

**Table 1.** Evaluation of 1–4 as polyethylene catalysts and polymer properties

Catalyst	Activity* (kg PE/moleTi/h/bar)	M <sub>n</sub> ** (× 10 <sup>5</sup> )	Polydispersity**
1	1600	3.1	2.1
2	40	2.7	4.5
3	25.9	0.27	Bimodal
4	11.5	0.33	22.7

\*All catalytic runs carried out in toluene (200 ml) at 298 K under 1 bar ethylene for 1 h with MAO as the cocatalyst (Al:Ti is 100:1).

\*\*Measured by high temperature GPC with polyethylene as standard.

number of average molecular weights of polyethylene obtained with 1 and 2 is approximately one order of magnitude higher than those obtained with 3 and 4. There is a notable reduction in the polydispersity of the polymers obtained with 1 and 2 as the catalysts, compared to those obtained by using 3 and 4. As shown in Figure 1b, a broad and distinctly bimodal molecular weight distribution function, a characteristic feature of many multisite catalysts is obtained for 3. However with 1, a narrow unimodal molecular weight distribution function is obtained.

When combined with the cocatalyst, complexes 3 and 4 like the well-established first generation Ziegler–Natta system are expected to give rise to *multisite colloidal* catalysts resulting in the formation of polymers with high polydispersity. On the other hand, majority of the surface catalytic sites in the supported catalysts have identical structures. These species are therefore expected to behave more as single site catalysts giving polymers of comparatively low polydispersity<sup>2b,7</sup>.

Polymerization reactions where MAO is used as the co-catalyst have been reported to involve the cleavage of metal alkoxide bonds<sup>8</sup>. It is very likely that with 1 and 2, cleavage of some of the dative Ti–O bonds does take place and as the reaction proceeds some of the anchored titanium species are leached out of the support. However, the leached out dissolved species, as well as 2, 3 and 4 probably undergo quick deactivation. In contrast, the diffusion barrier between the solution and the insoluble support, and the steric crowding in Cp<sub>2</sub>TiCl<sub>2</sub> make the supported catalytic species of 1 resistant towards deactivation. This would explain why the molecular weight of polymer obtained with 1 is notably higher than that of all the others.

In conclusion, we have shown that PES is versatile support material for polymerization catalysts. Complexes Cp<sub>2</sub>TiCl<sub>2</sub> and TiCl<sub>4</sub> can be supported on PES through dative O–Ti bonds. The supported materials in combination with MAO give polymers of low polydispersity indicating single site behaviour.

- (a) Ittel, S. D. and Johnson, L. K., *Chem. Rev.*, 2000, **100**, 1169; (b) Britovsek, G. J. P., Gibson, V. C. and Wass, D. F., *Angew. Chem., Int. Ed. Engl.*, 1999, **38**, 428.
- (a) Welch, M. B. and Hsieh, H. L., In *Handbook of Polyolefins. Synthesis and Properties* (eds Vasile, C., Seymour, R. B. and Dekker,

M.), New York, 1993, pp. 21–37 and references therein; (b) *Encyclopedia of Chemical Technology*, 1996, vol. 17, 4th edn, pp. 702–819.

- Hlatky, G. G., *Chem. Rev.*, 2000, **100**, 1347.
- Jerico, S., Schuchardt, U., Joekes, I., Kaminsky, W. and Noll, A., *J. Mol. Catal. A*, 1995, **99**, 167.
- Magni, E. and Somorjai, G. A., *J. Phys. Chem. B*, 1998, **102**, 8788.
- (a) Viossat, V., Lemoine, P., Dayan, E., Dung, N. and Viossat, B., *Polyhedron*, 2003, **22**, 1461; (b) Khenkin, A. M., Shimon, L. Z. W. and Neumann, R., *Inorg. Chem.*, 2003, **42**, 3331.
- (a) Brintzinger, H. H., Fischer, D., Malhaupt, R., Rieger, B. and Waymouth, R. M., *Angew. Chem.*, 1995, **107**, 1255–1283; *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1143; (b) Gupta, V. K., Satish, S. and Bhardwaj, I. S., *Rev. Macromol. Chem. Phys. C*, 1994, **34**, 439; (c) Boor, Jr. J., In *Ziegler-Natta Catalysis and Polymerization*, Academic Press, New York, 1979; (d) *Ziegler Catalysts* (eds Fink, G., Malhaupt, R. and Brintzinger, H. H.), Springer-Verlag, Heidelberg, 1994.
- Wang, W., Fujiki, M. and Nomura, K., *Macromol. Rapid Commun.*, 2005, **25**, 504.

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## Cloning of synthetic VP2 gene of infectious bursal disease virus in a mammalian expression vector and its use as DNA vaccine in chicken

S. Chauhan<sup>1</sup>, A. Rai<sup>1\*</sup>, N. Rai<sup>1</sup>, N. Dighe<sup>2</sup>, B. Roy<sup>2</sup>, P. K. Gupta<sup>1</sup> and Divya<sup>1</sup>

<sup>1</sup>National Biotechnology Centre, Indian Veterinary Research Institute, Izatnagar, Bareilly 243 122, India

<sup>2</sup>Bangalore Genei, Bangalore 560 058, India

**The 1367 bp VP2 gene of infectious bursal disease virus was synthesized in three phases and cloned in pUC18 vector. All the three clones having the inserts were bi-directionally sequenced and minor mutations were repaired. The three selected clones were sequentially cloned in pUC29 vector. The 1367 bp gene was released from the pUC29 and re-cloned in pVAX1 vector. The recombinant plasmid was designated as pVAX1.ibdvp2. It was used to transfect chick embryo fibroblast culture and found to express VP2 protein, as detected by immunoperoxidase test. The recombinant plasmid was used as DNA vaccine in chicken using 50 µg per chick, injected intramuscularly. It was found to give 100% protection, while vector inoculated or healthy control chicken died/suffered from the disease. The present work showed that the recombinant plasmid can be used as DNA vaccine against infectious bursal disease in chicken.**

\*For correspondence. (e-mail: raial@rediffmail.com)

INFECTIOUS bursal disease (IBD) in chicken was first described by Cosgrove<sup>1</sup>. It is a fatal disease of young chicken that is responsible for heavy economic losses to the poultry industry worldwide<sup>2</sup>. Infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus* of the family *Birnaviridae*<sup>3</sup>. The genome consists of two segments, A and B of double stranded RNA, which are localized within a single-shelled, icosahedral capsid of 60 nm diameter. The large segment A of 3.3 kb encodes a poly protein of approximately 100 kDa, which is autocleaved into mature protein<sup>4</sup> VP2, VP3 and VP4. The second open reading frame (ORF) overlapping the first ORF of same segment A, codes for a 21 kDa VP5 protein of unknown function<sup>5,6</sup>. The smaller segment B of 2.7 kb encodes a single 90 kDa protein considered to be viral protease<sup>7</sup>. The main structural protein VP2, bears host-protective conformational and virus neutralizing epitopes<sup>8</sup>.

In India, after the first report of virulent viruses in 1993, the virus caused severe economic losses by spreading rapidly throughout the country, with high mortality (10–75%) of even vaccinated flocks of growing layers of 3–16 weeks age<sup>9</sup>.

The insect cell-expressed recombinant VP2 gene of IBDV conferred protection against the disease in chicken<sup>10</sup>. The IBDV VP2 gene expressed by a fowlpox virus recombinant conferred protection against IBD in chicken<sup>10</sup>, while the VP2 gene expressed in a recombinant baculovirus also conferred protection in chicken<sup>11</sup>. A recombinant Marek's disease virus expressing IBDV VP2 gene protected 55% of the chickens after challenge with virulent IBDV, whereas all of the chickens vaccinated with conventional IBD vaccine showed no clinical signs and were protected<sup>12</sup>. In another experiment, VP2 gene was expressed in baculovirus system giving rise to large quantity of recombinant VP2 protein, which was 453 amino acids in length with a molecular weight of about 48 kDa. It produced a high level of antibodies in vaccinated birds and gave full protection against virulent challenge, with no mortality or weight changes in the bursa of fabricius in vaccinated birds, whereas in the negative control, birds, 50% mortality was found<sup>13</sup>.

The aim of this study was to synthesize and clone the VP2 gene, which could be used as DNA vaccine against IBD.

The VP2 gene sequence of IBDV was derived from the GenBank accession no. D00499. The 1367 bp gene was synthesized in three phases. In phase I, the first fragment from bases 1 to 519 was synthesized following standard recursive PCR protocol and cloned in *EcoRI/NdeI* site of pUC18 (clone I). In phase II, the fragment from bases 520 to 1081 was synthesized and cloned in *NdeI/BamHI* sites of pUC18. In phase III, the fragment from bases 1082 to 1367 was synthesized and cloned in *BamHI/XbaI* sites of pUC18 (clone III). The clones were detected by white selection and confirmed by release of insert following double-restriction digestion. Selected clones were sequenced bidirec-

tionally and the best clone having minimum number of mutations was selected for repair following the standard Genei *in vitro* site-directed mutagenesis protocol<sup>14</sup>. Finally the insert was released from the respective clones and cloned sequentially in pUC29 using JM109 strain of *Escherichia coli* and ampicillin 100 µg/ml. The final clone pUC29 containing the 1367 bp insert was confirmed by multiple restriction digestion and release of inserts of expected sizes. Data obtained from bidirectional sequencing, were blasted with the original sequence data to confirm the sequence. The insert was then recloned in pVAXI vector at the *EcoRI/XbaI* site using *E. coli* JM109 and kanamycin, 50 µg/ml. The transformed colonies were checked by restriction enzyme analysis.

*E. coli* JMI09 having recombinant plasmid was grown in LB broth containing 50 µg/ml kanamycin at 37°C in an orbital shaking incubator and plasmid DNA was isolated using HiSpeed Midi kit (QIAGEN) using the kit protocol. The concentration of DNA was estimated spectrophotometrically and was found to be 200 µg/ml.

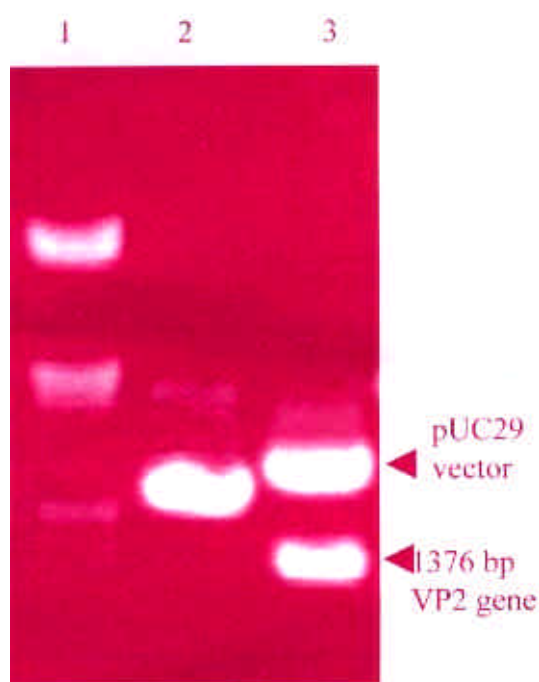
The expression of recombinant plasmid was assayed using the protocol for transient transfection of adherent cells (QIAGEN) using the effectene transfection reagent (QIAGEN) for one well of a six-well culture plate. The reagents used per well were: DNA, 0.4 µg; enhancer, 3.2 µg; final volume of DNA in DNA-condensation buffer, 100 µg; volume of effectene reagent, 10 µg; volume of medium to add to cells, 1600 µg; volume of medium to add to complexes, 600 µg. One 25 cm<sup>2</sup> flask containing a complete monolayer of chick embryo fibroblast cells was taken, and the cells were dispersed using trypsin–versene solution and finally suspended in 10 ml growth medium. The cell suspension was distributed into a six-well culture plate, with 1.6 ml in each well. The plate was kept in a CO<sub>2</sub> incubator under 5% CO<sub>2</sub> at 37°C for 24 h. On the day of transfection, 16 µl DNA (1.6 µg) was diluted with the DNA-condensation buffer to a total volume of 400 µl in a 15 ml tube. Then 12.8 µl of enhancer was added and mixed by vortexing. After incubation at room temperature (25°C) for 5 min, it was given a short spin. Next 40 µl of effectene transfection reagent was added to the DNA-enhancer mixture and mixed by pipetting up and down five times. It was incubated for 10 min at room temperature to allow transfection-complex formation. The medium was pipetted out from six-well culture plate without disturbing the cells and washed with growth medium. Next 1.6 ml growth medium was added in control wells and 1280 µl growth medium was added in the test wells to be transfected. Then 2400 µl of growth medium containing FCS and antibiotic was added to the tube having transfection complexes and mixed by pipetting up and down twice. Next 750 µl of transfection complex was added dropwise into each well in the culture plate, leaving the controls. The plate was gently swirled to ensure uniform distribution of the transfection-complexes. The cells were incubated for 48 h in 5% CO<sub>2</sub> incubator at 37°C, after which the protein expressed was detected by immunoperoxidase staining.

**Table 1.** Immunological study of IBD DNA vaccine in chicks\*

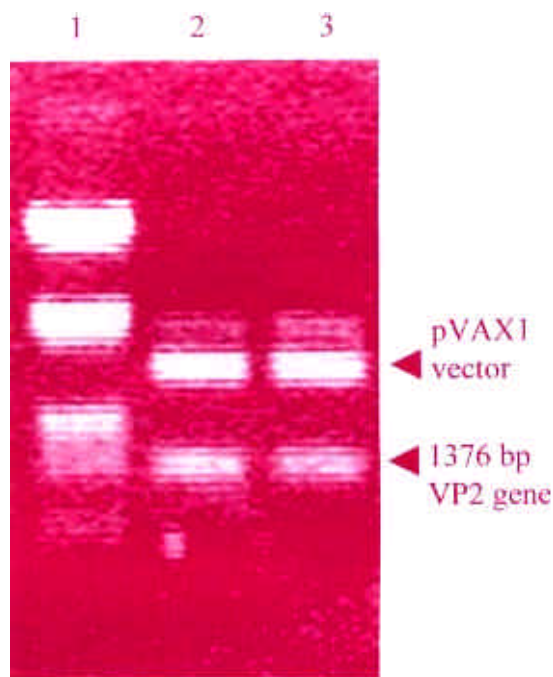
Groups	Dose (µg)	No. of chicks	SN antibody titre**	No. of chicks protected	Percentage protection
pVAX1 ibdvp2 DNA	50	10	256	10	100
pVAX1 ibdvp2 DNA + ISA 50 (10% v/v)	50	10	512	10	100
pVAX1 vector + ISA50	50	10	0	0	0
Healthy control	-	30	0	0	0

\*DNA was given intramuscularly in 0.25 ml to 3-day-old chicks and all the chicks, including healthy controls were challenged 21 days post-vaccination with 10,000 ID<sub>50</sub> of virulent bursa passaged IBD virus intramuscularly.

\*\*SNT was done in microtitre plates using 100 TCID<sub>50</sub> cell culture-adapted IBD virus.

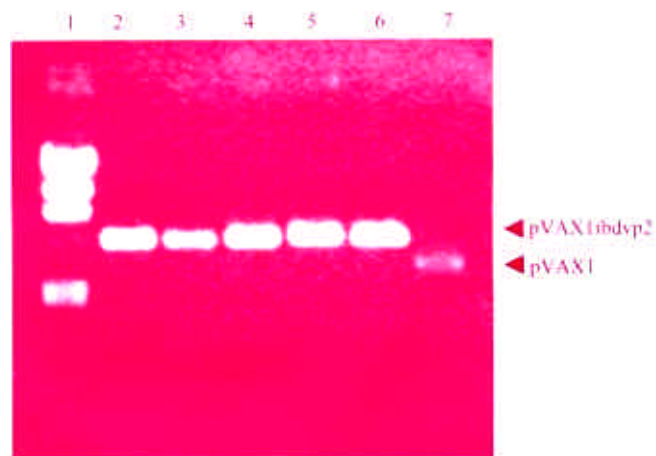


**Figure 1.** Cloning of IBD VP2 gene in pUC29. Lane 1, I DNA *HindIII/EcoRI* cut; lane 2, pUC29 ibdvp2 recombinant plasmid DNA uncut; lane 3, pUC29 ibdvp2 digested with *EcoRI/XbaI* showing 1376 bp VP2 gene and vector.



**Figure 2.** Subcloning of IBD VP2 gene in pVAX1 vector. Lane 1: I DNA *HindIII/EcoRI* cut; lanes 2 and 3, pVAX1 ibdvp2 cut with *EcoRI/XbaI* showing VP2 gene insert and vector.

The procedure described by Rai<sup>15</sup> was followed. The medium from the wells of culture plate was removed, the monolayer washed with PBS, pH 7.2, and the cells were fixed with chilled acetone at 4°C for 10 min and then air-dried. Then a few drops of chicken anti-IBD antiserum was added and incubated at 37°C for 1 h in a humid chamber. Two wells served as control. Then the wells were washed with PBS. Few drops of the affinity isolated rabbit anti-chicken IgG-peroxidase conjugate (Sigma) were added and incubated at 37°C for 1 h in a humid chamber. The wells were washed three times with PBS and once with distilled water, and then air-dried. Three drops of freshly prepared Nadi reagent were added and allowed to react for 2 min, the preparation was rinsed briefly with PBS and treated for about 1 min with each of the following: 70%, 90% and 100% alcohol followed by a quick wash with xylene. The monolayers were observed under a microscope.



**Figure 3.** Subcloning of IBD VP2 gene in pVAX1 vector. Lane 1, I DNA *HindIII* cut; lanes 2-6, pVAX1ibdvp2 recombinant plasmid DNA; lane 7, pVAX1 plasmid DNA.

The recombinant plasmid DNA, 50 µg in 0.25 ml with ISA 50 adjuvant per chick and the other group without any adjuvant was inoculated intramuscularly in the thigh of three-day-old chicks. Another group of chicks was inoculated with vector alone and a group was kept as healthy control. All the chicks were free from IBD antibody as determined by serum neutralization (SN) test using sera samples. At 21 days post-vaccination, all the birds were challenged with 10,000 ID<sub>50</sub> of virulent IBD virus per bird, intramuscularly. The birds were observed for 14 days for sign of disease or death.

VP2 gene was synthesized and cloned successfully in pUC29 and recloned in pVAXI (Figures 1–3). The recombinant plasmid containing the VP2 gene expressed the VP2 protein in CEF cell culture as detected by immunoperoxidase test. The recombinant plasmid when used as DNA vaccine in chicken was found to induce protective immunity (Table 1). It gave 100% protection in the chicken. The SN antibody titre was found to be 256 and 512 in vaccinated groups, while no antibody level was detected in vector alone and healthy control groups. The study corroborated the observations of previous workers<sup>10</sup>, who found protection against the disease from insect cell-derived VP2 of IBD. The VP2 gene expressed in baculovirus recombinant plasmid was also found to protect the chickens from the disease<sup>8</sup>. About 55% protection was found by expressing IBD VP2 gene in recombinant Marek's disease virus and using it as vaccine<sup>12</sup>. In our study, 100% protection was observed, which showed that the DNA vaccine developed in the present study was highly effective.

It is evident from the present work that recombinant, plasmid DNA can be used as DNA vaccine against IBD. Since DNA vaccine needs no cold chain and it is cheaper to produce on a large scale, it can be widely used as an effective vaccine against IBD under Indian conditions.

14. Hegde, K. V., Hegde, V. S. and Roy, B., *Curr. Sci.*, 2003, **85**, 1523–1525.
15. Rai, A., *Methods in Cell Culture and Virology*, Allied Publishers, New Delhi, 1985, pp. 223–230.

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## Isolation and characterization of a mouse embryonic stem cell line that contributes efficiently to the germ line

Jomini Liza Alex, D. Partha Sarathi, Sandeep Goel and Satish Kumar\*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

**Embryonic stem (ES) cells have proven vital for introduction of pre-defined genetic modifications into mouse germ line. Here, we describe the isolation of a mouse ES cell line namely, R1.9 through sub-cloning and karyotyping. R1.9 ES cells have normal chromosomal complement and contribute extensively to chimaerism and germ line. We have been successfully using R1.9 ES cells in our laboratory for creation of novel strains of mouse through gene targeting.**

THE isolation and genetic manipulation of embryonic stem (ES) cells has been a major achievement in mammalian developmental biology with far-reaching implications in almost all areas of mammalian biology. Murine embryonic stem cells are pluripotent cells harvested from the inner cell mass of mouse blastocysts and grown *in-vitro* under conditions inhibiting their spontaneous differentiation<sup>1,2</sup>. ES cells are now widely used for introduction of genetic alterations into the mouse germ line. This is achieved in a number of stages. First, a targeting vector, containing the genetic modifications to be introduced as well as genes conferring drug resistance or sensitivity for selection is constructed. This targeting vector containing the desired mutation is electroporated into ES cells. In most cells, the targeting vector inserts randomly into the ES genome. However, in a few cells, the targeting vector pairs with the homologous sequences and transfers the mutation to the corresponding endogenous gene on the chromosome. Screening procedures are then used to identify the rare ES cell in which the targeted event has occurred. Next, the tar-

1. Cosgrove, A. S., *Avian Dis.*, 1962, **9**, 385–389.
2. Faragher, J. T., *Vet. Bull.*, 1972, **42**, 361–369.
3. Murphy, F. A. *et al.*, Springer, New York, 1995, pp. 240–244.
4. Hudson, P. I., Mckern, N. M., Power, B. E. and Azad, A. A., *Nucleic Acids Res.*, 1986, **14**, 5001–5012.
5. Bayliss, C. D., Spies, D., Shaw, K., Peters, R. W., Papageorgiou, A., Muller, H. and Bournsnel, M. E. G., *J. Gen. Virol.*, 1990, **71**, 1303–1312.
6. Kibenge, F. S. V., Dhillon, A. S. and Russell, R. G., *J. Gen. Virol.*, 1988, **69**, 1757–1775.
7. Azad, A. A., Barrett, S. A. and Fahey, K. I., *Virology*, 1985, **143**, 35–44.
8. Vakharia, V. N., He, J., Ahmed, B. and Snyder, D. B., *Virus Res.*, 1994, **31**, 265–273.
9. Sah, R. L., Kataria, J. M., Arya, S. C. and Verma, K. C., *Indian J. Comp. Microbiol. Immunol. Infect. Dis.*, 1995, **16**, 7–13.
10. Heine, H. G. and Boyle, D. B., *Arch. Virol.*, 1993, **131**, 3–4.
11. Vakharia, V. N., Snyder, B. D., He, I., Edwards, G. H., Savage, P. K., Mengel and Whereat, S. A., *J. Gen. Virol.*, 1991, **74**, 1201–1206.
12. Tsukamoto, Kenji, Kojima, C., Komori, Y., Tanimura, N., Mase, M. and Yamaguchi, S., *Virology*, 1999, **257**, 352–362.
13. Pitcovski, I. *et al.*, *Avian Dis.*, 1996, **40**, 753–761.

\*For correspondence. (e-mail: satishk@ccmb.res.in)