ab	>256,000	56,000	4,000	1,000
Sp	>256,000	112,000	6,000	2,000
Ja	2,000	1,000	>256,000	1,500
N 1	12,000	6,000	24,000	192,000

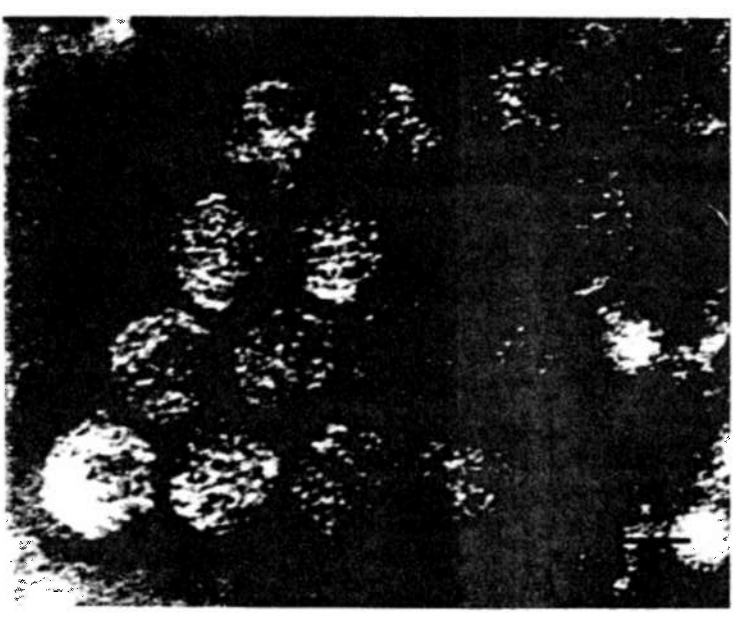

Fig. 1. Electron micrograph of 2 per cent potassium phosphotungstate stained IPN N 1 virus. Bar = 100 nm

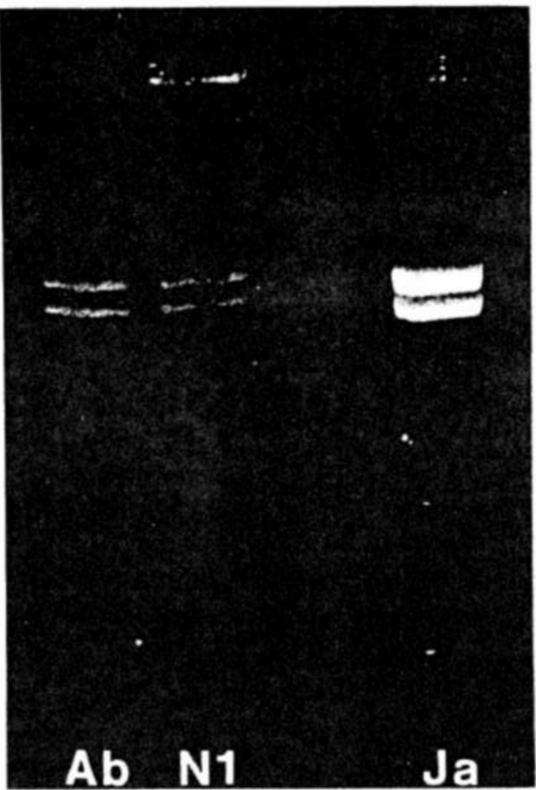

Fig. 2. Agarose slab gel electrophoresis of purified dsRNAs from the IPNV Ab, N 1, and Jasper (*Ja*) strains, stained with ethidium bromide

A

5'-TAC.TAC.ATG.ATA.ACG.GGC.AGA.GTC.CCA.

AAC.CCC.GGA.GAA.GAG.TAC.GAG.GAC.TAT.G-3'

B

3'-G.CTC.CTG.ATA.CAC.GCT.TTT.GGC.TAT.TGG.

GCT.GGT.TGG.CTG.TAC.CTG.TTT.TAG.TCT.-5'

Fig. 3. DNA sequences of the synthetic IPNV (Jasper) probe used for nucleic acid hybridization. **A** Plus strand sequence of segment A base number 2,679 to 2,733. **B** Negative strand sequence of segment A base number 2,723 to 2,777. The overlapping sequence of the probe is underlined

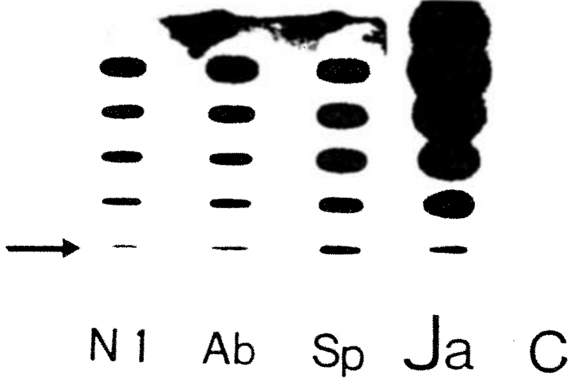

Fig. 4. Autoradiographs of nucleic acid hybridization of purified IPNV RNA of strains N 1, Ab, Sp, and Jasper using a ^{32}P-labelled synthetic IPNV Jasper probe. *C* RNA isolated from uninfected cells. The arrow represents application of approximately 70 pg of RNA

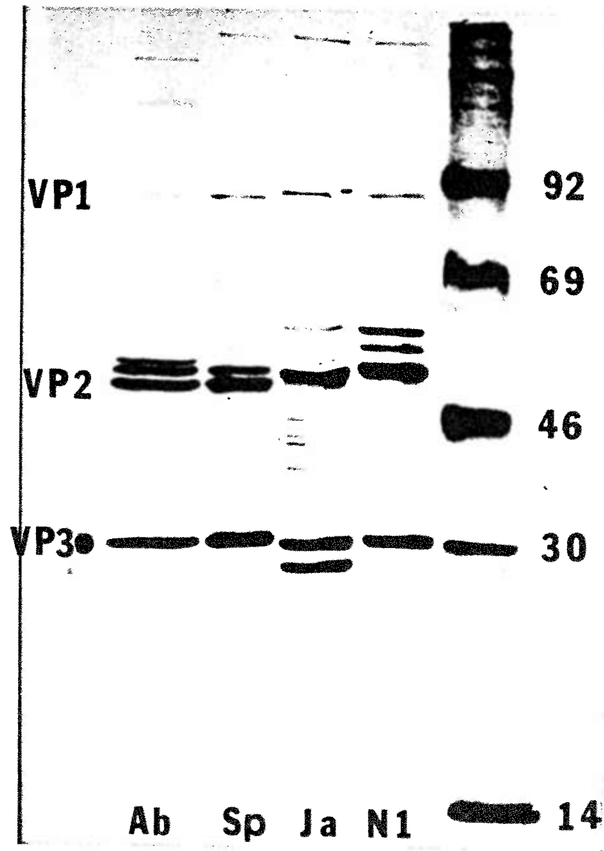

Fig. 5. Autoradiographs of ^{35}S-methionine-labelled IPNV polypeptides of purified virus strains Ab, Sp, Jasper, and N 1 analyzed on a 7.5–20 per cent SDS-PAGE gradient gel together with molecular weight marker proteins of 14, 30, 46, 69, and 92 kD

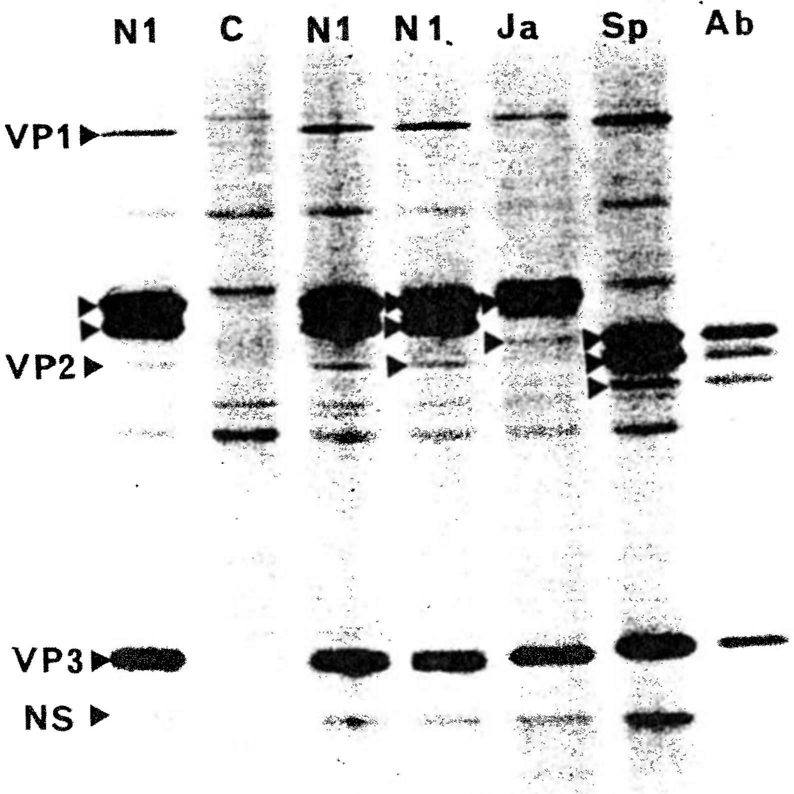

Fig. 6. Autoradiographs of non-infected cells (*C*) and cells infected with IPNV strains N 1, Jasper (*Ja*), Sp, and Ab analyzed on a 12.5 per cent SDS-PAGE gel. The cells were labelled with ^{35}S-methionine between 22–28 h p.i.

(Fig. 2). Nucleic acid hybridization, using a ^{32}P-labelled synthetic DNA-probe representing the Jasper VP 3 sequence (Fig. 3) and low stringency washing conditions, gave positive results with all IPNV strains tested (Fig. 4). Control RNA isolated from non-infected CHSE cells did not hybridize with the probe. The electronic microscopy (Fig. 1), the MW of the RNA-segments (Fig. 2) and the result of the hybridization assay (Fig. 4) show that the N 1 virus belongs to the birnavirus group.

During SDS-PAGE of radio-labelled purified virus two polypeptides with MW 92 kD (VP 1) and 31 kD (VP 3) co-migrated for all virus strains tested (Fig. 5). Cells infected with the Ab strain contained an additional polypeptide in the range of 90-100 kD (Fig. 6). This polypeptide may represent a dimer of VP 2 as Dobos has shown for the VR 299 strain [6].

The major structural virus protein (VP 2) was detected primarily in the purified virus preparations, and only minor quantities of this polypeptide were observed in virus infected cells (Fig. 6). The MW of VP 2 was 54 kD for the Jasper strain, 52 kD for the N 1 strain and 50 kD for the Ab and Sp (Figs. 5 and 6).

The precursor proteins of VP 2 were demonstrated primarily in virus infected cells (Fig. 6), but small amounts were detected in the purified virus preparations (Fig. 5). Cells infected with the three European IPNV strains contained two precursor proteins of VP 2 (Figs. 5 and 6). The MWs of these polypeptides were about 60 and 62 kD for the N 1 strain and 55 kD and 54 kD for the Ab and Sp strains. However, only one polypeptide, representing preVP 2, was detected in cells infected with Jasper virus. The MW of this polypeptide was about 62 kD.

A polypeptide with MW about 29 kD was demonstrated in virus-infected cells for all IPNV strains tested. This polypeptide represents NS, the non-structural virus protein. Purified Jasper virus contained an additional polypeptide not detected in the other virus strains. This polypeptide (VP 4) has a MW about 29 kD and is a degradation product of VP 3.

A minor band representing a polypeptide with MW below 29 kD was detected in purified virus of all strains tested. The MW of this polypeptide was about 24 kD for the Jasper strain, 25 kD for the N 1 strain and 26 kD for Ab and Sp (Figs. 5 and 6).

Discussion

Our isolate of IPNV, called the N 1 strain, was isolated from Atlantic salmon during an episode of increased mortality in fish with histopathological lesions of IPN.

The low cross reaction obtained by cross neutralization assay between the N 1 strain and the Jasper, Ab, and Sp strains shows that the N 1 strain differs serologically from the three classical serotypes of IPNV. In addition, recent experiments with the IPNV serotype 1 strains West Buxton and VR 299 show that these viruses are not neutralized by immune serum raised against the IPNV N 1 strain. These results indicate that the N 1 strain represents a new serotype of IPNV.

The polypeptide pattern of N 1 virus differed from that of the three other IPNV strains. The MW of VP 2 was larger for the N 1 strain than for the Ab and Sp strain, but smaller than for the Jasper strain. Cells infected with the N 1 strain contained two preVP 2 compared to one preVP 2 detected in cells infected with the Jasper strain. Purified N 1 virus particles did not contain VP 4 which was present in purified Jasper virus particles. All the IPNV strains tested contained a minor polypeptide, probably representing a degradation product of NS. The MW of this polypeptide was smaller for the N 1 virus than for the other IPNV strains. Recent analysis of the IPNV strains West Buxton and VR 299 show that the size of VP 2 for these strains is identical to the size of Jasper VP 2 (data not shown). The different protein pattern observed for N 1 virus, strongly supports the assumption that this represents a new serotype of IPNV.

Complete cross reaction between the Ab and Sp strains was observed by cross neutralization assay. The polypeptide patterns of these strains also were identical. However, phenotypic differences between the Ab and Sp strains may

be demonstrated by a 10 fold reduction in virus yield and about 50% reduction in the plaque diameter obtained with the Ab virus compared to the Sp virus (data not shown). Serological cross reaction between the Ab and Sp has been reported earlier [19] and is supposed to depend on the immunization route [20]. However, our results using both immunological and biochemical analysis suggest that the Ab and Sp strain may be very closely related.

A new serotype of IPNV isolated in Canada has been proposed based on examinations using monoclonal antibodies against the IPN West Buxton strain (serotype 1) [3]. No further characterization of this isolate has been reported. Comparative studies of this virus isolate and the N 1 isolate will be of great interest.

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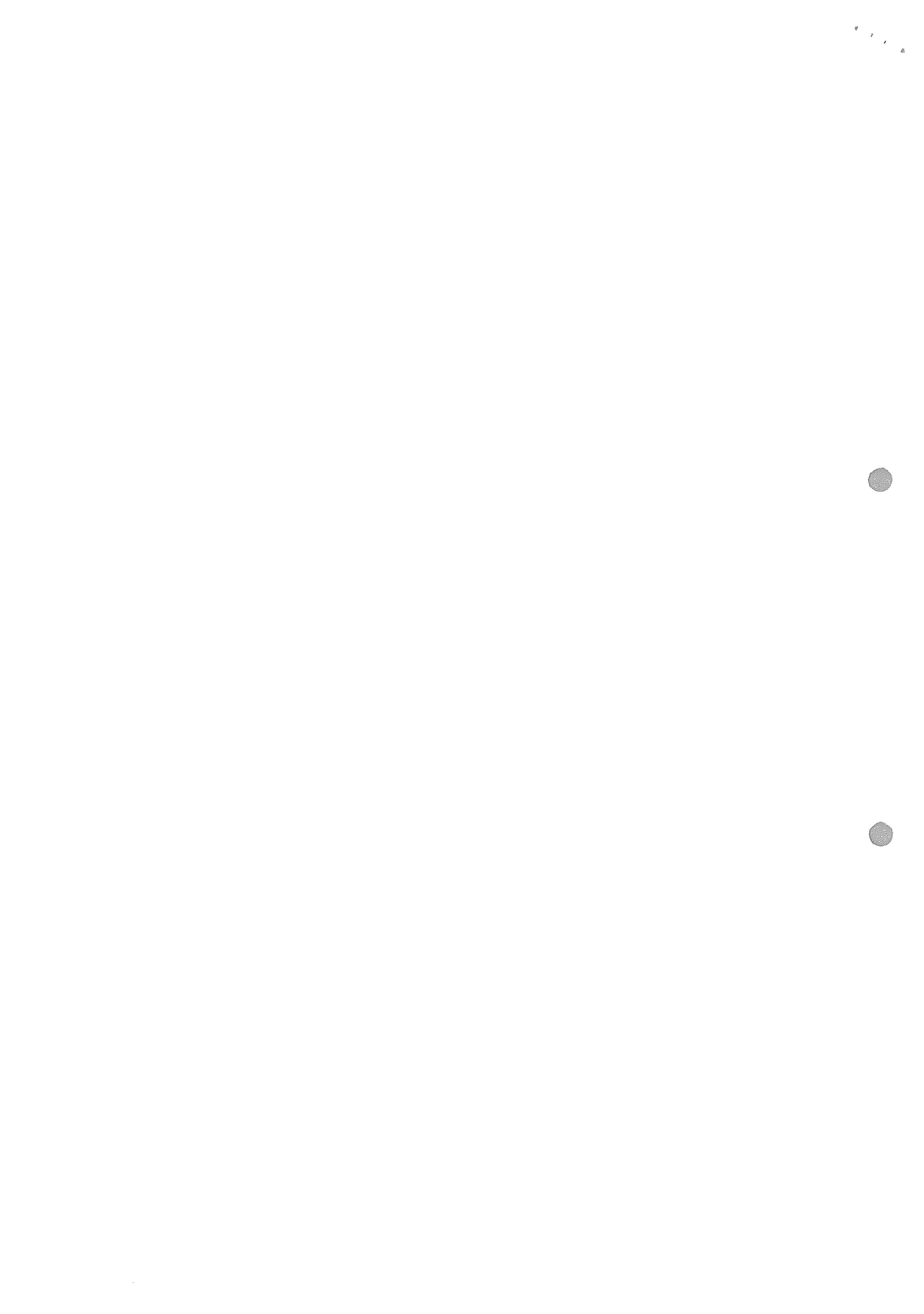

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Sequence of the large double-stranded RNA segment of the N1 strain of infectious pancreatic necrosis virus: a comparison with other *Birnaviridae*

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The cDNA sequence of the large dsRNA segment (segment A) of the N1 strain of infectious pancreatic necrosis virus (IPNV) has been determined. The nucleotide and deduced amino acid sequences were compared to the sequences of segment A of the Jasper strain of IPNV and to the sequences of segments A and B (5′ and 3′ flanking regions) of the 002-73 strain of infectious bursal disease virus (IBDV). The comparison demonstrated that the precursor protein of the major structural polypeptide, pVP2, is highly conserved at the N and C termini, whereas the amino acid sequence of an internal segment shows greater diversity between the strains. This internal segment probably carries the serotype-specific epitopes of birnaviruses. An alternative open reading frame (ORF) (444 bp) partly overlapping with the large ORF (2916 bp) of segment A was found to be conserved among the IPNV strains and is probably also present in the 002-73 strain of IBDV. This small ORF may encode a novel birnavirus polypeptide with an M_r of 17K. SDS–PAGE of radiolabelled purified IPNV particles revealed a band corresponding to the possible novel 17K polypeptide. Short terminal inverted repeats are found in segment A of the N1 and Jasper strains of IPNV and in segment B of the 002-73 strain of IBDV. Segment A of IPNV and segment B of IBDV also contain adjacent inverted repeats at their 5′-terminal flanking regions.

Introduction

Infectious pancreatic necrosis virus (IPNV) is the prototype of a family of viruses called the *Birnaviridae* (Dobos *et al.*, 1979). Birnaviruses also include infectious bursal disease virus (IBDV) of domestic fowl (Müller *et al.*, 1979) and drosophila X virus of *Drosophila melanogaster* (Teninges *et al.*, 1979). IPNV causes an acute, contagious disease in juvenile salmonids, e.g. brook trout (*Salvelinus fontinalis*) and rainbow trout (*Salmo gairdneri*) (Hill, 1982). IBD is a highly contagious disease of young chickens, characterized by the destruction of the lymphoid cells in the bursa of Fabricius (Chevill, 1967).

Several strains of IPNV and IBDV with different immunological and/or biochemical properties have been described (Becht *et al.*, 1988; Caswell-Reno *et al.*, 1986; Kibenge *et al.*, 1988). In this article only the birnavirus strains that have been characterized by cDNA cloning and nucleotide sequence analysis will be considered. In addition to the N1 strain, these include the Jasper strain of IPNV (Duncan & Dobos, 1986) and the Australian IBDV strain 002-73 (Azad *et al.*, 1985; Hudson *et al.*, 1986; Morgan *et al.*, 1988).

Birnaviruses possess a bisegmented, dsRNA genome contained within an unenveloped, icosahedral capsid (Dobos & Roberts, 1983). The larger genome segment A (approx. 3100 bp) of IPNV (N1 and Jasper strains) encodes three proteins in a single large open reading frame (large ORF): the 60K to 62K precursor (pVP2) of the 52K to 54K major structural protein VP2, the 29K non-structural protein (NS) and the 31K minor structural protein VP3 (Duncan *et al.*, 1987; Nagy *et al.*, 1987). The corresponding M_r of the segment A proteins of IBDV (strain 002-73) are 50K to 60K (precursor to VP2), 41K to 37K (VP2), 29K (VP4) and 32K (VP3) (Fahey *et al.*, 1985; Kibenge *et al.*, 1988). The smaller B segment (approx. 2900 bp) of birnaviruses encodes a single gene product (VP1) with an M_r of approximately 90K, presumed to be the viral RNA polymerase (Gorbalenya & Koonin, 1988; Morgan *et al.*, 1988). Nucleotide and peptide sequence analyses have shown that the large ORF of the A segment of IPNV is monocistronic and encodes a polyprotein in which three viral polypeptides are arranged in the order N–pVP2–NS–VP3–C (Duncan & Dobos, 1986; Hudson *et al.*, 1986; Nagy *et al.*, 1987). However, the precise borders of the three coding regions have not yet been defined (Duncan *et al.*, 1987). The same applies to IBDV, apart from the fact that the protein equivalent to pVP2 is called VPX and the protein equivalent to NS is called VP4 (Kibenge *et al.*, 1988).

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VP4 is involved in the processing of the precursor polyprotein (large ORF gene product), in cleaving between VPX and VP4 and between VP4 and VP3 (Azad *et al.*, 1987; Jagadish *et al.*, 1988).

The Jasper strain of IPNV was originally isolated from rainbow trout of the Jasper river in Alberta, Canada. Recently we isolated a new serotype of IPNV from Atlantic salmon (*Salmo salar*) (Christie *et al.*, 1988). This serotype, called the N1 strain, was detected in young hatchery-reared salmon from western Norway. We report here the cDNA cloning of the N1 strain and sequencing of the A segment of the N1 genome. The nucleotide and deduced amino acid sequences were compared to the corresponding sequences of the Jasper and the 002-73 strains. We also present results indicating that a conserved ORF, overlapping with the N-terminal part of VP2, encodes a fifth birnavirus polypeptide (VP5).

Methods

Cells and viruses. The culturing of the chinook salmon embryo cells (CHSE-214) and the propagation and purification of IPNV have been described previously (Christie *et al.*, 1988). The Ab and Sp reference strains were obtained from P. E. V. Jørgensen (Denmark) and the Jasper strain was obtained from P. Dobos (Canada).

Construction of a lambda gt10 cDNA library. Synthesis of double-stranded cDNA from N1 genomic dsRNA was performed using random primers essentially as described by Azad *et al.* (1985). A lambda gt10 cDNA cloning system (Amersham) was used to construct a recombinant lambda gt10 library from the cDNA. Briefly, the cDNA was methylated with *Eco*RI methylase. *Eco*RI linkers were ligated to the cDNA and the cDNA was ligated to *Eco*RI-digested and phosphatase-treated lambda gt10 arms. The products of the ligation were packaged using packaging extracts from Amersham.

Isolation of cDNA clones. The lambda library was initially screened with two 5′ end-labelled synthetic oligonucleotides (40-mers). Their nucleotide sequences were taken from the 5′ and 3′ ends of segment A of the Jasper strain [nucleotides (nt) 31 to 70 and 3050 to 3089, Fig. 2]. Standard hybridization and washing conditions were used. Lambda phage DNA was isolated by using LambdaSorb phage adsorbent (Promega).

cDNA sequencing. Isolated virus clones were subcloned in both orientations into the pGEM-7Zf(+) vector (Promega). When necessary, DNA subclones of varying lengths were generated using the Erase-a-Base system (Promega). ssDNA, prepared by infection with the M13 K07 helper phage, was sequenced according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using the Sequenase system (United States Biochemical Corporation). Both strands of the cDNA were sequenced completely. The sequence was compiled from independent, overlapping clones. In the sequence presented in Fig. 1 each base has been confirmed from at least two overlapping independent clones, except for the terminal 87 bp at the 5′ end and the terminal 25 bp at the 3′ end. Ambiguous or compressed sequence regions were resolved by substitution of dITP for dGTP in the sequencing reactions. Sequences were assembled and analysed using Staden-Plus software (Amersham). The hydrophobicity and charge plots were made using the method of Kyte & Doolittle (1982) with a window size of 13, as implemented in the Staden-Plus software. Sequence alignment was performed using the Align program (Scientific & Educational Software). Mismatch penalty, open gap penalty and extend gap penalty were the recommended values of 2, 4 and 1, respectively.

SDS–PAGE of radiolabelled virus particles. A monolayer of CHSE-214 cells was infected with 1 p.f.u. per cell (25 cm² flasks, Costar). The medium was exchanged 22 h post-infection with methionine-free medium containing 16 μCi [^{35}S]methionine (Amersham) per ml. The infection was allowed to proceed to complete c.p.e. and the virus was harvested and purified as described earlier (Christie *et al.*, 1988). SDS-PAGE was performed as originally described by Laemmli (1970). The M_r values of the viral polypeptides were estimated from their electrophoretic mobilities relative to ^{14}C-labelled M_r markers (Amersham) run in parallel.

Results

Nucleotide sequences of segment A

The cDNA sequence of genome segment A of the N1 strain of IPNV is given in Fig. 1. The sequence is 3104 bp long and contains a large ORF of 2916 bp. The large ORF of the N1 strain is identical in length to the one already described for the Jasper strain (Duncan & Dobos, 1986). We found an identity of 79·5% between the nucleotide sequences of the A segments of the N1 and Jasper strains. A comparison with segment A of the 002-73 strain resulted in 54% identity (Table 1).

To look for possible new virus-encoded proteins we searched for conserved alternative ORFs on the A segments of the IPN viruses. Only one such perfectly conserved ORF was found, which is 444 bp long and overlaps with that encoding the N-terminal part of pVP2 (Fig. 1). This small ORF has the potential of encoding a 17K peptide containing 148 amino acids. The initiation codon of the small ORF is located 79 bp from the 5′ terminus of the A segment. It is closer to the 5′ terminus than the ATG codon of the large ORF, which is located 131 bp from the 5′ end of segment A (Fig. 1). Presumably, the 002-73 strain of IBDV also contains this small ORF. Unfortunately, much of the 5′ flanking region is missing from the sequence published by Hudson *et al.* (1986). Therefore, 15 bp of the small ORF is not available and it is not possible to tell whether there is a correctly positioned initiation codon at the N-terminal end of the small ORF of the 002-73 strain.

The optimal sequence for initiation by eukaryotic ribosomes has been determined to be ACCATGG (Kozak's rule) (Kozak, 1986). At the least a functional initiation codon should be flanked by a neighbouring purine (usually A) at position −3 and/or a G at position +4. Neither the large nor the small ORF of segment A contains a perfect Kozak consensus sequence at their start region (Fig. 2). The large ORF start region contains

Table 1. *Comparison between corresponding nucleic acid and amino acid sequences of the N1 and Jasper strains of IPNV and the 002-73 strain of IBDV**

	Segment A	ORF A	pVP2/VPX†	NS/VP4	VP3	VP5
N1 Jasper	NA‡ 79·5	–	80·9	76·6	77·3	84·2
	AA§ –	84·0	88·8	79·9	78·2	63·5
N1 IBDV	NA 53·8	–	56·6	49·0	51·2	60·1
	AA –	35·4	44·6	18·6	33·1	35·4
Jasper IBDV	NA 54·2	–	55·8	49·0	53·1	60·8
	AA –	34·7	43·6	21·1	31·3	28·0

* Results are given as percentage of identity.
† The large ORFs were divided into three segments corresponding to the gene products pVP2/VPX, NS/VP4 and VP3; the putative novel peptide VP5 was also included in the analysis.
‡ NA, nucleic acid.
§ AA, amino acid.

the important purine in the −3 position, but lacks a G in position +4. For the small ORF it is the other way around.

The large ORF of segment A of the N1 strain is bracketed by 5′- and 3′-terminal flanking regions consisting of 130 bp and 55 bp, respectively (Fig. 1). These regions are highly conserved within the IPNV strains (Fig. 2). A comparison between the corresponding flanking regions of the A segments of IPNV and IBDV reveal no significant sequence similarity (data not shown).

Terminal inverted repeats were found 10 bp from the 5′ terminus (nt 11 to 32) and 37 bp from the 3′ terminus (nt 3047 to 3067) of segment A of the N1 strain (Fig. 3*a*). Segment A of the Jasper strain has identical sequences at its 5′ and 3′ termini. It is not possible to determine whether segment A of IBDV has equivalent inverted repeats, because sequence information for the 5′ terminus is missing. On the other hand, segment B of the 002-73 strain of IBDV contains terminal inverted repeats (nt 67 to 78 and nt 2746 to 2757; Fig. 3*b*). The terminal inverted repeats of the two segments contain similar sequences and have the motif 5′ AAGAG 3′ in common, but the repeats on segment B (5′ TCCTCTTCTT 3′ 3′ AGGAGAAGAA 5′) are inverted relative to the repeats on segment A (5′ AGAAAGAGAG 3′/3′ TCTTTCTCTC 5′) (Fig. 3*a* and *b*; 5′ ends). In addition, adjacent inverted repeats are also found at the 5′-terminal flanking regions of segment A of IPNV (nt 41 to 82; open arrows, Fig. 3*a*) and segment B of IBDV (nt 17 to 61; open arrows, Fig. 3*b*).

Another puzzling sequence structure of IPN viruses is found approximately half way between the initiation codons of the large and the small ORF (Fig. 2). In the N1 strain this sequence can be read forwards and backwards to give the same sense (5′ TCTAACAAACAAACAAA-CA[illegible]CT 3′). The corresponding Jasper sequence has four mismatches compared to the N1 sequence (Fig. 2). All mismatches are positioned to the right side of the axis of symmetry when the sequence is written in the 5′ to 3′ direction.

Deduced amino acid sequences

The alignment of the deduced amino acid sequences of the large and small ORFs of the N1, Jasper and 002-73 strains is presented in Fig. 4 and Fig. 5, respectively. When the amino acid sequences of the N1 and Jasper polypeptides are compared, pVP2 proves to be the most conserved viral protein (88·8% identity), whereas the possible new gene product VP5 is least conserved (63·5% identity). On the other hand, if the IPNV gene products are compared to those of the 002-73 strain of IBDV, pVP2/VPX is still the most conserved polypeptide (approx. 44% identity), but the least conserved one has now changed to NS/VP4 (approx. 20% identity, Table 1).

The pVP2/VPX protein consists of two strongly conserved segments (the N and C termini) and a less conserved internal segment (I). The amino acid positions (Fig. 4) for these three segments are for the N1 and Jasper strains of IPNV 1 to 182 (N), 183 to 338 (I) and 339 to 470 (C), and for the 002-73 strain of IBDV 1 to 185 (N), 186 to 330 (I) and 331 to 469 (C). A comparison between the segments of the two strains of IPNV shows approximately 94% identity for the end segments and 78% identity for the internal segments. Similarly, a comparison between the IPNV strains and the 002-73 strain of IBDV resulted in approximately 50% and 25% identity for the end segments and the internal segments, respectively. The internal segments of the IPNV strains in particular have many amino acid replacements at positions 234 to 264 (Fig. 4). The same region also contains several possible *N*-glycosylation sites (N-X-S/T).

Charge plots of the putative VP5 polypeptides

```
                                                                                 M  A  K  A  L  S  N  K  Q  T  N  N  L  Y
GTGTGTTTGACAGAAAGAGAGTTTCAACGTTAGTGGTAACCCACGAGCGGAGAGCTCTTACGGAGGAGCTCTCCGTCGATGGCGAAAGCCCTTTCTAACAAACAAACAAACAATCTATAT
        10        20        30        40        50        60        70        80        90       100       110       120
 S  I  Q  D  E  H  K  Q  G  N  R  N  L  L  E  I  H  Y  A  S  R  D  W  T  S  K  H  P  G  R  H  N  G  E  T  H  P  K  T  R
          M  N  T  N  K  A  T  A  T  Y  L  K  S  I  M  L  P  E  T  G  P  A  S  I  P  D  D  I  T  E  R  H  I  L  K  Q  E
TCAATACAAGATGAACACAAACAAGGCAACCGCAACTTACTTGAAATCCATTATGCTTCCAGAGACTGGACCAGCAAGCATCCCGGACGACATAACGGAGAGACACATCCTAAAACAAGA
       130       140       150       160       170       180       190       200       210       220       230       240
 D  L  V  I  Q  P  R  G  L  R  I  R  K  W  H  S  C  L  F  P  W  G  T  R  L  T  D  R  C  T  L  Q  M  E  C  E  P  D  G  A
 T  S  S  Y  N  L  E  V  S  E  S  G  S  G  I  L  V  C  F  P  G  A  P  G  S  R  I  G  A  H  Y  R  W  N  A  N  Q  T  G  L
GACCTCGTCATACAACCTAGAGGTCTCCGAATCAGGAAGTGGCATTCTTGTTTGTTTCCCTGGGGCACCAGGCTCACGGATCGGTGCACACTACAGATGGAATGCGAACCAGACGGGGCT
       250       260       270       280       290       300       310       320       330       340       350       360
 G  V  R  P  V  A  G  D  V  A  G  P  E  E  S  L  Q  L  R  E  A  D  L  K  E  I  R  H  P  K  L  H  T  T  G  R  S  L  C  S
 E  F  D  Q  W  L  E  T  S  Q  D  L  K  K  A  F  N  Y  G  R  L  I  S  R  K  Y  D  I  Q  S  S  T  L  P  A  G  L  Y  A  L
GGAGTTCGACCAGTGGCTGGAGACGTCGCAGGACCTGAAGAAAGCCTTCAACTACGGGAGGCTGATCTCAAGGAAATACGACATCCAAAGCTCCACACTACCGGCCGGTCTCTATGCTCT
       370       380       390       400       410       420       430       440       450       460       470       480
 E  R  D  A  Q  R  C  H  L  R  R  Q  S  V  *
 N  G  T  L  N  A  A  T  F  E  G  S  L  S  E  V  E  S  L  T  Y  N  S  L  M  S  L  T  T  N  P  Q  D  K  V  N  N  Q  L  V
GAACGGGACGCTCAACGCTGCCACCTTCGAAGGCAGTCTGTCTGAGGTGGAGAGCCTGACCTACAACAGCCTGATGTCCCTAACAACGAACCCCCAGGACAAAGTCAACAACCAGCTGGT
       490       500       510       520       530       540       550       560       570       580       590       600
 T  K  G  V  T  V  L  N  L  P  T  G  F  D  K  P  Y  V  R  L  E  D  E  T  P  Q  G  L  Q  S  M  N  G  A  K  M  R  C  T  A
GACCAAAGGAGTCACAGTCCTGAATCTACCAACAGGGTTCGACAAACCATACGTCCGCCTAGAGGACGAGACACCCCAGGGTCTCCAGTCAATGAACGGGGCCAAGATGAGGTGCACAGC
       610       620       630       640       650       660       670       680       690       700       710       720
 A  I  A  P  R  R  Y  E  I  D  L  P  S  Q  R  L  P  P  V  P  A  T  G  T  L  T  T  L  Y  E  G  N  A  D  I  V  N  S  T  T
TGCAATTGCACCGCGGAGGTACGAGATCGACCTCCCATCCCAACGCCTACCCCCCGTTCCTGCGACAGGAACCCTCACCACTCTCTACGAGGGAAACGCCGACATCGTCAACTCCACAAC
       730       740       750       760       770       780       790       800       810       820       830       840
 V  T  G  D  I  N  F  S  L  A  E  Q  P  A  N  E  T  K  F  D  F  Q  L  D  F  M  G  L  D  N  D  V  P  V  V  T  V  V  S  S
AGTGACGGGAGACATAAACTTCAGTCTGGCAGAACAACCCGCGAACGAGACCAAGTTCGACTTCCAGCTGGACTTCATGGGCCTTGACAACGACGTCCCAGTTGTCACAGTGGTCAGCTC
       850       860       870       880       890       900       910       920       930       940       950       960
 V  L  A  T  N  D  N  Y  R  G  V  S  A  K  M  T  Q  S  I  P  T  E  N  I  T  K  P  I  T  R  V  K  L  S  Y  K  I  N  Q  Q
CGTGCTGGCCACAAATGACAACTACAGAGGAGTCTCAGCCAAGATGACCCAGTCCATCCCGACCGAGAACATCACAAAGCCGATCACCAGGGTCAAGCTGTCATACAAGATCAACCAGCA
       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
 T  A  I  G  N  V  A  T  L  G  T  M  G  P  T  T  V  S  F  S  S  G  N  G  N  V  P  G  V  L  R  P  I  T  L  V  A  Y  E  K
GACAGCAATCGGCAACGTCGCCACCCTGGGCACAATGGGTCCGACTACCGTCTCCTTCTCATCAGGGAACGGAAATGTCCCCGGCGTGCTCAGACCAATCACACTGGTGGCCTATGAGAA
      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
 M  T  P  L  S  I  L  T  V  A  G  V  S  N  Y  E  S  Y  P  N  P  E  L  L  K  N  M  V  T  R  Y  G  K  Y  D  P  E  G  L  N
GATGACACCGCTGTCCATCCTGACCGTAGCTGGAGTGTCCAACTACGAGTCGTACCCAAACCCAGAACTCCTCAAGAACATGGTGACACGCTATGGCAAGTACGACCCCGAAGGTCTCAA
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
 Y  A  K  M  I  L  S  H  R  E  E  L  D  I  R  T  V  W  R  T  E  E  Y  K  E  R  T  R  V  F  N  E  I  T  D  F  S  S  D  L
CTATGCCAAGATGATCCTGTCCCACAGGGAAGAGCTGGACATCAGGACAGTGTGGAGGACAGAGGAGTACAAGGAGAGGACCAGAGTCTTCAACGAAATCACGGACTTCTCCAGTGACCT
      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
 P  T  S  K  A  W  G  W  R  D  I  V  R  G  I  R  K  V  A  A  P  V  L  S  T  L  F  P  M  A  A  P  L  I  G  M  A  D  Q  F
GCCCACGTCAAAGGCATGGGGCTGGAGAGACATAGTCAGAGGAATTCGGAAAGTCGCAGCTCCTGTACTGTCCACGCTGTTTCCAATGGCAGCACCACTCATAGGAATGGCAGACCAATT
      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

 I  G  D  L  T  K  T  N  A  A  G  G  R  Y  H  S  M  A  A  G  G  R  H  K  D  V  L  E  S  W  A  S  G  G  P  D  G  K  F  S
CATTGGAGATCTCACCAAGACCAACGCAGCAGGCGGAAGGTACCACTCCATGGCCGCAGGAGGGCGCCACAAAGACGTGCTCGAGTCCTGGGCAAGCGGAGGGCCCGACGGAAAATTCTC
      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
 R  A  L  K  N  R  L  E  S  A  N  Y  E  E  V  E  L  P  P  P  S  K  G  V  I  V  P  V  V  H  T  V  K  S  A  P  G  E  A  F
CCGAGCCCTCAAGAACAGGCTGGAGTCCGCCAACTACGAGGAAGTCGAGCTTCCACCCCCCTCAAAAGGAGTCATCGTCCCTGTGGTGCACACAGTCAAGAGTGCACCAGGCGAGGCATT
      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
 G  S  L  A  I  I  I  P  G  E  Y  P  E  L  L  D  A  N  Q  Q  V  L  S  H  F  A  N  D  T  G  S  V  W  G  I  G  E  D  I  P
CGGGTCCCTGGCAATCATAATTCCAGGGGAGTACCCCGAGCTTCTAGATGCCAACCAGCAGGTCCTATCCCACTTCGCAAACGACACCGGGAGCGTGTGGGGCATAGGAGAGGACATACC
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
 F  E  G  D  N  M  C  Y  T  A  L  P  L  K  E  I  K  R  N  G  N  I  V  V  E  K  I  F  A  G  P  I  M  G  P  S  A  Q  L  G
CTTCGAGGGAGACAACATGTGCTACACTGCACTCCCACTCAAGGAGATCAAAAGAAACGGGAACATAGTAGTCGAGAAGATCTTTGCTGGACCAATCATGGGTCCCTCTGCTCAACTAGG
      1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040
 L  S  L  L  V  N  D  I  E  D  G  V  P  R  M  V  F  T  G  E  I  A  D  D  E  E  T  I  I  P  I  C  G  V  D  I  K  A  I  A
ACTGTCCCTACTTGTGAACGACATCGAGGACGGAGTTCCAAGGATGGTATTCACCGGCGAAATCGCCGATGACGAGGAGACAATCATACCAATCTGCGGTGTAGACATCAAAGCCATCGC
      2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160
 A  H  E  P  G  L  P  L  I  G  N  Q  P  G  V  D  E  E  V  R  N  T  S  L  A  A  H  L  I  Q  T  G  T  L  P  V  Q  R  A  K
AGCCCATGAACCAGGGCTGCCACTCATCGGCAACCAACCAGGAGTGGACGAGGAGGTGCGAAACACATCCCTGGCCGCACACCTGATCCAGACCGGAACCCTGCCCGTACAACGAGCAAA
      2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280
 G  S  N  K  R  I  K  Y  L  G  E  L  M  A  S  N  A  S  G  M  D  E  E  L  Q  R  L  L  N  A  T  M  A  R  A  K  E  V  Q  D
GGGCTCCAACAAGAGGATCAAGTACCTGGGAGAGCTGATGGCATCAAATGCATCCGGGATGGACGAGGAACTGCAACGCCTCCTGAACGCCACAATGGCACGGGCCAAAGAAGTCCAGGA
      2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
 A  E  I  Y  K  L  L  K  L  M  A  W  T  R  K  N  D  L  T  D  H  M  Y  E  W  S  K  E  D  P  D  A  L  K  F  G  K  L  I  S
CGCCGAGATCTACAAACTTCTTAAGCTCATGGCATGGACCAGAAAGAACGACCTCACCGACCACATGTACGAGTGGTCAAAAGAGGACCCCGATGCACTAAAGTTCGGAAAGCTCATCAG
      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520
 T  P  P  K  H  P  E  K  P  K  G  P  D  Q  H  H  A  Q  E  A  R  A  T  R  I  S  L  D  A  V  R  A  G  A  D  F  A  T  P  E
CACGCCACCAAAGCACCCTGAGAAGCCCAAAGGACCAGACCAACACCACGCCCAAGAGGCGAGAGCCACCCGCATATCACTGGACGCCGTGAGAGCCGGGGCGGACTTCGCCACACCGGA
      2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640
 W  V  A  L  N  N  Y  R  G  P  S  P  G  Q  F  K  Y  Y  L  I  T  G  R  E  P  E  P  G  D  E  Y  E  D  Y  I  K  Q  P  I  V
ATGGGTCGCGCTGAACAACTACCGCGGCCCATCTCCCGGGCAGTTCAAGTACTACCTGATCACTGGACGAGAACCAGAACCAGGCGACGAGTACGAGGACTACATAAAACAACCCATTGT
      2650      2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760
 K  P  T  D  M  N  K  I  R  R  L  A  N  S  V  Y  G  L  P  H  Q  E  P  A  P  E  E  F  Y  D  A  V  A  A  V  F  A  Q  N  G
GAAACCGACCGACATGAACAAAATCAGACGTCTAGCCAACAGTGTGTACGGCCTCCCACACCAGGAACCAGCACCAGAGGAGTTCTACGATGCAGTTGCAGCTGTATTCGCACAGAACGG
      2770      2780      2790      2800      2810      2820      2830      2840      2850      2860      2870      2880
 G  R  G  P  D  Q  D  Q  M  Q  D  L  R  E  L  A  R  Q  M  K  R  R  P  R  N  A  D  A  P  R  R  T  R  A  P  A  E  P  A  P
AGGCAGAGGTCCCGACCAGGACCAAATGCAAGACCTCAGGGAGCTCGCAAGACAGATGAAACGACGACCCCGGAACGCCGATGCACCACGGAGAACCAGAGCGCCAGCGGAACCGGCACC
      2890      2900      2910      2920      2930      2940      2950      2960      2970      2980      2990      3000
 P  G  R  S  R  F  T  P  S  G  D  N  A  E  V  *
GCCCGGACGCTCAAGGTTCACCCCCAGCGGAGACAACGCTGAGGTGTAACGACTACTCTCTTTCCTGACTGATCCCCTGGCCAAAACCCCGGCCCCCCAGGGGG
      3010      3020      3030      3040      3050      3060      3070      3080      3090      3100
```

Fig. 1. cDNA plus-strand sequence of segment A of the N1 strain and deduced amino acid sequences of the small ORF (nt 79 to 522) and the large ORF (nt 131 to 3046).

```
N1       5'-GTGTGTTTGACAGAAAGAGAGTTTCAACGTTAGTGGTAACCCACGAGCGGAGAGCTCTTA
                       :::::::::::::::::::::::::::::::::::::::::::::::
Jasper             5'-GGAAAGAGAGTTTCAACGTTAGTGGTAACCCACGAGCGGAGAGCTCTTA
                               20        30        40        50        60

                               *
N1          CGGAGGAGCTCTCCGTCGATGGCGAAAGCCCTTTCTAACAAACAAACAAACAATCTATAT
            ::::::::::::::::::::::::::::::::::::::::::::: :::  : :::
Jasper      CGGAGGAGCTCTCCGTCGATGGCGAAAGCCCTTTCTAACAAACAACCAACAATTCTTATC
                     70        80        90        100       110       120

                      *
N1          TCAATACAAGATGAAC- - - - - - - - -Large ORF - - - - - - - - - -
            :  ::  :  :::
Jasper      TACATGAATCATGAGC- - - - - - - - -Large ORF - - - - - - - - - -
                     130

            *
N1          TAACGACTACTCTCTTTCCTGACTGATCCCCTGGCCAAAACCCCGGCCCCCAGGGGG-3'
            ::::  ::::::::::::::::::::::::::::::::  ::::::::::::::::::::
Jasper      TAACAGCTACTCTCTTTCCTGACTGATCCCCTGGCCGTAACCCCGGCCCCCAGGGGGCCCC-3'
            3050      3060      3070      3080      3090      3100
```

Fig. 2. Alignment of the 5′ and 3′ flanking regions of segment A of the N1 and Jasper strains of IPNV. The sequence for the Jasper strain of IPNV is from Duncan & Dobos (1986).

demonstrate that the patterns of the N1, Jasper and 002-73 strains are quite similar. A comparison between the corresponding VP5 hydrophobicity plots shows that the IPNV strains have very similar plots, whereas the hydrophobicity plot of VP5 of the 002-73 strain of IBDV shows hardly any resemblance to the IPNV plots (Fig. 6).

SDS–PAGE of [^{35}S]methionine-labelled viral proteins

SDS–PAGE of radiolabelled purified IPNV particles (Ab. Sp, Jasper and N1 strains) revealed the polypeptide pattern shown in Fig. 7. In addition to bands corresponding to VP1, pVP2, VP2 and VP3, there are bands at the bottom of the gel with M_r of approximately 25K and 17K. The 17K bands of the Jasper and N1 lanes (Fig. 7) are poorly visible in the photograph.

Discussion

Results obtained by Azad *et al.* (1985) upon *in vitro* translation of IBDV RNA (strain 002-73) indicate that segment A may code for a 16K polypeptide in addition to PVX, PV3 and PV4 (Azad *et al.*, 1985). A second small ORF in segment A of IPNV, capable of encoding a 17K polypeptide, has also been mentioned by Duncan *et al.* (1987). However, these observations have not been discussed further, as the existence of a 17K polypeptide has never been corroborated. We consider it unlikely that the positions of the start and stop codons of the small ORF should be so perfectly conserved between the strains unless it encodes a functional gene product. No other ORFs on segment A are conserved in that way. Alignment of the amino acid sequences of the putative VP5 polypeptides of the N1, Jasper and 002-73 strains revealed some remarkably well conserved cysteine residues and demonstrated that many of the non-identical amino acids are conservative amino acid replacements (Fig. 5). Likewise, the comparison of hydrophobicity and charge shown in Fig. 6 demonstrated a greater similarity in physicochemical properties than would be expected from just considering matching amino acids. Furthermore, the autoradiograph shown in Fig. 7 revealed a band which corresponded to the theoretically calculated M_r of VP5. Since the SDS–PAGE gel was loaded with [^{35}S]methionine-labelled purified virus, the 17K band is probably of viral origin. Based on this new information we now think it is reasonable to believe that the small ORF on segment A encodes a fifth birnavirus protein (VP5). However, further investigation is required to prove the existence of VP5 and to understand its biological function. For that reason we are now preparing synthetic oligopeptides in order to make monospecific antisera against VP5. These antisera will be used to study the production of VP5 *in vivo*.

(*a*) N1 strain

```
5'-GTGTGTTTGACAGAAAGAGAGTTTCAACGTTAG
3'-CACACAAACTGTCTTTCTCTCAAAGTTGCAATC
           10          20          30

TGGTAACCCACGAGCGGAGAGCTCTTACGGAGGAGCTCTCCGTCGATGGCGAAAGCCCTT
ACCATTGGGTGCTCGCCTCTCGAGAATGCCTCCTCGAGAGGCAGCTACCGCTTTCGGGAA
    40         50                                         90

TCTAACAAACAAACAAACAATCTATATTCAATACAAGATG - - - Large ORF - - -
AGATTGTTTGTTTGTTTGTTAGATATAAGTTATGTTCTAC - - - Large ORF - - -
    100        110        120        130

TAACGACTACTCTCTTTCCTGACTGATCCCCTGGCCAAAACCCCGGCCCCCCAGGGGG-3'
ATTGCTGATGAGAGAAAGGACTGACTAGGGGACCGGTTTTGGGGCCGGGGGGTCCCCC-5'
                                 3080       3090       3100
```

(*b*) 002-73 strain

```
5'-CTCTGGAATCACGCAATAACGTGGCTACTAGGGGTGATG
3'-GAGACCTTAGTGCGTTATTGCACCGATGATCCCCACTAC
            10         20         30

CCCACCGCTAGCTGCCACGTTAAGCGTTCCTCTTCTTGATGATTCTGCCACCATG - -
GGGTGGCGATCGACGGTGCAATTCGCAAGGAGAAGAACTACTAAGACGGTGGTAC - -
                                 70         80         90

- - -ORF- - - TAGCCATGATGGGAACCATTCAAGAAGAGGACACTAACCCCAGACC
- - -ORF- - - ATCGGTACTACCCTTGGTAAGTTCTTCTCCTGTGATTGGGGTCTGG
              2730       2740                          2770

CCGTATCCCCGCCCTCTCGCCCTG-3'
GGCATAGGGGCGGGAGAGCGGGAC-5'
    2780       2790
```

Fig. 3. (*a*) Sequence of the 5′ and 3′ flanking regions of segment A of the N1 strain of IPNV. (*b*) Sequence of the 5′ and 3′ flanking regions of segment B of the 002-73 strain of IBDV. The sequence for the B segment of the 002-73 strain of IBDV is from Morgan *et al.* (1988). Symbols: *, initiation and stop codons; open arrows, adjacent inverted repeats; black arrows, terminal inverted repeats; :, base pairs.

The mechanism of replication has not been well studied in the *Birnaviridae* group, but evidence suggests that birnavirus replication is initiated independently at the ends of the segments and proceeds by strand displacement (Bernard, 1980; Mertens *et al.*, 1982; Spies *et al.*, 1987). All birnaviruses probably use identical, or at least very similar, mechanisms for replicating and packaging their genomes. It is also to be expected that segment A is replicated and packaged in the same way as segment B. The finding of terminal inverted repeats in both segment A of IPNV and segment B of IBDV indicates that these sequences are somehow essential to birnaviruses and raises a number of questions pertaining to function. We suggest that the terminal inverted repeats play important roles in birnavirus dsRNA replication and/or packaging.

Stem-and-loop structures might be expected to form at the 5′ untranslated regions of the mRNAs transcribed from segment A of IPNV and segment B of IBDV (Fig. 3*a* and 3*b*, adjacent inverted repeats). Several studies have demonstrated that a stem-and-loop structure 5′ to the start codon of the mRNA significantly decreases

```
Jasper  MSTSKATATYL-----------RSIMLPENGPASIPDDITERHILKQETSSYTLEVSESGSGLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLKKAFNYGRLI   (99)
N1      MNTNKATATYL-----------KSIMLPETGPASIPDDITERHILKQETSSYNLEVSESGSGILVCFPGAPGSRIGAHYRWNANQTGLEFDQWLETSQDLKKAFNYGRLI   (99)
IBDV    M-------TNLSDQTQQIVPFIRSLLMPTTGPASIPDDTLEKHTLRSETSTYNLTVGDTGSGLIVFFPGFPGSIVGAHYTMQSN-GNYKFDQMLLTAQNLPASYNYCRLV  (102)

Jasper  SRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGFDKPYVRLEDETPQGPQSMNGARMRCTAAIAPRRYEIDLP  (209)
N1      SRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVESLTYNSLMSLTTNPQDKVNNQLVTKGVTVLNLPTGFDKPYVRLEDETPQGLQSMNGAKMRCTAAIAPRRYEIDLP  (209)
IBDV    SRSLTVRSSTLPGGVYALNGTINAVTFQGSLSELTDVSYNGLMSATANINDKIGNVLVGEGVTVLSLPTSYDLGYVRLGDPIP-AIGLDPKMVATCDSSDRPRVYTITAA  (211)
                                                                                           ↑
Jasper  SERLPTVAATGTPTTIYEGNADIVNSTAVTGDITFQLEAEPVNETRFDFILQFLGLDNDVPVVTVTSSTLVTADNYRGASAK---------FTQSIPTEMITKPITRVKLA  (311)
N1      SQRLPPVPATGTLTTLYEGNADIVNSTTVTGDINFSLAEQPANETKFDFQLDFMGLDNDVPVVTVVSSVLATNDNYRGVSAK---------MTQSIPTENITKPITRVKLS  (311)
IBDV    DDYQFSSQYQPGGVTITLFSANIDAIT-----------NLSVGGELVFQTSVQGLVLNATIYLVGFDGTTVTTRAVAAGNGLTAGTDNLMPFNLVIPTSEITQPVTSIKL-  (310)

Jasper  YQLNQQTAIANAATLGAKGPASVSFSSGN--------GNVPGVLRPITLVAYEKMTPQSILTVAGVSNYELIPNPDLLKNMVTKYGKYDPEGLNYAKMILSHREELDIRTV  (414)
N1      YKINQQTAIGNVATLGTMGPTTVSFSSGN--------GNVPGVLRPITLVAYEKMTPLSILTVAGVSNYESYPNPELLKNMVTRYGKYDPEGLNYAKMILSHREELDIRTV  (414)
IBDV    -------EIVTSKSGGQAGDQMSWLASGNLAVTIHGGNYPGALRPVTLVAYERVATGSVVTVAGVSNFELIPNPELAKNLVTEYGRFDPGAMNYTKLILSERDRLGIKTV  (413)
                                  ↑
Jasper  WRTEEYKERTRAFKEITDFTSDLPTSKAWGWRDLVRGIRKVAAPVLSTLFPMAAPLIGAA----DQFIGDLTKTNSAGGRYLSHAAGGRYHDVMDSWASGSEAGSYSKHL  (520)
N1      WRTEEYKERTRVFNEITDFSSDLPTSKAWGWRDIVRGIRKVAAPVLSTLFPMAAPLIGMA----DQFIGDLTKTNAAGGRYHSMAAGGRHKDVLESWASGGPDGKFSRAL  (520)
IBDV    WPTREYTDFREYFMEVADLNSPLKIAGAFGFKDIIRAIRRIAVPVVSTLFPPAAPLAHAIGEGVDYLLGDEAQAASGTARAASGKA----------RAASGRIRQLTLAAD  (514)
                                                                         VP2 <|------------------------------|> NS
Jasper  KTRLESNNYEEVELPKPTKGVI-------------------------FPVVHT-VESA--PGEAFGSLVVVIPGAYPELLDPNQQVLSYFKNDTGCVWGIGEDIPFEGDDMCY  (605)
N1      KNRLESANYEEVELPPPSKGVI-------------------------VPVVHT-VKSA--PGEAFGSLAIIIPGEYPELLDANQQVLSHFANDTGSVWGIGEDIPFEGDNMCY  (605)
IBDV    KGYEVVANLFQVPQNPVVDGILASPGVLRGAHNLDCVLREGATLFPVVITTVEDAMTPKALNSKMFAVIEGVREDLQPPSQRGSFIRTLSGHRVYGYAPDGVLPLETGRD  (624)

Jasper  TALPLKEIKRNGNIVVEKIFAGPAMGPSSQLALSLLVNDIDEGIPRMVFTGEIADDEETVIPICGVDIKAIAAHEHGLPLIGCQPGVDEMVANTSLASHLIQSGALPVQK  (715)
N1      TALPLKEIKRNGNIVVEKIFAGPIMGPSAQLGLSLLVNDIEDGVPRMVFTGEIADDEETIIPICGVDIKAIAAHEPGLPLIGNQPGVDEEVRNTSLAAHLIQTGTLPVQR  (715)
IBDV    YTVVPIDDVWDDSIMLSKDPIPPIVGNSGNLAIAYMDVFRPKVPIHVAMTGALNAYGE-VEKVSFRSTKLATAHRLGLKLAG--PGAFDINTGPNWATFIKRFPHNPRDW  (731)

Jasper  AQGACRRIKYLG------QLMRTTASGMDAELQGLLQATMAR-AKEVKDAEVFKLLKLMSWTRKNDLTDHMYEWSKEDPDAIKFGRLVSTPPKHQEKPK-------------  (807)
N1      AKGSNKRIKYLG------ELMASNASGMDEELQRLLNATMAR-AKEVQDAEIYKLLKLMAWTRKNDLTDHMYEWSKEDPDALKFGKLISTPPKHPEKPK-------------  (807)
IBDV    DRLPYLNLPYLPPSAGRQYHLAMAASEFKETPELESAVRAMEAAADVDPLFQSALSVFMWLEENGIVTDMANFALSDPNAHRMRNFLANAPQAGSKSQRAKYGTAGYGVE  (841)
        NS <|----------------|> VP3
Jasper  --GPDQHTAQEAKATRISLDAVKAGADFASPEWIAENNYRGPSPGQFKYYMITGRVPNPGEEYEDYVRKPITRPTDMDKIRRLANSVYGLPHQEPAPDDFYQAVVEVFAE  (915)
N1      --GPDQHHAQEARATRISLDAVRAGADFATPEWVALNNYRGPSPGQFKYYLITGREPEPGDEYEDYIKQPIVKPTDMNKIRRLANSVYGLPHQEPAPEEFYDAVAAVFAQ  (915)
IBDV    ARGPTPEEAQREKDTRISKKMEAMGIYFATPEWVALNGHRGPSPGQLKYWQNTREIPDPNEDYLDYVHAEKSRLASEEQILRAATSIYGAPGQAEPPQAFIDEVAKVYEI  (951)

Jasper  NGGRGPDQDQMQDLRDLARQMKRR-PRPAETRRQTKTPPRAATSSGSRF-----TPSGDDGEV
N1      NGGRGPDQDQMQDLRELARQMKRR-PRNADAPRRTRAPAEPAPPGRSRF-----TPSGDNAEV
IBDV    NHGRGPNQEQMKDLLLTAMEMKHRNPRRAPPKPKPKPNAPSQRPPGRLGRWIRTVSDEDLE-
```

Fig. 4. Alignment of the amino acid sequences of the large ORFs of the N1 and Jasper strains of IPNV and the 002-73 strain of IBDV. The approximate borders of the three viral polypeptides N–pVP2–NS–VP3–C (IBDV: N–VPX–VP4–VP3–C) encoded by the large ORFs are indicated by the open arrows. The dotted lines indicate that the precise boundaries of the coding regions have not been determined. The region between the black arrows is the hypervariable segment of VP2. Potential *N*-linked glycosylation sites (N–X–T/S) are underlined. The sequences for the A segments of the Jasper strain of IPNV and the 002-73 strain of IBDV are from Duncan & Dobos (1986) and Hudson *et al.* (1986), respectively.

translational efficiency in eukaryotes (Pelletier & Sonenberg, 1985; Kozak, 1988). Thus, it seems logical to predict that the stem-and-loop structures of the 5′ untranslated regions of segments A and B regulate viral protein synthesis by decreasing or blocking translation. Presumably, specific cellular or viral factors disrupt the secondary structure to relieve the translation inhibition.

Current evidence indicates that VP2 of birnaviruses carries the serotype-specific epitopes responsible for the induction of neutralizing protective antibodies and that

```
Jasper  MAKALSNKQPTILIYMNHEHIQGNRNLLEIHYASRE-WASKHSGRHNREA  (49)
        :::::::::   :  ...:: ::::::::::::::.-:.:::.:::: :.
N1      MAKALSNKQTNNLYSIQDEHKQGNRNLLEIHYASRD-WTSKHPGRHNGET  (49)
             :  :::.    .  . .     .  . .  . .:. ::::: ::.
IBDV    -----SRDQTNDRSDDKPVRSNPADCSVYTEPSDANNRTGVHPGRHPGEA  (45)

Jasper  YTKTRDLVIHLRGIRIRKWASCLLPRSSWIQGRCPLQVESEPDGTRIRPV  (99)
          :::::::. ::.::::: :X:.:..... .:X ::.: ::::. .:::
N1      HPKTRDLVIQPRGLRIRKWHSCLFPWGTRLTDRCTLQMECEPDGAGVRPV  (99)
        :.. ::: .:      :  . .X::::  ::  :X.:  .  :     :::
IBDV    HSQVRDLNLQFDCGGHRVRANCLFPWIPRLNCRCSL-HDAEQWELQVRPD  (94)

Jasper  ARDVTGPKEGIQLRETDLTEIRHPELNPSRWSVCTQWDPERCHLRRKSV  (148)
        : ::.:: :..::::.:: ::::: :. . .:.X...:..:X::::.::
N1      AGDVAGPEESLQLREADLKEIRHPKLHTTGRSLCSERDAQRCHLRRQSV  (148)
        : : . :   ::: .:   :      ::  . :X. .. .: .: :..
IBDV    APDGSEPTSELQLLQASESESNSKVKHTPWWRLCTKWHHKRRDLPRKPE  (143)
```

Fig. 5. Alignment of the amino acid sequences of the small ORFs (VP5) of the N1 and Jasper strains of IPNV and the 002-73 strain of IBDV. Symbols: X, conserved cysteine residues; :, identical amino acids; ., conserved amino acid replacements [scores greater than zero in the mutation probability data matrix (Dayhoff, 1978)]. The amino acid sequences of the small ORFs of the Jasper strain of IPNV and 002-73 strain of IBDV were deduced from their respective nucleic acid sequences (Duncan & Dobos, 1986; Hudson *et al.*, 1986).

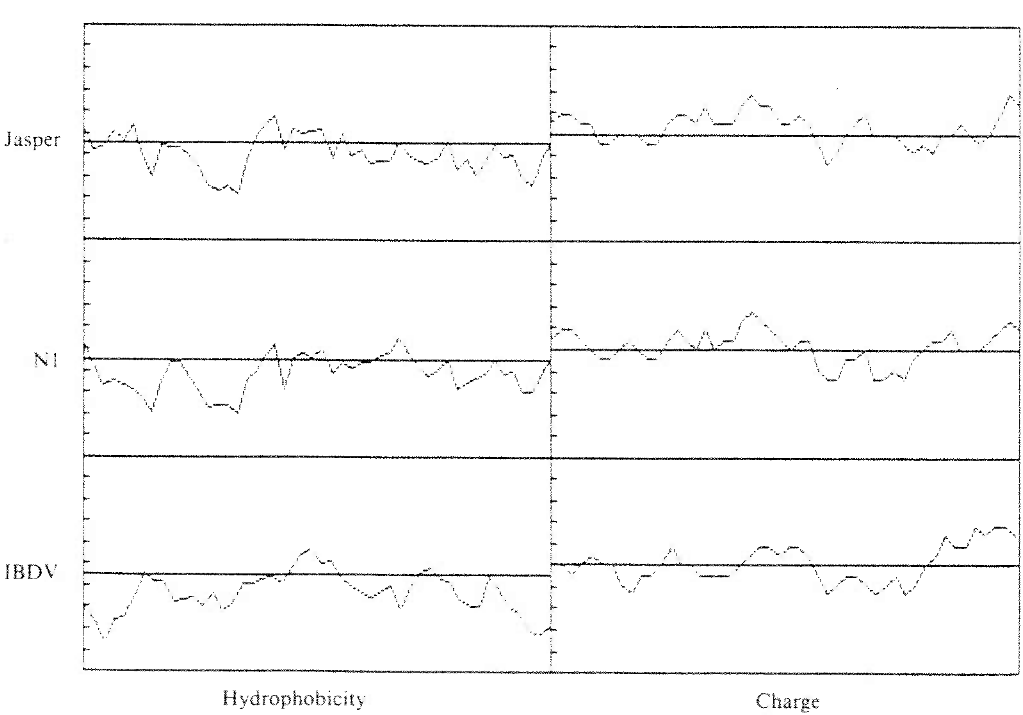

Fig. 6. Plots of hydrophobicity and charge of VP5 by the method of Kyte & Doolittle (1982). Comparison between the N1 and Jasper strains of IPNV and the 002-73 strain of IBDV. The amino acid sequences of the small ORFs (VP5) of the Jasper strain of IPNV and the 002-73 strain of IBDV used in these analyses were deduced from their respective nucleic acid sequences (Duncan & Dobos, 1986; Hudson *et al.*, 1986).

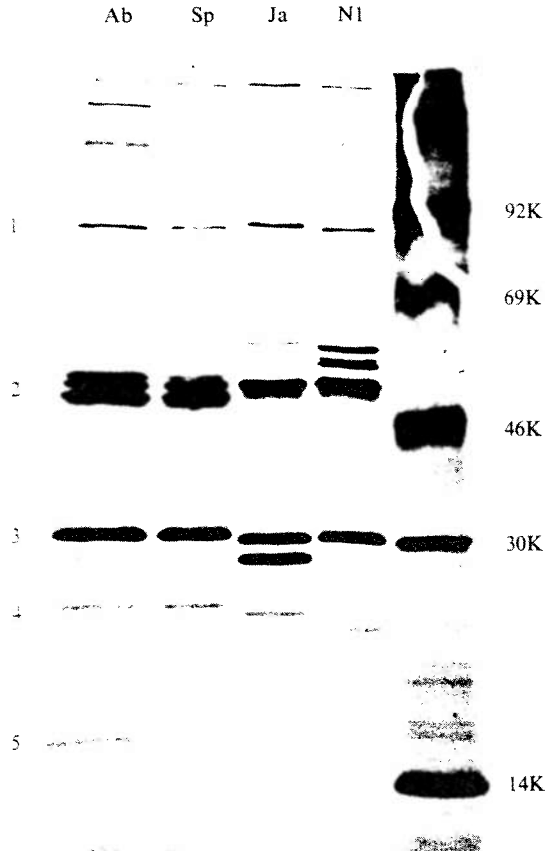

Fig. 7. Autoradiography after SDS–PAGE of [35S]methionine-labelled purified IPNV particles: strains Ab, Sp, Jasper and N1. Bands: 1, Vp1; 2, pVP2 and VP2; 3, Vp3; 4, the 25K band; 5, the putative novel 17K polypeptide VP5. The M_r of the marker proteins on the right-hand side range from 14K to 92K.

these epitopes are highly conformation-dependent (Azad *et al.*, 1987; Becht *et al.*, 1988; Caswell-Reno *et al.*, 1986; Christie & Håvarstein, 1988). Deletion expression analyses of VP2 of IBDV were carried out by Azad *et al.* (1987) to define the minimum region within VP2 recognized by the virus-neutralizing monoclonal antibody (MAb) 17/82. They demonstrated that the conformational epitope recognized by MAb 17/82 is contained within a 145 amino acid polypeptide (IBDV; amino acid positions 206 to 350, Fig. 4). By comparing the VP2 amino acid sequences of the IPNV strains with VP2 of the IBDV strain (Fig. 4) we have found that an internal segment of VP2, approximately 150 amino acids long, constitutes a hypervariable part of the molecule. A comparison between the VP2 sequence of the N1 strain and the VP2 sequence of the Jasper strain identified the same internal segment of VP2 as being the most variable part of the molecule. Particularly interesting, in that respect, is a part of VP2 of the N1 and Jasper strains (position 234 to 264, Fig. 4) where 11 out of 30 amino acids differ between the two serotypes of IPNV. It turns out that the hypervariable internal segment identified by amino acid comparison analysis is identical to the segment recognized by MAb 17/82. On the basis of these two independent observations we suggest that the approximately 150 amino acid long hypervariable internal segment of VP2 carries serotype-specific epitopes of both IPNV and IBDV.

As far as we know it has not yet been determined whether VP2 of IPNV is glycosylated. Müller & Becht (1982) made attempts to demonstrate carbohydrates in IBDV-specific polypeptides and found that no appreciable amounts of common carbohydrates are present in IBDV. However, the conservation in all three birnavirus strains of several potential *N*-glycosylation sites in VP2 (Fig. 4), especially in the heterogeneous region mentioned above, suggests that it should not be completely ruled out that oligosaccharides may form part of VP2 of birnaviruses.

Azad *et al.* (1987) have shown that expression in *Escherichia coli* of the large ORF of segment A of IBDV results in autocatalytic cleavage of the polyprotein. Furthermore, Jagadish *et al.* (1988) demonstrated by mutagenesis studies that the only mutants which affected processing at the VPX/VP4 and VP4/VP3 junctions were those in which pieces of DNA were either inserted into or deleted from VP4. We have expressed a region of the large ORF of the N1 strain (positions 452 to 972; Fig. 4) in *E. coli* as a β-galactosidase fusion protein. Subsequent Western blot analysis using specific antisera revealed bands with M_r values corresponding to those of NS and VP3, indicating autocatalytic cleavage of the fusion protein (not shown). It is puzzling that the NS polypeptide of IPNV and VP4 polypeptide of IBDV, which carry out equivalent cleavage reactions, have diverged to such an extent that there is very little homology left between their amino acid sequences (Table 1). However, the NS protein is comparatively well conserved between the two strains of IPNV. The adaptation of IPN and IBD viruses to different hosts appears to have increased the rate of evolution of the NS/VP4 proteins relative to the other gene products of segment A.

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Technical Bulletin

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HOSTS AND VECTORS FOR EXPRESSION IN THE pET SYSTEM

HMS174 and BL21

The bacterial hosts for cloning and expression are the *E. coli* K12 strain HMS174 (F⁻ *recA* r_{K12}^- m_{K12}^+ Rif^r) (12) and the B strain BL21 (F⁻ *ompT* $r_B^- m_B^-$) (7,13). HMS174 is used as the host for initial cloning of target DNA into pET vectors and for maintaining plasmids. As an expression strain, BL21 has the potential advantage that, as a B strain, it should be deficient in the *lon* protease, and it also lacks the *ompT* outer membrane protease that can degrade proteins during purification (13). Thus, at least some target proteins might be expected to be more stable in BL21 than in host strains that contain these proteases. HMS174 has the potential disadvantage that rifampicin cannot be used to inhibit transcription by the host RNA polymerase in cases where a reduction of background synthesis of host RNA and proteins may be desirable.

BL21(DE3) and HMS174(DE3) lysogens

Bacteriophage DE3 is a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lac*I gene, the *lac*UV5 promoter, the beginning of the *lac*Z gene, and the gene for T7 RNA polymerase (7). This fragment is inserted into the *int* gene, and, because the *int* gene is inactivated, DE3 needs a helper for either integration into or excision from the chromosome. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). (T7 RNA polymerase is produced from its own translation start and not as a fusion to the beginning of the *lac*Z protein.) Addition of 0.4 mM IPTG to a growing culture of either the BL21(DE3) or HMS174(DE3) lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in the plasmid.

plysS and pLysE

Target genes whose products are sufficiently toxic cannot be established in BL21(DE3) or HMS174(DE3) because the basal level of T7 RNA polymerase activity will promote some transcription of the target gene in the uninduced cell. One way to reduce this basal activity (and thereby increase the range of target genes that can be maintained and expressed in these cells) is through the use of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (14,15).

T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall (16), and it binds to T7 RNA polymerase and inhibits transcription (14). When produced from the cloned gene, relatively high levels of T7 lysozyme can be tolerated by *E. coli*, apparently because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer. Treatments that disrupt the inner membrane but do not normally cause lysis, such as addition of chloroform or mild detergents, induce rapid lysis of cells that contain even small amounts of T7 lysozyme.

T7 lysozyme can be provided to the cell from a clone of the T7 lysozyme gene in the *Bam*HI site of pACYC184 (17). The cloned fragment we have used (bp 10,665-11,296 of T7 DNA(2)) also contains the *ϕ3.8* promoter for T7 RNA polymerase immediately following the lysozyme gene. A plasmid having this fragment oriented so that the lysozyme gene is expressed from the *tet* promoter of pACYC184 is referred to as pLysE; cells carrying this plasmid accumulate substantial levels of lysozyme. A plasmid having the fragment in the opposite orientation is referred to as pLysS; cells carrying this plasmid accumulate much lower levels of lysozyme. These plasmids confer resistance to chloramphenicol and are compatible with the pET vectors for cloning target genes

(described below). Neither lysozyme plasmid interferes with transformation of cells that contain it; pLysS has little effect on growth rate but pLysE causes a significant decrease in the growth rate of cells that carry it.

The presence of either pLysS or pLysE increases the tolerance of BL21(DE3) or HMS174(DE3) for toxic target plasmids: unstable plasmids become stable, and plasmids that would not otherwise be established can be maintained and expressed. Some target plasmids are too toxic to be established in the presence of pLysS but are able to be established in the presence of pLysE, and a few are too toxic to be established even in the presence of pLysE.

The low level of lysozyme provided by pLysS usually has little effect on expression of target genes upon induction of T7 RNA polymerase, except for a short lag in the appearance of target gene products. Apparently, more T7 RNA polymerase is induced than can be inhibited by the small amount of lysozyme. (The level of lysozyme might be expected to increase somewhat upon induction, since T7 RNA polymerase should be able to transcribe completely around the pLysS plasmid from the *ϕ3.8* promoter to make lysozyme mRNA; however, the *ϕ3.8* promoter is relatively weak (18), and most transcription should be from the much stronger *ϕ10* promoter used in the target plasmids.)

The higher level of lysozyme provided by pLysE can substantially increase the lag and substantially reduce the maximum level of expression of target genes upon induction of T7 RNA polymerase. This damping of expression is sufficient that cells containing a target gene whose product is relatively innocuous can continue to grow indefinitely in the presence of IPTG, a property that may be useful in some circumstances. (In contrast, the high level of expression in the absence of lysozyme or in the presence of pLysS almost always prevents continued growth of the cell.) Because of this damping of expression, most target genes will be expressed to higher levels by CE6 infection (described below) than by induction in the presence of pLysE.

The presence of pLysS (or pLysE) has the further advantage of facilitating the preparation of cell extracts. After the target protein has accumulated, the cells are collected and suspended in a buffer such as 50 mM Tris-Cl, 2 mM Na_3EDTA, pH 8.0. Simply freezing and thawing, or adding 0.1% triton X-100, will allow the resident lysozyme to lyse the cells efficiently. This property can make it advantageous to carry pLysS in the cell even when it is not required for stabilizing the target plasmid.

Bacteriophage CE6

Target plasmids that are too toxic to be established in DE3 lysogens (even in the presence of pLysE) can be expressed by infecting with a bacteriophage that provides T7 RNA polymerase to the cell. No T7 RNA polymerase will be present in the cell before infection, so any target DNA that can be cloned under control of a T7 promoter should be expressible in this way.

A convenient bacteriophage for delivering T7 RNA polymerase to the cell is CE6, a lambda derivative that carries the gene for T7 RNA polymerase under control of the phage p_L and p_I promoters and also has the cI857 thermolabile repressor and the *Sam7* lysis mutations (7). When CE6 infects HMS174, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed. Comparable levels of target RNAs and proteins are produced whether T7 RNA polymerase is delivered to the cell by induction or by infection.

GROWTH MEDIA

BL21 and HMS174 grow on minimal or complex media, and presumably a wide range of growth media would be suitable for growth of these strains and expression of target DNAs. For routine growth of cultures we use ZB medium (10 g N-Z-amine A and 5 g NaCl in 1 liter of water). N-Z-amine A is obtained from Sheffield Products (P.O. Box 398, Memphis, TN 38101); Bacto Tryptone (Difco) in place of N-Z-amine A in any of the media described gives essentially equivalent results. Defined media are usually M9 medium, containing 1 g NH_4Cl, 3 g KH_2PO_4, 6 g Na_2HPO_4, 4 g glucose and 1 ml 1 M $MgSO_4$ in 1 liter of water, or B2 medium, which is essentially M9 medium in which all but 0.16 mM of the phosphate is replaced by salts and bis-Tris buffer (26). M9mal and B2mal are the equivalent media in which glucose is replaced by maltose. Richer media include M9ZB, which contains the components of both M9 and ZB; ZY (10 g N-Z-amine A, 5 g Bacto yeast extract (Difco), and 5 g NaCl in 1 liter of water); or ZYG (ZY medium plus 4 g glucose per liter). Sugars, $MgSO_4$, and phosphate solutions are autoclaved separately and added to the media after cooling.

The many small (1-3 ml) cultures generated in cloning DNA fragments and isolating recombinant plasmids are usually grown in M9ZB + antibiotic in standing 13x100 mm culture tubes in a 37 °C incubator. After overnight incubation of standing cultures, HMS174 remains largely dispersed throughout the culture whereas BL21 cells mostly settle to the bottom of the tube. Larger cultures are grown in shaking flasks at 37 °C. When cultures are grown overnight in shaking flasks, the growth medium is usually ZB, because continued shaking after saturation in rich media containing glucose (such as M9ZB or ZYG) leads to some lysis of BL21.

Dilutions for titering bacteria or phage are made in ZB. Plating is done by mixing samples with 2.5 ml of melted top agar (ZB containing 0.7% agar) and spreading on standard 100 x15 mm plastic Petri dishes containing 20 ml of hardened bottom agar (ZB containing 1% agar).

Where antibiotics are added to liquid growth media or bottom agar of Petri dishes, ampicillin is typically 20 μg/ml and chloramphenicol or kanamycin 25 μg/ml. It is not necessary to use pre-formed antibiotic plates: where bottom agar contains no antibiotic, selection can be accomplished by adding 200 μg of ampicillin or 250 μg of chloramphenicol or kanamycin per ml of top agar at the time of plating.

For induction of T7 RNA polymerase in BL21(DE3) or HMS174(DE3), a growing culture is made 0.4 mM in IPTG. For induction on plates, top agar is made 1 mM in IPTG at the time of plating.

STORAGE OF STRAINS

For long term storage, 1.5 ml of a growing or saturated culture is placed in a cryovial, mixed with one-tenth volume of 80% glycerol, and the tube is stored directly in a -75 °C freezer. We avoid higher concentrations of glycerol because they become increasingly toxic to cells at room temperature. Plasmid-bearing strains, particularly those having any tendency toward instability, are titered at the time of freezing to be sure that the vast majority of cells in the culture have the intended host-plasmid combination (see section on TOXIC GENES AND PLASMID INSTABILITY). To inoculate a culture from the frozen stock, a few μl is scraped or melted from the surface, typically with a sterile pipette or plastic culture loop, and the remainder is returned to the freezer without thawing. Cells stored at -75 °C in this way have remained viable for several years and presumably will remain viable for very long periods. (In our experience, cells survive for many months in a -20 °C freezer, but survival for longer periods is variable.)

CLONING TARGET DNAS

Target DNAs are cloned into the pET vectors by standard techniques (27). We use HMS174 as the host for initial cloning and analysis of plasmids, because plasmid DNAs remain monomers in the *rec*A background and expression of the target DNA is minimal in the absence of T7 RNA polymerase. Any easily transformable, preferably *rec*A strain should be suitable for this purpose. BL21 is not appropriate because it is *rec*A$^+$ and it has a somewhat lower transformation efficiency. DE3 lysogens should not be used for initial cloning because of potential problems from expression of the target gene by the small amounts of T7 RNA polymerase present in the uninduced cell.

Once the desired plasmid is obtained, the target DNA can be expressed by infection with CE6, or, if the target plasmid is stable, by induction in BL21(DE3) or HMS174(DE3) or in one of these strains carrying pLysS. To test for stability, transformations with the target plasmid are attempted in a set of four strains: BL21; BL21(DE3); BL21(DE3)pLysS; and BL21(DE3)pLysE, or the equivalent set based on HMS174. Plasmids having no target gene, or whose target gene is relatively innocuous, will give transformants with about equal frequency in all four hosts; at some level of toxicity, plasmids will fail to transform the DE3 lysogen itself (where the basal activity of T7 RNA polymerase is highest); at somewhat higher toxicity the lysogen containing pLysS will also fail to be transformed; and a few target plasmids are so toxic that even the lysogen containing pLysE cannot be transformed, although the host that lacks the gene for T7 RNA polymerase will be transformed at normal frequency.

A few of the target genes we have worked with are more stable in HMS174(DE3) or its derivatives than in the equivalent derivative of BL21(DE3). A possible explanation for this difference (suggested by Stewart Shuman, personal communication) is that small amounts of some target gene products induce the SOS response of *E. coli*. This in turn induces the prophage and kills BL21(DE3); but the *rec*A deficiency of HMS174 prevents induction of the prophage and killing of HMS174(DE3).

EXPRESSING TARGET DNA BY IPTG INDUCTION OF BL21(DE3) OR HMS174(DE3)

If a target plasmid can be established in BL21(DE3), HMS174(DE3) or in one of these strains containing pLysS, induction of T7 RNA polymerase by IPTG is a convenient way to direct expression of the target DNA. We usually grow the cells in M9 or M9ZB containing the selective antibiotic (and also 25 µg chloramphenicol/ml if the cells carry pLysS), and make the culture 0.4 mM in IPTG when the culture reaches an OD_{600} of 0.6-1.

Immediately before induction, the culture is titered to determine the fraction of cells that carry inducible plasmid. This involves plating on four plates, which differ in the composition of the top agar used in plating. Typically, the culture would be plated at a dilution of 10^5 on plates that have both IPTG and antibiotic or just IPTG added to the top agar, and at a dilution of 2×10^6 on plates that have just antibiotic or nothing added to the top agar. This test and its interpretation is described more fully in the next section. (We usually do not test for the relatively stable pLysS.)

If appropriate attention is paid to possible problems of plasmid instability, more than 98% of the cells in the culture will usually contain expressible target plasmids. Cells are usually harvested 2-3 hr after induction, enough time for substantial accumulation of target protein but before the culture can be overgrown with cells that have lost plasmid or are otherwise unproductive. However, some target proteins continue to accumulate for much longer times.

Occasional results have suggested that the basal activity of T7 RNA polymerase in uninduced cells may be somewhat lower when the growth medium contains glucose than when it does not. Induction of the *lac*UV5 promoter (which directs transcription of the T7 RNA polymerase gene in the DE3 lysogens) is not subject to catabolite repression (28) but perhaps the repressed promoter retains some sensitivity. We have not analyzed this effect in detail, but we suspect that media containing glucose (such as M9 and M9ZB) may be more suitable than media without glucose (such as ZY) for growing DE3 lysogens for the induction of target genes.

TOXIC GENES AND PLASMID INSTABILITY

Plasmid pBR322 and many of its derivatives are relatively stable and are retained by a very high fraction of host cells even after growth for many generations in the absence of a selective antibiotic. However, problems of plasmid instability can arise when a gene whose product is toxic to the host cell is cloned in the plasmid. The level of expression may be such that the plasmid can be maintained but growth of the cell is impaired; segregation of cells lacking plasmid may also be increased because of decreased copy number or for other reasons. In such a situation, cells that lack the plasmid can rapidly overgrow the culture whenever selective antibiotic is lacking. If the plasmid is to be maintained in a significant fraction of the cells, the culture must not be allowed to grow in the absence of selection for the plasmid.

Use of ampicillin as a selective antibiotic requires special care, because β-lactamase is made in substantial amounts and is secreted into the medium, where it can destroy all of the ampicillin. This means that a culture whose cells carry an unstable plasmid will be growing under ampicillin selection only until enough β-lactamase has been secreted to destroy the ampicillin in the medium; from that point on, cells that lack plasmid will not be killed and will begin to overgrow the culture. For a typical pBR322-based plasmid growing in a medium containing 20 μg ampicillin per ml, this point is reached when the culture is barely becoming turbid, perhaps around 10^7 cells per ml. Growth in the presence of 200 μg ampicillin per ml delays this point to a slightly higher cell density, but given the catalytic activity of β-lactamase, it would not be feasible to add enough ampicillin to the medium to keep the cells under selection all the way to saturation.

A further complication is that certain toxic genes, while having little effect on cells that are growing logarithmically, kill cells at saturation. Almost all cells retain plasmid until saturation, but upon continued incubation, fewer and fewer plasmid-containing cells survive and, because no ampicillin remains, cells that lack plasmid overgrow the culture.

A culture grown to saturation from selective conditions will have secreted a considerable amount of β-lactamase into the medium even if it becomes substantially overgrown by cells that lack plasmid. Subcultures might typically be grown from dilutions of 200 to 1000 fold into fresh ampicillin-containing medium. However, enough β-lactamase is typically present in the saturated culture that, even at these dilutions, enough remains to destroy all of the ampicillin before the cells that lack plasmid can be killed. Therefore, the subculture will grow completely in the absence of selection. The inoculum may already have had a substantial fraction of cells lacking plasmid, and by the time the subculture has grown to a density where expression of the target gene is induced, it is quite possible that only a minor fraction of the cells will contain the target plasmid. Failure to appreciate these potential problems can easily lead to the erroneous conclusion that certain target genes are poorly expressed, when in fact only a small fraction of cells in the cultures that were tested contained plasmid.

Most of the pET vectors described here have ampicillin as the selective antibiotic, and simple precautions are advisable to maximize retention of plasmid through the procedures for isolating, maintaining and expressing target plasmids. We use the following isolation protocol, which usually produces the highest possible fraction of cells containing functional target plasmid. A colony from the transformation plate is inoculated into 2 ml M9ZB + ampicillin and incubated for a few hr, until the culture becomes lightly turbid, when a sample is streaked on a plate containing ampicillin to obtain a single colony. As soon as the colony develops (usually overnight at 37 °C), it is inoculated into 2 ml of M9ZB + ampicillin and grown almost to saturation, when 1.5 ml of culture is mixed with 0.15 ml of 80% glycerol in a cryovial and stored in a -75 °C freezer. If there is any question about the possible stability of the plasmid, the culture is titered at the time of freezing to determine what fraction of the cells contain functional target plasmid.

For cells that carry a plasmid but no source of T7 RNA polymerase, titering in the presence and absence of ampicillin (200 µg/ml in the top agar) determines the fraction of cells that have plasmid. When the target plasmid is carried in BL21(DE3) or HMS174(DE3), the fraction of cells able to express the target gene can be tested by including 1 mM IPTG in the top agar, which will prevent colony formation by any cell that has both the inducible gene for T7 RNA polymerase and a functional target plasmid (but will not prevent growth of cells that lack plasmid or mutants that have lost the ability to express target DNA). In the presence of pLysS, IPTG also prevents colony formation (except in rare cases, including pET-3 itself). In the presence of pLysE, IPTG usually does not prevent colony formation unless the target gene product is toxic.

In practice, DE3 lysogens that carry a target plasmid that confers ampicillin resistance are titered on four plates, which have ampicillin, IPTG, both, or neither added to the top agar: all viable cells will grow on the plate with no additive; only cells that retain plasmid will grow in the presence of ampicillin; only cells that have lost plasmid or mutants that have lost the ability to express target DNA will grow in the presence of IPTG; and only mutants that retain plasmid but have lost the ability to express target DNA will grow in the presence of both ampicillin and IPTG. In a typical culture useful for producing target proteins, almost all cells will form colonies both on plates without additives and on plates containing only ampicillin; less than 2% of the cells will form a colony on plates containing only IPTG; and less than 0.01% will form a colony on plates containing both ampicillin and IPTG. With unstable target plasmids, the fraction of cells that have lost plasmid will be reflected by an increase in colonies on the IPTG plate and a decrease on the ampicillin plate. Mutants that retain plasmid but have lost the ability to express target DNA arise in some cases, but relatively infrequently.

If the plasmid is stable, cultures for expressing the target gene can be grown from the freezer stock without special precautions: even if the ampicillin in the fresh medium is destroyed or if the culture is incubated overnight at saturation, almost all of the cells will retain the target plasmid. However, if the target plasmid is unstable, cultures are grown from a dilution of 10^4 or higher from the freezer stock and grown directly to the density used for expression. Because of the potential for loss of plasmid, we always determine the composition of the cells in the culture by plating immediately before induction. This simple test can be invaluable in interpreting any unusual properties of an induction and in making sure that effort is not wasted on processing cells that had suboptimal levels of expression.

Some of the problems outlined here might be circumvented by using the pET-9 series of vectors, which provide resistance to kanamycin rather than ampicillin. However, the general principles and procedures should be useful in dealing with the problems of cloning toxic genes in any vector.

FACTORS THAT AFFECT PRODUCTION OF TARGET PROTEINS

This T7 expression system has produced substantial amounts of target protein from a wide variety of genes, both prokaryotic and eukaryotic. However, some proteins are made in disappointingly small amounts, for reasons that are obvious in some cases and obscure in others. We here summarize briefly some of the known or likely reasons for obtaining low levels of expression.

The target protein itself may interfere with gene expression or with the integrity of the cell. Sometimes pulse labeling shows a gradual or rapid decrease in the rate of protein synthesis as target protein accumulates, or sometimes all protein synthesis stops before any target protein can be detected. Occasionally, considerable lysis of a culture is observed.

One might expect that instability of target mRNA might limit expression in some cases, although in each case we have examined, substantial amounts of target mRNA seem to accumulate. This apparent stability of target mRNA could be due to the stem-and-loop structures at both ends of RNA initiated at the usual *ϕ10* promoter and terminated at Tϕ or cut at R*1.1*; or the mRNA may be relatively inaccessible to exonucleases by being embedded in the long RNAs produced by T7 RNA polymerase in the absence of Tϕ; or perhaps so much RNA is produced that the normal mRNA degradation system is overloaded.

Instability of certain target proteins might also be expected, although BL21 is apparently deficient in the *lon* and *ompT* proteases and many proteins produced in this strain are quite stable. Some relatively short proteins produced by out-of-frame fusions are also quite stable in this strain whereas others are so rapidly degraded as to be undetected by pulse labeling.

Many target proteins seem to be made in equivalent amounts whether or not the Tϕ transcription terminator is present in the vector. In some cases, however, having Tϕ behind the target gene increases the production of target protein. In the cases we have encountered, the target mRNA is translated from its own translation initiation signals rather than from the strong T7 gene *10* signals. A possible interpretation is that some translation initiation signals do not compete well against the *bla* mRNA, which is made along with the target mRNA, and that Tϕ, by reducing the amount of this competing mRNA, allows more target protein to be made. In the pET-9 vectors, where the *kan* gene and the target gene have opposite orientations, no competing mRNAs are known to be made along with the target mRNA.

Some target proteins are made in relatively small amounts even though both the mRNA and protein appear to be relatively stable and the coding sequence is joined to the efficient T7 translation initiation signals. The cause of the poor translation of these mRNAs is not well understood but could perhaps be due to factors such as unfavorable distributions of rare codons, relatively high levels of translational frameshifting, or interfering structures in the mRNA.

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Aanvullende gegevens bij kennisgeving ingeperkt gebruik

TITEL: GROOTSCHALIGE PRODUKTIE VAN INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV) VP2 ANTIGEEN

Volgens artikel 11, tweede lid, van het besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen, dienen in geval van Categorie B handelingen met Groep II GGO's in ieder geval de gegevens bedoeld in bijlage 4, onderdeel 3, bij dit Besluit, aangeleverd te worden.
De gevraagde gegevens worden hieronder verstrekt, waarbij de nummering verwijst naar bedoelde bijlage 4. Tevens worden de vereiste gegevens zoals vermeld in bijlage 4, ondereel 4, verstrekt.

(3.a.)
2.a. n.v.t.

2.b. Zie kennisgeving.

2.c. Zie kennisgeving.

2.e. Grootschalige kweken en downstream processing voor vaccin produktie.

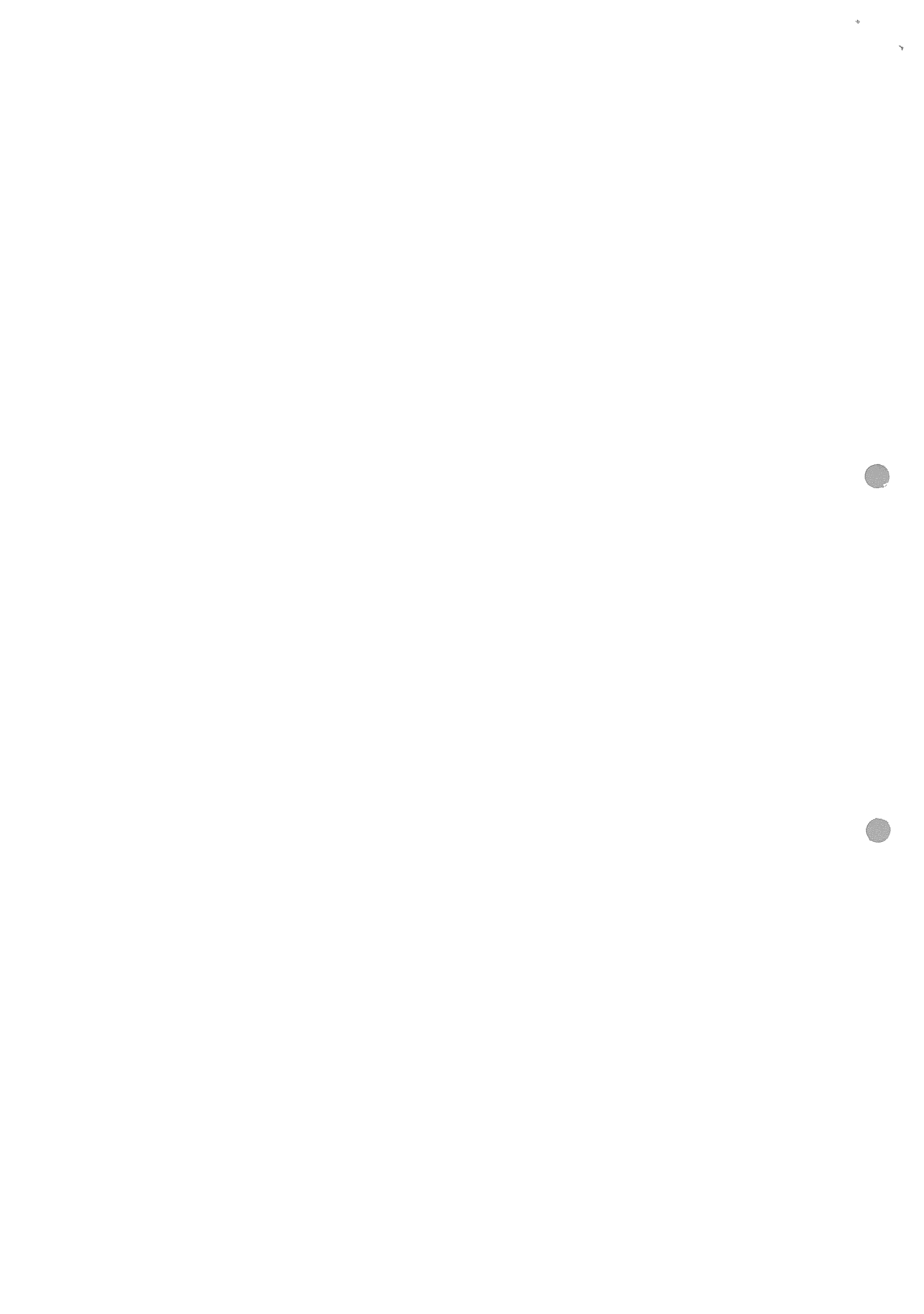
2.f. Zie punt 4.g. hieronder.

2.g. Kweekvolumes 70 L en 300 L.

3.b. Het GGO wordt vanuit een workingseed geënt in een voorkultuur, en vervolgens gekweekt in een fermentor. Het VP2 eiwit wordt in de vorm van "inclusion bodies" gezuiverd middels sonificatie en centrifugatie, gebruik makend van verhitting tot 60°C en toevoeging van 4 M ureum. Het GGO wordt tijdens het proces geïnactiveerd met chlorocresol of ß-propiolactone.

3.c. Zie Addendum I (aanvraag Hinderwet vergunning) en Addendum II (Veiligheidsvoorschrift voor het werken met GGO's in de afdeling Bacteriologische Productie; S.O.P. no.0212-5519-002).

3.d. Het betreft hier een Groep II GGO (zie kennisgeving punt 4.1), waarop inperkingscategorie GS-I van toepassing is.
Wat betreft de voorzieningen voor afvalstoffen beheer en de veiligheidsmaatregelen, wordt verwezen naar Addendum I (aanvraag Hinderwet vergunning) en Addendum II (Veiligheidsvoorschrift Bacteriologische Produktie).



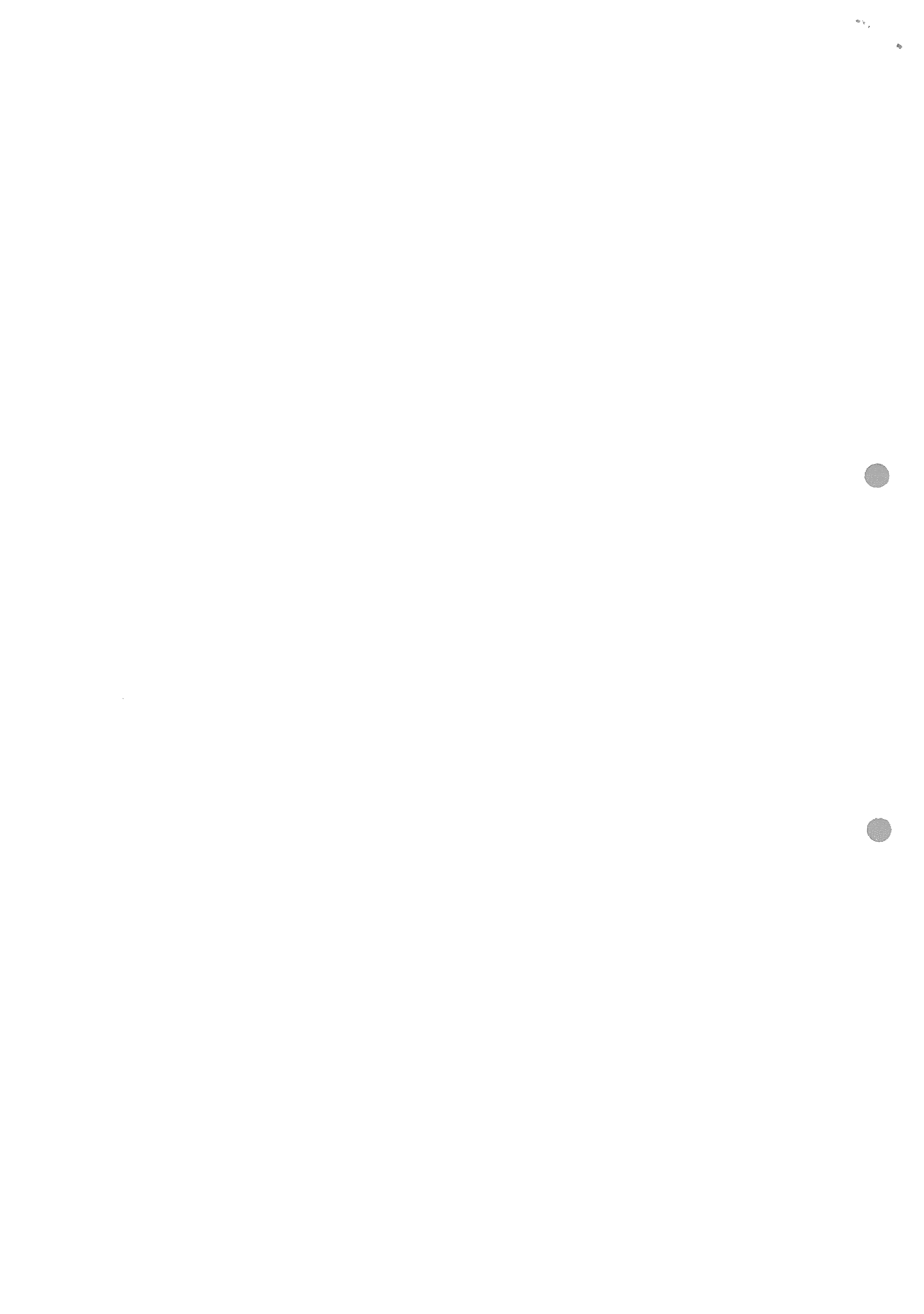
4.b. Zie de kennisgeving (punt 5).

4.d. Produktie vindt plaats op de afdeling Bacteriologische Produktie van Intervet International BV te Boxmeer. Het GGO wordt gekweekt in roestvrijstalen fermentoren van 70 tot 300 liter. De fermentoren zijn voorzien van "fixed piping" en "mechanical seals". Beluchting en afgassing van de fermentoren vindt plaats via absoluut filters. Downstream processing vindt plaats in gesloten systemen, via roestvrijstalen containers en/of pijpleidingen.

4.e. Zie Addendum I (aanvraag Hinderwet vergunning) en Addendum II (Veiligheidsvoorschrift Bacteriologische Productie). Verder zijn milieu-aspecten van emissies opgenomen in het Handboek Intern Milieuzorg Systeem Intervet, december 1991 (niet bijgevoegd).

4.f. Zie Addendum II (Veiligheidsvoorschrift Bacteriologische Productie), Addendum III (Ongevalsprogramma Bacteriologische Productie; S.O.P. no.0212-5519-003), en Addendum IV (Ontruimingsprocedures; uit Veiligheidsvoorschriften Intervet lokatie Boxmeer, VGWM-dienst, mei 1991).

4.g. Zie kennisgeving, deel 3A-1 punt 4.



ADDENDUM I.

Uit: aanvraag Hinderwet vergunning d.d. 26 november 1993

1.2. Bacteriologische produktie

1.2.1. Specificatie van het produktie proces

In de afdeling bacteriologische produktie, gebouw nr. 40, worden vaccins gemaakt met behulp van fermentoren. Een fermentor is een gesloten vat met een roerwerk.
Er wordt gestart met een medium (waterige oplossing van voedingszouten) waaraan vervolgens een micro-organisme wordt toegevoegd.
Micro-organismen hebben voor hun groei een aantal randvoorwaarden nodig namelijk voldoende voedingsstoffen, voldoende water, optimale zuurgraad, optimale temperatuur en voldoende zuurstofspanning in het medium.
Na afloop van de kweek worden micro-organismen afgedood met physische of chemische methoden, waarna opwerking plaatsvindt d.m.v. centrifugatie en/of filtratie. De diverse totaalprocessen binnen de afdeling bacteriologische produktie lopen in principe op de volgende manier af:
- bereiding van medium;
- opkweken van micro-organisme;
- opwerken van cultuur, verzamelen van micro-organismen of supernatant;
- zuiveren van de gewenste component;
- bereiden van antigeenfase.

Na afloop van elk deelproces moet alle daarbij gebruikte apparatuur inclusief de procestanks gereinigd en gesteriliseerd worden. De 2000 l fermentor en bijbehorende bulktanks en apparatuur worden na afloop van het bereidingsproces via een C.I.P.-installatie (Cleaning In Place) schoongemaakt en via een S.I.P.-installatie (Steaming In Place) gesteriliseerd. Voor het reinigen van fermentoren, centrifuges en tanks wordt een detergent gebruikt. Na gebruik wordt dit geloosd op het riool.
Alle overgebleven besmette materialen worden gedesinfecteerd alvorens de produktieomgeving te verlaten.

Produktieruimtes worden na afloop van een produktiesessie schoongemaakt.

Waar met levende cultures wordt gewerkt staan de ruimtes onder negatieve luchtdruk, waar met geïnactiveerd materiaal wordt gewerkt staan de ruimtes onder positieve druk. Er zijn ook aseptische ruimtes in het gebouw.

Recombinant DNA-vaccins

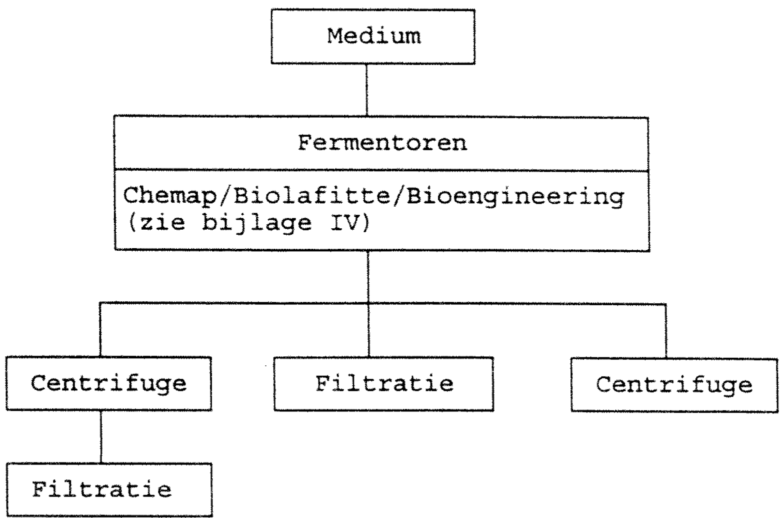
In het Bacteriologische Produktie gebouw wordt op een produktieschaal tot een niveau van 2000 liter gewerkt.
Het gebouw staat los van alle andere produktie-, research- en administratiegebouwen. De ruimten waar met levende recombinant DNA micro-organismen gewerkt wordt, staan onder een lichte onderdruk ten opzichte van de omgeving (-15 Pascal t.o.v. de gang).
De constructie van het gebouw en de daarin aanwezige laboratoria staat werk op GS-1 (te vergelijken met C-1 op R & D-niveau) niveau toe, terwijl in de praktijk normaliter op GILSP (te vergelijken met VMT op R & D-niveau) niveau wordt gewerkt, gezien de aard van de biologische inperking van de te gebruiken r-DNA stammen.

Beschrijving van de werkzaamheden en de daarbij gebruikte technieken en aanwezige recombinantstammen

- De kleinschalige technieken die gebruikt worden in het laboratorium beperken zich tot het kweken van micro-organismen in vloeibare cultures in volumina van 1 tot 500 ml in schudkolven. Dit ter opschaling van entcultures voor de fermentoren.
- De fermentoren zijn gezien hun fysische inperkingsniveau geschikt voor het werken op GS-1 niveau. De fermentoren zijn ontworpen voor volumina tot maximaal 2000 liter cultuurvloeistof.
- Elke fermentor staat in een eigen ruimte met gladde wanden, vloeren en plafonds. Deze ruimtes worden individueel op een negatieve druk van ongeveer -15 Pascal ten opzichte van de gang gehouden.
- Alvorens over te gaan tot de zogenaamde downsteam processing wordt het r-DNA micro-organisme in de fermentor of in een gesloten roestvrijstalen vat afgedood met behulp van een gevalideerde methode.

De aanwezige recombinantstammen zijn allen ingeschaald op GILSP niveau.

Blokschema bacteriologisch produktieproces



1.2.2. Milieu-aspecten

1.2.2.1. *Bodem*

In de bacteriologische produktie afdeling vindt alleen de voor het produktieproces noodzakelijke opslag plaats van:
- grondstoffen zoals zouten, glaswerk, etc.
- chemicaliën in een losse kast. Hoeveelheid chemicaliën die hier wordt opgeslagen is minder dan 100 kg.
- verbruiksartikelen zoals tissues, zeep, etc.
- halffabrikaten die nog niet zijn vrijgegeven.

Alle andere voorraden worden opgeslagen in het magazijn. Alle produktieprocessen vinden plaats in gebouwen met vloeistofdichte vloeren zodat bodem- en grondwaterverontreiniging is uitgesloten. Alle eindprodukten en/of geïnfecteerde afval materialen worden gedesinfecteerd voordat ze de produktie afdeling verlaten door middel van oppervlakte desinfectie of door autoclaveren (121° C, 30 min.).

In de keuken van de bacteriologische produktie worden alleen gedecontamineerde filters schoongemaakt en gespoeld. Verder worden hier de media t.b.v. de eigen afdeling bereid. Tevens worden hier de produktie ketels schoongemaakt en gedesinfecteerd. De keuken afvoeren en alle afdelingswasbakken zijn aangesloten op het Intervet rioleringssysteem en deze loost op het gemeentelijk riool. Zie lozingsvergunning W.V.O. d.d. 8 maart 1988.

1.2.2.2. *Lucht*

Om de benodigde positieve en/of negatieve luchtdruk ten opzichte van de omgeving te handhaven, zijn verschillende luchtbehandelingsinstallaties geïn-stalleerd. Alleen de installaties t.b.v. de clean rooms zijn voorzien van Hepa filters (99,99 %) op zowel de ingaande als uitgaande lucht (zie bijlage IV). Om de juiste werking te controleren worden deze filters jaarlijks gedoptest.
Doptest-resultaten en verslaglegging van eventueel genomen akties zijn centraal opgeslagen bij de Technische Dienst. Indien noodzakelijk vanwege contaminatie van of produkt of omgeving, wordt inperkende apparatuur zoals fermentoren en biosafety cabinets gebruikt (zie bijlage IV).
Door de genomen maatregelen is luchtverontreiniging uitgesloten. Gassen, dampen en stank komen niet vrij bij het bacteriologisch produktieproces, alleen lage concentraties formaline dat gebruikt wordt bij desinfectie.

1.2.2.3. *Geluid*

Gezien de aard van de opgestelde apparatuur (zie bijlage IV) en de in de regel in dagdienst uitgevoerde werkzaamheden, zijn de niveaus van geluid, trillingen, licht en straling van geen betekenis en vallen ruimschoots binnen de daarvoor geldende normen.

1.2.2.4. *Gevaar*

Aan het bacteriologisch produktieproces zijn geen specifieke gevaren verbonden.
Calamiteiten borging vindt plaats vanwege inspecties door:
- Biosafety officer
- Chemical safety officer
- Eigen organisatie
- Veterinaire inspecties

1.2.2.5. *Afval*

Buiten het huishoudelijk afval (gebruiksartikelen) worden door het bacteriologische produktieproces de volgende afvalstromen geproduceerd:

- Bedrijfsafval:
  Dit bestaat uit halffabrikaten of eindprodukten die door de kwaliteitscontrole worden afgekeurd. Indien mogelijk worden deze afgekeurde produkten in andere produkten opgewerkt, anders worden ze bij onbesmet materiaal (vnl. eiwitten) geloosd op het riool en bij besmet materiaal ter vernietiging afgevoerd naar de algemene vuilverbranding in Rijnmond (A.V.R.), na eerst een desinfectie te hebben ondergaan op 121° C gedurende 30 minuten.

- Bacteriologisch afval:
  De ten gevolge van het bacteriologisch produktieproces ontstane niet bruikbare delen worden met formaline afgedood en vervolgens geloosd op het riool.

- Klein chemisch afval:
  Klein chemisch afval zoals batterijen, enz. wordt apart ingezameld en afgevoerd naar de A.V.R..

HINDERWETAANVRAAG INTERVET INTERNATIONAL B.V., BOXMEER 1993

INTERVET INTERNATIONAL B.V.
BOXMEER

RUIMTEBENAMING MET OVERZICHT BRANDBLUSAPPARATUUR / AFWERKSTAAT

Building Number:	P401 Bacto produktie	**Drawing Number:**	P401BH01

Rnr.	Omschrijving	Brandblus- apparatuur	Vloer- afwerking	Wand- afwerking	Plafond- afwerking
01	Cellar		01	11	21

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INTERVET INTERNATIONAL B.V.
BOXMEER

RUIMTEBENAMING MET OVERZICHT BRANDBLUSAPPARATUUR / AFWERKSTAAT

Building Number:	P401 Bacto produktie	**Drawing Number:**	P401BH01

Rnr.	Omschrijving	Brandblus- apparatuur	Vloer- afwerking	Wand- afwerking	Plafond- afwerking
01	Entree		04	14/17	24/26
02	Luchtsluis		04	14/17	24/26
03	Kantoor		04	14/17	23/24/26
04	Technische ruimte	Koolzuurblusser 5 kg	01	11	24/26
05	Toiletten		04	17	24/26
06	Luchtsluis keuken		04	17	24/26
07	Keukenruimte	Koolzuurblusser 5 kg	02	14/17	23/24/26
08	Steriele ruimte		04	14/17	24/26
09	Luchtsluis		04	17	24/26
10	Gang	Brandslanghaspel 19mm/20m 3x Koolzuurblusser 5 kg (5x)	04	14/17	24/26
11	Lab	Koolzuurblusser 5 kg	04	14/17	24/26
12	Fermentor/ Centrifugeruimte	Koolzuurblusser 5 kg	04	14/17	24/26
17	Fermentorruimte		04	14/17	24/26
18	Centrifugeruimte		04	14/17	24/26
19	Fermentorruimte		04	14/17	24/26
20	Fermentorruimte		04	14/17	24/26
21	Fermentorruimte		04	14/17	24/26
22	Filtratieruimte		04	14/17	24/26
23	Aseptische ruimte		04	14/17	24/26
23a	Luchtsluis personeel		04	17	24/26

23b					
23c	Luchtsluis goederen		04	17	24/26
24	Isolatorenruimte		04	17	24/26
25	Luchtsluis personeel		04	17	24/26
26					
27	Steriele ruimte		04	14/17	24/26
27a	Luchtsluis goederen		04	17	24/26
28	Koude kamer		04	17	24/26
29	Koude kamer		04	17	24/26
30	Warme kamer		04	17	24/26
31	Koude kamer		04	17	24/26
32	Incubatieruimte		04	17	24/26
33	Warme kamer		04	17	24/26
34	Luchtsluis goederen		04	17	24/26
35	Luchtsluis personeel		04	17	24/26
36					
37	Aseptische ruimte		04	14/17	24/26

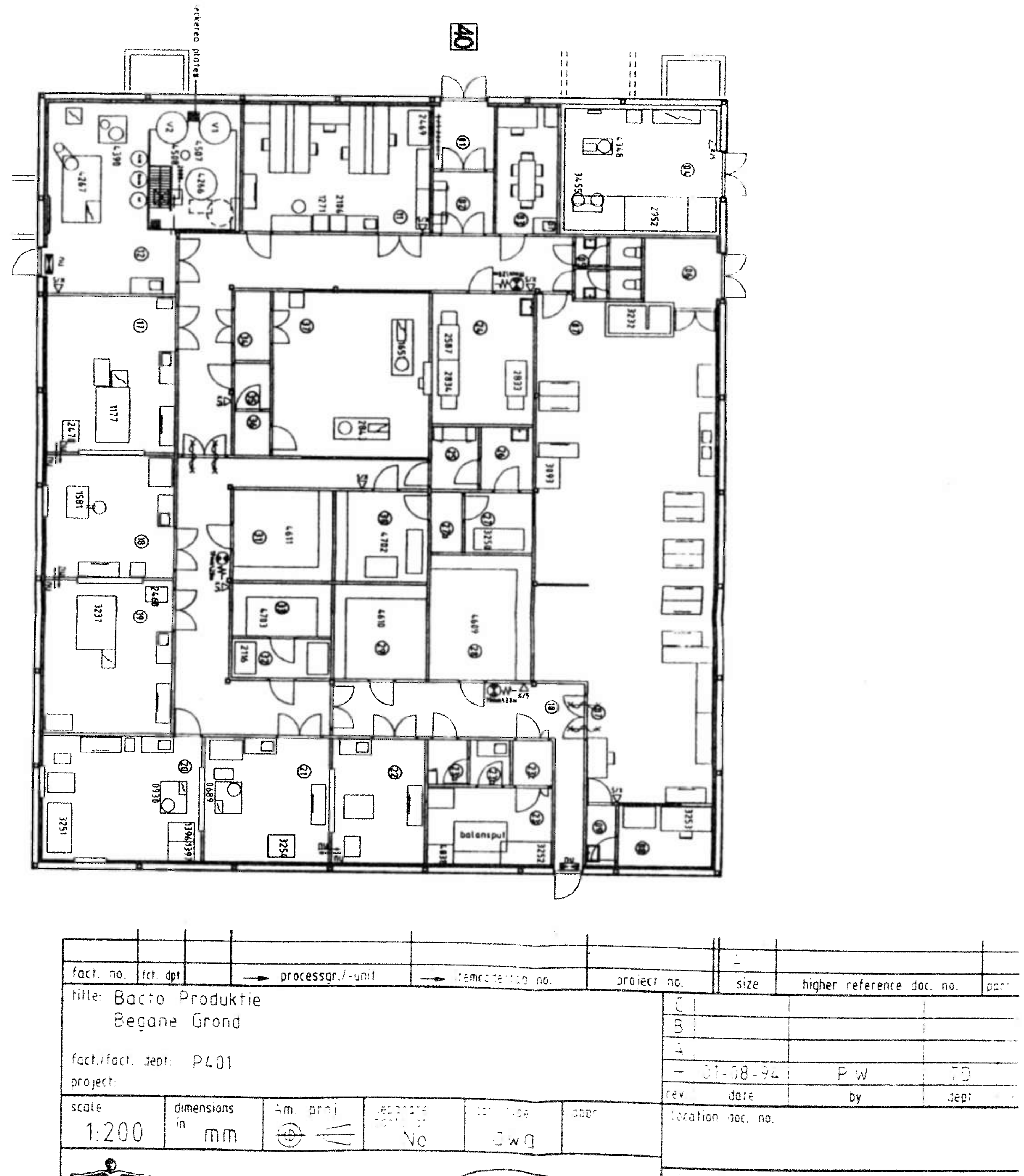
HINDERWETAANVRAAG INTERVET INTERNATIONAL B.V., BOXMEER 1993

INTERVET INTERNATIONAL B.V.
BOXMEER

RUIMTEBENAMING MET OVERZICHT BRANDBLUSAPPARATUUR / AFWERKSTAAT

Building Number:	P402 Bacto produktie verdieping	**Drawing Number:**	P402BH01

Rnr.	Omschrijving	Brandblus-apparatuur	Vloer-afwerking	Wand-afwerking	Plafond-afwerking
01	Technische ruimte		01/04	17	26



fact. no.	fct. dpt	processgr./-unit	itemcodering no.	project no.	size	higher reference doc. no.	part

title: Bacto Produktie
Begane Grond

fact./fact. dept: P401
project:

rev	date	by	dept
C			
B			
A			
–	01-08-94	P.W.	TD

scale	dimensions in	Am. proj			
1:200	mm		No	Dwg	

location doc. no.

AKZO © Intervet International bv Boxmeer The Netherlands

Intervet

size / doc. no.: A3-P401BH02 sh. 1

93-01 ACAD12/GEN7.01

ADDENDUM II

WERKVOORSCHRIFTEN S.O.P.			
Onderwerp:	Veiligheidsvoorschrift voor het werken met GGO's in de Afdeling Bacteriologische Productie		S.O.P. no: 0212-5519-002 Pag: 1 van: 14
Auteur:	V. Rijnierse		Interne code:
Uitgifte: 23-08-1993	Version No: 1 Copy No:		Goedgekeurd: VR: BVF: QA:
Betrokken Afdelingen:	Bacteriologische Productie		

ALGEMEEN

De Veiligheidsvoorschriften voor het werken met pathogene micro-organismen zijn voor de Afdeling Bacteriologische Productie van toepassing. Deze voorschriften worden hieronder nogmaals kort samengevat inclusief een aantal specifieke voorschriften die van belang zijn voor het werken met genetisch gemodificeerde micro-organismen (GGO's) op GS-I niveau.

1. Toegang tot het gebouw is uitsluitend voorbehouden aan daartoe bevoegde personen. Het werken met GGO's is voorbehouden aan personen die op de hoogte zijn van de aard en risico's van de werkzaamheden en voor welke personen de BVF toestemming heeft verleend. In dit kader kan het volgen van een VMT-cursus verplicht worden gesteld.

2. De toegangsdeuren tot de Afdeling zijn altijd dicht en buiten de werkuren afgesloten.

3. Voor omkleedprocedures wordt verwezen naar S.O.P. Nr. 0212-5519-001.

4. Het dragen van ringen, armbanden en polshorloges is niet toegestaan. Persoonlijke zaken (zoals b.v. handtassen e.d.) mogen niet mee naar binnen worden genomen.

5. Alle werkzaamheden worden genoteerd op de productie protocollen. Zij worden ook genoteerd in de bij de diverse apparaten behorende logboeken en afgeparafeerd. In de logboeken worden ook alle bijzonderheden als storingen, reparaties, ongevallen etc. vermeld.

6. Het is niet toegestaan om te eten, te drinken of te roken in de werkruimtes. Handen dienen vóór het verlaten van een werkruimte, of direct nadat er mogelijk contact is geweest met besmet materiaal, gewassen te worden met zeep, waarna hand-desinfectie met Sterilium plaats vindt. Bij mogelijke contaminatie van de werkplaats dient direct gedesinfecteerd te worden met Bacillol. Materialen als tissues e.d., die bij dergelijke werkzaamheden worden gebruikt worden in RVS-tonnen gedeponeerd voor decontaminatie.

7. Het is niet toegestaan met de mond te pipetteren of een andere handeling te verrichten waarbij het gevaar bestaat voor inwendige contaminatie. Contact tussen handen en gezicht dient vermeden te worden en voor neussnuiten kunnen tissues gebruikt worden.

8. Verspreiding van micro-organismen dient zo veel mogelijk voorkomen te worden zoals bij het aanraken van besmette randen van kweekflessen of schalen, buitenzijde van pipet, etc. of het onbeschermd neerleggen van mogelijk besmette materialen.

9. Voor alle werkzaamheden geldt dat het risico van aërosolen tot een minimum beperkt dient te worden. Het gebruik van injectienaalden is alleen dan toegestaan indien er geen alternatief voorhanden is.

10. Werkzaamheden met GGO's worden zo veel mogelijk in een biosafety kast uitgevoerd of in zelf-inperkende apparatuur, zoals fermentoren, tanks en gesloten centrifuge-systemen.

11. Alle materialen waarin zich GGO's kunnen bevinden dienen duidelijk geëtiketteerd te zijn met naam, datum, microörganisme en batchnummer.

<table>
<tr><td colspan="3">WERKVOORSCHRIFTEN S.O.P.</td></tr>
<tr><td>Onderwerp:</td><td>Veiligheidsvoorschrift voor het werken met GGO's in de Afdeling Bacteriologische Productie</td><td>S.O.P. no: 0212-5519-002
Pag: 3 van: 3 4</td></tr>
<tr><td>Auteur:</td><td>V. Rijnierse</td><td>Interne code:</td></tr>
<tr><td>Uitgifte:

23-08-1993</td><td>Version No: 1

Copy No:</td><td>Goedgekeurd:

VR: BVF: QA:</td></tr>
<tr><td>Betrokken Afdelingen:</td><td colspan="2">Bacteriologische Productie</td></tr>
</table>

12. Vervoer van GGO's binnen het laboratorium, b.v. naar het IPC, dient in afgesloten containers plaats te vinden. Wanneer deze GGO's naar een lokatie buiten het laboratorium worden vervoerd moeten op de container de navolgende gegevens aangegeven zijn: afzender, geadresseerde, datum, soort monster en de fysische inperkingscondities waarbinnen de verpakking geopend mag worden (b.v. CI, DII of VMT). Voorgedrukte stickers zijn via de BVF verkrijgbaar.

13. Het bacterieel afval dat achterblijft na het centrifugeren wordt na inactiveren (indien dit nog nodig is) geloosd op het openbare riool.

14. Afvoeren van alle verdere biologische afval of wegwerp materiaal, dat hiermee besmet is, gebeurt in r.v.s. tonnen met daarin autoclaveerbare plastic zakken. Na sterilisatie in een doorgeef-autoclaaf kan dit materiaal als normaal bedrijfsafval verder verwerkt worden.

15. Alle glaswerk en instrumenten worden eveneens geautoclaveerd alvorens het materiaal voor hergebruik klaar te maken.
Materialen of apparaten die niet bestand zijn tegen autoclaveren dienen gedesinfecteerd te worden, b.v. met behulp van Bacillol, alvorens ze worden afgevoerd.

16. Fermentoren, tanks en containers, waarin zich nog levende GGO's bevinden worden gedesinfecteerd met behulp van stoom, alvorens ze worden geopend.

17. Alle tonnen en containers worden, alvorens de Afdeling in gesloten toestand te verlaten, aan de buitenzijde gedesinfecteerd met Bacillol.

18. De werkplaats wordt na afloop van een experiment ontsmet met Bacillol. Gebruikte materialen en apparaten worden eventueel gereinigd, ontsmet en weer opgeruimd. Verder is het de gezamenlijke verantwoordelijkheid van alle personeel werkzaam in de afdeling om er voor te zorgen dat de laboratoria ten alle tijde netjes en schoon gehouden worden.

19. Alle ruimtes in de afdeling worden schoongemaakt door personeel van de afdeling zelf. Dit betekent dat er geen "vreemd" personeel toegelaten wordt op de afdeling om schoonmaakwerkzaamheden te verrichten, tenzij op uitdrukkelijk verzoek van de afdelingsleiding.

20. Desinfectie van de ruimtes m.b.v. Formaline vindt plaats wanneer dat door de afdelingsleiding nodig wordt geoordeeld. Dit is met name dan het geval wanneer het vermoeden bestaat dat er tijdens werkzaamheden een GGO-aërosol zou kunnen zijn ontstaan.

21. Voor de wijze van handelen in geval van een ongeval wordt verwezen naar het ongevalsprogramma zoals dat is omschreven in S.O.P. Nr. 0212-5519-003.

ADDENDUM III

WERKVOORSCHRIFTEN S.O.P.		
Onderwerp:	Ongevalsprogramma Bacteriologische Productie	S.O.P. no: 0212-5519-003 Pag: 1 van: 2
Auteur:	V. Rijnierse	Interne code:
Uitgifte: 16-11-1994	Version No: 1 Copy No:	Goedgekeurd: VR: BVF: QA:
Betrokken Afdelingen:	Bacteriologische Productie	

1. <u>BRAND</u>

   1. In geval van kleine brand in de Bacteriologische Productie: zelf proberen te blussen. In ieder geval daarna de Afdelingschef of BSA waarschuwen.

   2. In geval van grote brand, dreigende grote brand in de Bacteriologische Productie of indien de Bacteriologische Productie om andere redenen moet worden verlaten (algemeen alarm):

   3. Zet overdruk van de ketel af.

   4. Zet luchttoevoer af.

   5. Verlaat daarna de Bacteriologische Productie zo snel mogelijk.

   6. Vang voor begeleiding de Brandweer en hulpdiensten buiten op.

   7. Waarschuw de afdelingschef of BSA.

2. <u>VRIJKOMEN KLEINE HOEVEELHEID CULTUUR</u> (milliliters)

   1. Stop de lekkage indien mogelijk.

   2. Neem het gemorste materiaal op met absorberend materiaal zoals watten of tissues, gedrenkt in desinfectans (handschoenen aan).

   3. Verzamel het opgenomen materiaal in een plastic zak en laat deze zak autoclaveren.

   4. Meld ieder voorval aan de afdelingschef of BSA.

<table>
<tr><td colspan="3">WERKVOORSCHRIFTEN S.O.P.</td></tr>
<tr><td>Onderwerp:</td><td>Ongevalsprogramma
Bacteriologische Productie</td><td>S.O.P. no: 0212-5519-003
Pag: 2 van: 2</td></tr>
<tr><td>Auteur:</td><td>V. Rijnierse</td><td>Interne code:</td></tr>
<tr><td>Uitgifte:

16-11-1994</td><td>Version No: 1

Copy No:</td><td>Goedgekeurd:

VR: BVF: QA:</td></tr>
<tr><td>Betrokken
Afdelingen:</td><td>Bacteriologische Productie</td><td></td></tr>
</table>

3. <u>VRIJKOMEN GROTE HOEVEELHEID CULTUUR</u> (liters).

1. Stop de lekkage indien mogelijk.

2. Zet de ruimteventilatie af.

3. Voorkom verspreiding van het gemorste materiaal door indammen met dweilen (handschoenen aan).

4. Waarschuw de afdelingschef of BSA.

5. Verlaat de besmette ruimte en houdt deze gesloten (aerosolen laten bezinken).
   Besmette kleding dient ter plekke te worden uitgedaan en gedeponeerd in een daarvoor bestemde bak.
   Lichaamsdelen, die besmet kunnen zijn geraakt, wassen. Zonodig schone noodkleding aantrekken.
   De besmette kleding dient direct geautoclaveerd te worden.

6. Wacht verdere instructies af.

ADDENDUM IV.

Uit: Veiligheidsvoorscgriften Intervet lokatie Boxmeer
VGWM-dienst, mei 1991.

4. ONTRUIMINGSPROCEDURES

4a Waarom ontruimingsprocedures ?

Alarmsituaties kunnen zich zowel door interne (Intervet) oorzaken als door externe (buiten Intervet) oorzaken voordoen.

Bij Intervet International wordt slechts sporadisch gewerkt met giftige, explosieve chemicaliën; daardoor is de kans niet groot dat zich bij Intervet een catastrofe zal voordoen.
In de buurt van het Intervet terrein bevinden zich echter drie hoofdtransportwegen, elk met hun eigen vervoersmogelijkheid : vervoer per boot, vervoer per spoor en vervoer per vrachtwagen, over respectievelijk de Maas, de spoorlijn en de A73 met toevoerwegen.
Op deze hoofdtransportroutes zou zich een ramp kunnen voordoen, waarbij bijvoorbeeld een wolk giftig gas zou kunnen ontsnappen, die dan de omgeving bedreigt. Mede daarom is een ontruimingsplan noodzakelijk.

4b Alarmering

Via politie of brandweer of intern wordt gewaarschuwd dat er extern of intern gevaar dreigt voor een calamiteit en dat het fabrieksterrein ontruimd moet worden.
Na opdracht via de Directie slaat de telefoon-centrale zo snel mogelijk alarm. Dit gebeurt telefonisch of mondeling (door de brandweer).

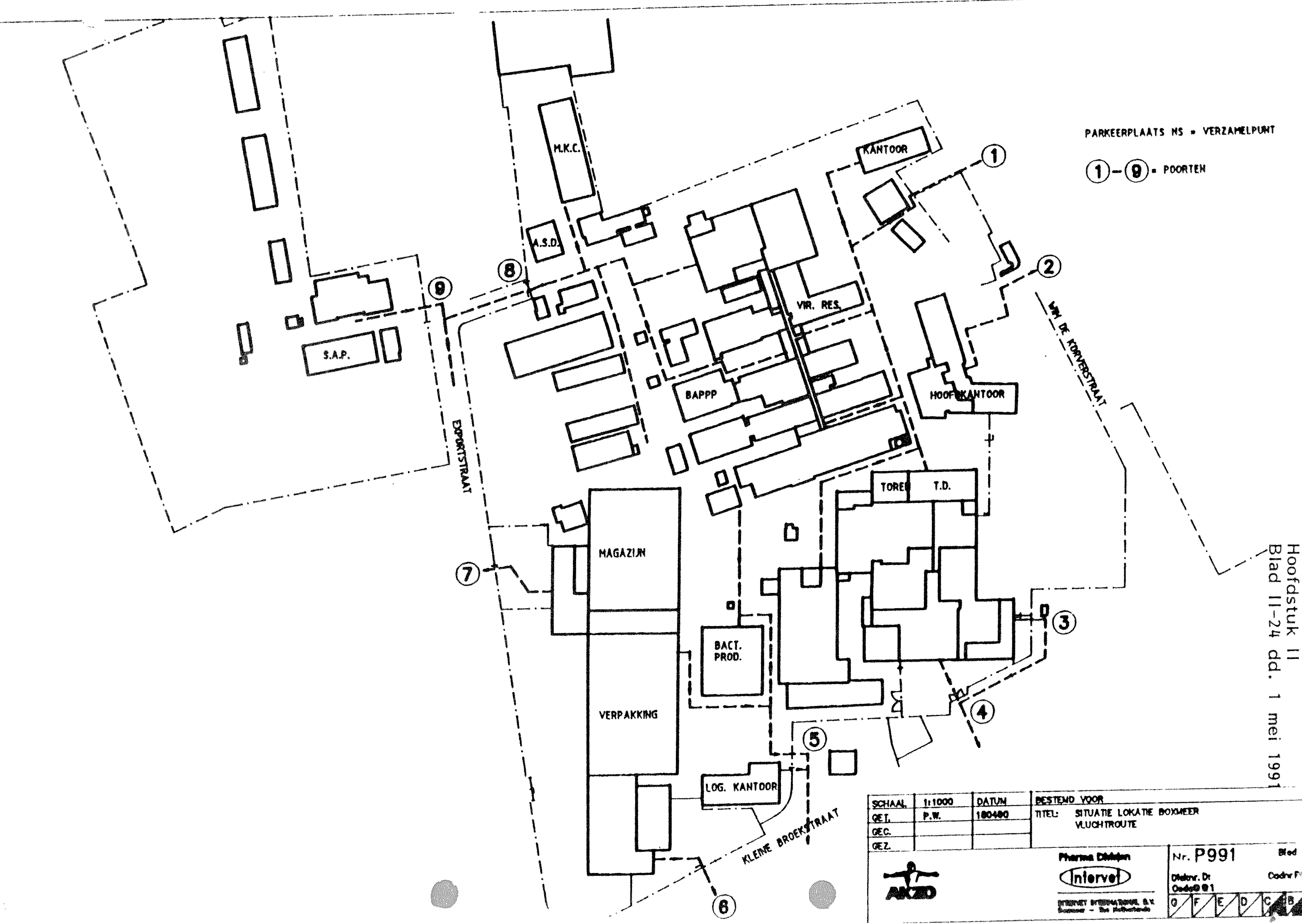
Bij eventuele stroomuitval worden alle electrische sloten van de hekken ontgrendeld.

4c Hoe te handelen

Indien men het signaal tot ontruiming krijgt, dient men :

- Te stoppen met de werkzaamheden waarmee men bezig is; alle apparatuur die handbediend functioneert buiten bedrijf te stellen.
- Indien daar gelegenheid toe is, alle waardevolle documenten of preparaten op te bergen in daarvoor beschikbare ruimten.
- Zo ordelijk mogelijk het fabrieksterrein te verlaten (zie vluchtwegenkaart, pagina II-24). Tenzij ook daar gevaar dreigt, is de verzamelplaats op het parkeerterrein tegenover het station. Daar dient men nadere instructies af te wachten.
- Als er direct gevaar dreigt (bv. gifwolk) verlaat men het terrein afhankelijk van de windrichting langs de kortste route via één van de vijf poorten in een veilige richting. Mensen met auto's dienen daarbij zoveel mogelijk andere mensen die te voet of met de fiets zijn, mee te nemen.
- De afdelingschef of diens plaatsvervanger (zie II-37 en II-38) controleert of de afdeling geheel verlaten is en meldt dit aan de brandweercommandant tst. : 333 brandweerloods.
  Vervolgens verlaat hij eveneens het terrein.
- De brandweer, met perslucht, controleert of het terrein verlaten is. De brandweercommandant verlaat zelf als laatste het terrein en bewaakt verder de toegang tot het terrein.

Bijlage : Plattegrond Intervet met routes naar de vijf uitgangen.


PARKEERPLAATS NS = VERZAMELPUNT
1 — 9 = POORTEN
1
2
3
4
5
6
7
8
9
W.H. DE KORVERSTRAAT
KANTOOR
HOOFDKANTOOR
T.D.
TOREN
VIR. RES.
BAPPP
BACT. PROD.
LOG. KANTOOR
KLEINE BROEKSTRAAT
MAGAZIJN
VERPAKKING
M.K.C.
A.S.D.
EXPORTSTRAAT
S.A.P.
SCHAAL 1:1000
GET. P.W.
GEC.
GEZ.
DATUM 180490
BESTEMD VOOR
TITEL: SITUATIE LOKATIE BOXMEER VLUCHTROUTE
Pharma Divisie
Intervet
Nr. P991
AKZO