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Research article

Molecular characterization, tissue expression, and antiviral activities of Bama minipig interferon-α subtypes

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ABSTRACT

Interferons play a major role in innate immunity and disease resistance. Porcine interferon alpha has 17 subtypes, and their gene sequences, tissue expression profiles, and antiviral activities have been primarily studied in domestic pigs but not in minipigs. Bama minipigs are genetically stable disease-resistant and making them as laboratory animal models for bioscience studies. To define the potential mechanism for disease resistance, in this study, we cloned 17 subtypes of Porcine interferon alpha genes in Bama minipigs using high fidelity polymerase chain reaction and subsequent sequencing. Sequence alignment showed that the 17 porcine interferon alpha subtypes were 98%–100 % homologous in those of domestic pigs. However, significantly different tissue expression profiles of PoIFN-α subtypes were found in the two pig species using real-time quantitative RT-PCR. Among the 10 different Bama minipig tissues tested, significant expression of multi-subtype porcine interferon alpha was detected in the lymph nodes and spleen, whereas no or low expression of fewer subtypes was detected in the heart, lung, brain, and small intestine. Sequence analysis revealed that the porcine interferon alpha promoters were almost similar between the two pig species. A cytopathic effect inhibition assay showed that the recombinant 17 porcine interferon alpha subtypes purified from mammalian cells had significantly different antiviral profile against vesicular stomatitis virus, porcine pseudorabies virus and porcine reproductive and respiratory syndrome virus compared with those in domestic pigs. Our findings provide evidence that porcine interferon alpha subtypes are highly conserved between Bama minipigs and domestic pigs but show varied tissue expression pattern and antiviral capabilities, which may contribute to their differences in disease resistance.

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1. Introduction

Interferons are cytokine clusters with growth-inhibitory properties and antiviral and immunomodulatory activities [1,2].

List of Abbreviations

AA	amino acid
Bp	base pair
cDNA	complementary DNA
CPE	cytopathic effect
gDNA	genomic DNA
IFN	interferon
kDa	kilodalton
NA	nucleic acid
ORF	open reading frame
OD	optical density
PoIFN	porcine interferon
PRRSV	porcine reproductive and respiratory syndrome virus
PRV	pseudorabies virus
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
rPoIFN-α	recombinant porcine interferon alpha
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
$TCID_{50}$	tissue culture infective dose

Three types of interferons (IFNs) have been detected in animals based on their molecular characteristics, signaling pathways, and receptor network [3]. Type II IFN made up of a single subunit, primarily produced by active cytotoxic T lymphocytes, natural killer cells, and hence plays an essential function in the arbitration of adaptive immunity [3]. In contrast, type I and III IFNs comprise multifunctional genes that are important in innate immunity and are crucial for linking cell mediated immunity to virus clearance [2]. Type I IFNs are important antiviral cytokines that have adapted to continuous changing in viral pressures throughout vertebrate evaluation and domestication [4]. The type I porcine interferon (PoIFN) family includes minimum 39 functional genes coding for 17 IFN-α, 11 IFN-δ, and 7 IFN-ω subtypes along with a single subtype of IFN-κ, IFN-ε, IFN-αω, and IFN-β [5]. Although these subtypes share high sequence similarity, their promoter sequence among most types of type I PoIFNs are relatively different [6], suggesting differential gene expression depending on different tissue types/cell types or stimuli [5]. Furthermore, these subtypes have been reported to exhibit distinct tissue expression profiles and antiviral properties in domestic pigs [4]. However, the type I PoIFNs expression level and antiviral properties of various PoIFN-α subtypes in swine species remain unelucidated, particularly in minipigs.

Minipigs recently, have received significant attention as laboratory animal models in bioscience studies [7,8], such as Minnesota minipigs Göttingen minipigs, Aachen minipigs, and Brazilian minipigs [9]. The Chinese Bama minipig is one of the rare minipig breeds worldwide that has been established by long-term selective inbreeding and has been developed as an experimental inbred line originated from Bama Xiang pigs, a nascent pig type free from artificial printing for commercial characteristics [10]. Because Bama minipigs are disease resistant, genetically stable [11,12], they have been used as experimental animal models for investigating viral diseases, xenotransplantation, pathogenesis, as well as for assessing vaccines [13]. In this project we cloned 17 subtypes of PoIFN-α genes from Bama minipigs and compared their tissue expression pattern and antiviral properties with those of domestic pigs to define their potential mechanism for disease resistance.

2. Materials and methods

2.1. Cells and viruses

Monkey kidney (MARC-145), pig kidney (PK-15), and bovine kidney cells (MDBK) (American Type Culture Collection, USA) were grwon in Dulbecco's modified Eagle's medium (Gibco, China) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10 % fetal bovine serum. Using previously described methods, the New Jersey strain of vesicular stomatitis virus (VSV) was cultivated and titrated on MDBK cells [14]. The Bartha K61 strain of pseudorabies virus (PRV) was grown and titrated on PK-15 cells [15],and porcine reproductive and respiratory syndrome virus (PRRSV) strain VR2332 was grown and titrated on MARC-145 cells [16], and for each virus tissue culture infective dose ($TCID_{50}$) was detected.

2.2. Animal housing

Three healthy female Bama minipigs each 6 months old were obtained from the Beijing Institute of Zoology, Chinese Academy of Sciences, and accommodated in a well-ventilated room (0.5sqft/kg of body weight space). Room temperature was maintained at 19 °C–29 °C with a humidity of 40 %–70 %. The diet was formulated according to a balanced diet for minipigs at the Yangzhou University Experimental Pig Farm.

2.3. Collection of tissue

Anesthesia was administered, as previously mentioned [17]. Briefly, atropine sulfate (1 mg) and ketamine (10 mg/kg) were injected intramuscularly as pre-anesthetic agents, followed by the administration of 1 % thiopental (0.5 ml/kg) to attain general anesthesia. After killing the model animals via femoral artery hemorrhage, we harvested the spleen, heart, Kidney, lung, uterus, lymph node, liver, intestine, skin, and brain. Each tissue was chopped into (1 g) and stored in liquid nitrogen until further investigation. All the experimental methods were established following the instructions of the Guide for the Care and Use of Laboratory Animals of Yangzhou University and after obtaining approval from the Medical Experimental Animal Center of Jiangsu Province (No. NSFC2020-dkxy.02, March 27, 2020).

2.4. Isolation of PoIFN-α genes

Genomic DNA (gDNA) was obtained from the pooled tissue by a Genomic DNA Extraction Kit (Tiangen Biotech, China) following the manufacturer's guidelines. Total RNAs were obtained from the pooled tissue by MiniBEST Universal RNA Extraction Kit (TaKaRa, China) and inversely transcribed into the first-strand cDNA by cDNA Synthesis Kit (TaKaRa) with random primers pair. The PoIFN-α genes sequences were amplified from gDNA or complementary DNA (cDNA) by a High-Fidelity Taq DNA Polymerase (TaKaRa) with subtype-common or gene-specific primers (Table S1). The polymerase chain reaction (PCR) parameters were as follows: 10 s of pre-denaturation at 94 °C; 30 cycles of denaturation for 10 s at 94 °C, following annealing for 10 s at 64 °C, and extension for 20 s at 72 °C; and final extension for 10 min at 72 °C.After TA tailing (TaKaRa), the amplified products were inserted into pMD19-T TA Cloning Vectors. A total of 121 clones were bidirectionally sequenced using vector sequencing primers (TaKaRa). The resultant sequence data were aligned into consensus sequences and then translated into protein sequences using the SeqMan and EditSeq programs, respectively, in Lasergene (DNAStar, USA). Both nucleotide and protein sequences were blasted in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the PoIFN-α sequences. The different PoIFN-α subtypes were defined according to their highest percent homology (ClustalW scores) the PoIFN-α subtypes of domestic pigs. The amino acid sequences of the PoIFN-α subtypes were further analyzed for conserved cysteine residues, signal peptides, putative N-glycosylation sites, and amino acid substitutions using the MegAlign program (DNAStar).

2.5. Cloning of virus-responsive elements (VREs)

To clone the VREs in the PoIFN-α promoters, we designed a forward primer according to the consensus sequence of the PoIFN-α1, α2, α3, α4, α5, α6, α8, α9, α10, α12, α13 and α14 promoters or PoIFN-α7/α11promoter [6]. We also designed a universal reverse primer based on the consensus sequence of all PoIFN-α promoters (Table S1). After 5-min pre-denaturation at 94 °C, 5-cycle touch-down PCR (annealing temperatures from 60 °C to 56 °C), and 30-cycle PCR were performed by High-Fidelity PCR Taq System as described. The resulting amplicons were then inserted into pMD19-T TA cloning vectors and aligned bidirectionally, as previously mentioned. The resultant sequences were sequenced for the four promoter modules to modulate the IFN promoter's properties in response to viral disease [6].

2.6. Quantitative PCR (qPCR) analysis

We analyzed the PoIFN-α subtypes' mRNA expression levels in different tissues using qPCR with optimized primers (Table S2) as previously described [5,18]. Melting curve analysis using the $2^{-\triangle\triangle Ct}$ method to investigate the relative PoIFN-α subtypes mRNA expression levels by standardization against the housekeeping gene *GAPDH*, with the expression index 1.0. Each sample was analyzed in triplicate and included a control with no template.

2.7. Recombinant protein preparation and identification

We obtained the coding open reading frame (ORF) of each PoIFN-α subtype from the pMD-19- cDNA-cloned vector using gene-specific primers (Table S3) and High-Fidelity Taq DNA polymerase. The resultant amplicons were cloned into a pcDNA3.0 vector (Invitrogen, China) using restriction enzymes *Xho*I and *Hind*III. Under the control of $P_{CMV,}$ early promoter and porcine recombinant protein were expressed as His-tagged protein. HEK-293 cells were transfected in T25 flasks with each recombinant vector (20 μg) using Lipofectamine ™ 3000 (Invitrogen, China) according to the manufacturer's guidelines. After 72 h of transfection, purified proteins were obtained from cell lysates using Ni-NTA affinity chromatography (CWBIO, China). The protein purity was assessed using 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).SDS-PAGE. After transfer onto a nitrocellulose membrane, the recombinant peptides were detected by western blotting with an mAb against the His-tag (Sangon Biotech, China), as previously described [18]. A highly sensitive ECL Chemiluminescence Detection Kit (Vazyme, China) was used to generate the hybridization signals.

2.8. Cytotoxicity and antiviral assay

Cytotoxicity of rPoIFN-α subtypes was detected by using MTS Assay Kit (Abcam, U.K.) following the manufacturer's instructions. The cytopathic effect (CPE) inhibition assay was used to assess the antiviral properties of different PoIFN-α subtypes against VSV,

PRRSV, and PRV in MDBK, MARC-145, or PK-15 cells, respectively [5,18]. In short, tested cells were grown in 96-well plates (4×10^5 cells/well) for 8 h and treated in eight duplicates with each PoIFN-α subtype (2.0 or 200 ng/ml in serum-free medium) at a volume of 0.1ml/well. After 24 h of incubation, the tested cells were infected with PRRSV (0.1 $TCID_{50}$), PRV (0.1 $TCID_{50}$), or VSV (0.2 $TCID_{50}$). Untreated, uninfected cells were used as the standard control, and untreated, virus-infected cells were used as the virus control. At 72 h after infection, the cells were extracted using 1 % acetic acid and 70 % ethanol and stained using 1 % crystal violet in 15 % ethanol. A Multifunctional ELISA Reader (BioTek, USA) was used to determine Optical density at 580 nm (OD_{580}). The CPE protection percentage was determined as follows: $(Vt - Vi)/(Vt - V0) \times 100$, where Vt, Vi, and V0 represent the maximum frequency of viral infection in mock-treated cells, the average OD_{580} results of IFN-tested cells, and the average OD_{580} results of cells without infecting with virus and IFN, respectively.

2.9. Statistical analysis

Statistical analyses and graphical representations were achieved using GraphPad Prism v9.0.1. We used *t*-test and one-way analysis of variance following Tukey post hoc multiple comparison test to assess differences in antiviral activity and gene expression levels [19]. The results were considered statistically significant with P-values <0.05.

3. Results

3.1. Isolation of PoIFN-α genes

By using the subtype-common primers for PoIFN-α, both 546-bp and 570-bp amplicons were amplified from the pooled cDNA of the spleen, liver, lung, and intestine from Bama minipigs. After cloning into the PCR cloning vector and transforming it into *E. coli*, a total of 121 clones were analyzed, and seven different consensus sequences were obtained. Blast analysis showed that the seven consensus sequences shared 99–100 % analogous at the nucleotide sequence level and 98–100 % analogous at the protein sequence level with published PoIFN-α1, PoIFN-α2, PoIFN-α8, PoIFN-α10, PoIFN-α12, PoIFN-α13, and PoIFN-α16, respectively (Table 1). By using the gene-specific primers for PoIFN-α7/11, a single 546-bp amplicon was amplified from the pooled cDNA, and two different consensus sequences were obtained. Blast analysis showed that the two consensus sequences shared 99–100 % similarity at the nucleotide or protein sequence level with published PoIFN-α7/11 (Table 1). Using the gene-specific primers for PoIFN-α14, a single 570-bp amplicon was amplified from the pooled cDNA, and one consensus sequence was obtained. Blast analysis showed that the consensus sequence shared 99–100 % homology with published PoIFN-α14 (Table 1). The remaining seven subtypes of PoIFN-α genes, namely PoIFN-α3, PoIFN-α4, PoIFN-α5, PoIFN-α6, PoIFN-α9, PoIFN-α15 and PoIFN-α17, were amplified from the gDNA using subtype-common primers. Blast analysis showed that the seven consensus sequences shared 99 % or 98%–100 % analogous at the nucleotide or amino acid level with published PoIFN-α3, PoIFN-α4, PoIFN-α5, PoIFN-α6, PoIFN-α9, PoIFN-α15, and PoIFN-α17 sequences, respectively (Table 1). Both the nucleotide and protein sequences of 17 PoIFN-α subtypes of Bama minipigs have been submitted to GenBank with accession numbers mentioned in Table 1.

3.2. Sequence analysis of PoIFN-α subtypes

The intact PoIFN-α ORFs of Bama minipigs encoded 189-aa pre-proteins, and the sequences with deletions of C-terminal encoded 181-aa pre-proteins, both with putative signal peptides of 23 aa (Fig. S1). After cleavage of signal peptides, the first residues of the

Table 1
Sequence homology (ClustalW scores) of the 17 PoIFN-α subtypes comparing domestic pigs with Bama minipigs.

Bama minipig		Domestic pig	
Subtype	GenBank accession	ORF %	Protein %
α1	OM066078	99–100	100
α2	OM066079	100	100
α3	OM066080	99	99
α4	OM066081	99	99
α5	OL697886	99	99
α6	OM066082	99	99
α7	OM066083	99	99
α8	OL697888	99	99
α9	OM066084	99	98
α10	OL697889	99	99
α11	OL697890	99–100	99–100
α12	OM066085	99	98
α13	OL697891	99	99
α14	OM066086	99–100	100
α15	OM066087	99	100
α16	OL697892	99	98
α17	OM066088	99	99

Table 2
Nucleic acid (NA) and amino acid (AA) sequence similarities between the 17 Bama minipig PoIFN-α subtypes.

AA	NA																
	α1	α2	α3	α4	α5	α6	α7	α8	α9	α10	α11	α12	α13	α14	α15	α16	α17
α1		97.6	97.8	97.9	97.4	98.2	97.8	97.4	97.2	97.6	97.8	98.6	97.7	98.4	97.5	97.2	97.1
α2	96.2	\	99.1	98.0	97.4	97.4	97.3	98.4	97.6	98.5	97.3	98.0	97.3	97.8	98.5	98.2	98.7
α3	96.2	98.9	\	97.8	97.3	97.4	97.4	98.5	97.4	99.5	97.4	97.8	96.7	97.3	98.3	98.4	98.5
α4	96.3	95.6	95.6	\	97.0	97.6	98.2	98.4	97.9	97.6	98.2	98.6	98.1	97.7	97.9	98.2	97.8
α5	94.2	95.1	95.1	94.2	\	96.9	96.5	97.5	97	97.1	96.5	97.4	96.8	96.8	97.7	97.4	96.9
α6	97.3	96.7	96.7	96.7	94.5	\	98.4	97.1	96.3	97.3	98.4	97.8	97.3	96.9	97.2	96.9	96.9
α7	95.1	94.5	94.5	95.6	93.4	97.8	\	97.4	96.3	97.6	99.6	97.4	97.4	96.5	96.9	97.3	97.1
α8	95.3	97.3	97.3	96.8	95.3	96.2	95.1	\	98.4	98.4	97.4	98.1	97.5	97.5	99.1	99.8	97.8
α9	95.3	95.6	95.6	95.8	94.2	94.5	92.3	95.8	\	97.3	96.3	97.5	96.7	97	98.2	98.2	97.1
α10	95.6	97.3	98.4	95.1	94	96.2	95.1	96.7	95.8	\	97.6	97.3	97.3	96.7	97.8	98.2	98
α11	95.1	94.5	94.5	95.6	92.3	97.8	98.9	95.1	96.7	95.1	\	97.4	97.4	96.5	96.9	97.3	97.1
α12	97.9	96.2	96.2	97.4	94.7	97.3	95.1	96.3	95.1	94.5	95.1	\	98.1	98.4	98.6	97.9	97.1
α13	96.8	95.6	95.6	96.3	93.7	96.7	94.5	96.3	96.3	95.1	94.5	97.9		97.2	97.4	97.4	96.3
α14	97.4	96.2	95.1	95.3	93.7	95.6	93.4	94.2	96.3	94	93.4	96.8	95.8	\	97.7	97.7	96.5
α15	95.8	97.8	97.8	96.3	95.8	96.7	94.5	98.4	94.2	96.1	94.5	97.9	96.8	94.7	\	98.9	97.6
α16	94.7	96.7	96.7	96.3	94.7	95.6	94.5	99.5	98.4	96.2	94.5	95.8	95.8	94.7	97.9		97.6
α17	95.1	97.8	97.8	96.7	94	95.6	94.5	97.3	99.5	97.3	94.5	95.1	94.5	94	96.7	96.7	\

mature peptides were always a cysteine. The other three cysteine residues involved in disulfide bond formation at positions 29, 99, and 139 were conserved in all of the PoIFN-α subtypes. In addition, one putative N-glycosylation site was found in all PoIFN-α subtypes. Further sequence alignment showed the high extensive conservation of different PoIFN-α subtypes of Bama minipigs, with 96.3%–99.8 % or 92.3%–99.5 % similarity at the nucleotide or amino acid level (Table 2).

3.3. Sequence analysis of virus-responsive elements in PoIFN-α promoters

By using the subtype-common or gene-specific primers for PoIFN-α promoters, both 98-bp and 116-bp amplicons were obtained from the spleen gDNA of Bama minipigs. After cloning into the PCR cloning vector, twenty-seven clones were sequenced, and nine different consensus sequences were obtained. The four modules in the nine consensus sequences were identical to those in published PoIFN-α promoters, including module AB corresponding to the IRF-7 attachment site, module C to the IRF-3 attachment site, and module D to the PRDI-like domain (Fig. 1). The sequences between modules C and D were highly conserved, and thus PoIFN-α subtypes could not be distinguished according to their promoter sequences.

3.4. Tissue expression profiles of different PoIFN-α subtypes

RT-PCR analysis revealed significant differences in tissue expression profiles among 17 PoIFN-α subtypes of Bama minipigs (Table 3). In lymph nodes, for example, 8 PoIFN-α genes were vastly detected, with a relative expression index higher than one. Multiple subtypes of PoIFN-α were also highly expressed in the spleen (5 genes) and uterus (3 genes). In lymph nodes, the expression level of PoIFN-α17 was the highest with a relative expression index of 22.11, followed by PoIFN-α8 (16.38), PoIFN-α9 (11.36), PoIFN-α2 (8.11), PoIFN-α15 (3.73), PoIFN-α1 (3.20) and PoIFN-α5/6 (2.65). In the spleen, the expression level of PoIFN-α8 was the highest with a relative expression index of 5.88, followed by PoIFN-α16 (3.37), PoIFN-α2 (3.03), PoIFN-α12 (1.96) and PoIFN-α9 (1.48). In contrast, no or low expression of fewer PoIFN-α subtypes was detected in the heart, lung, small intestine, and brain, with a relative expression index lower than one (Table 3).

3.5. Preparation and identification of different subtypes of rPoIFN-α

HEK-293 cells were transfected with pcDNA-PoIFN-α vectors under optimized conditions. After 72 h, the transfected cells were obtained and subjected to Ni-NTA affinity chromatography to obtain purified rPoIFN-α proteins, and SDS-PAGE analysis was performed to assess purity (Fig. 2). Western blotting revealed that all 17 recombinant proteins were recognized by the mAb against the C-terminal His-tag. The molecular weight of the recombinant proteins ranged from 21 to 24 kDa (kDa) (Fig. S2).

3.6. Antiviral activities of different PoIFN-α subtypes

MTS assay results showed that none of the Bama minipig rPoIFN-α subtypes were cytotoxic to tested cells (data not shown). The CPE inhibition assay results revealed that all Bama minipig PoIFN-α subtypes exhibited dose-dependent antiviral activities against VSV, PRV, and PRRSV. Among the 17 PoIFN-α subtypes, PoIFN-α5, PoIFN-α6, and PoIFN-α10 showed the highest antiviral activity against PRV, VSV, and PRRSV at a dose of 2 ng/ml, with 60 %, 68 % and 56 % CPE protection, respectively. In contrast, PoIFN-α3 exhibited the lowest antiviral activity against the three pig viruses, with only 6 %, 8 % and 4 % CPE protection, respectively (Fig. 3A). At a dose of 200 ng/ml, the PoIFN-α14 showed highest antiviral activity against VSV or PRRSV, with 85 % or 81 % CPE protection, whereas PoIFN-α5/7 exhibited highest anti-PRV activity with 85 % CPE protection. In contrast, the PoIFN-α12/13 showed the lowest antiviral activity against VSV, PRRSV, and PRV, with 40 %, 52 %, and 38 % CPE protection, respectively (Fig. 3B).

4. Discussion

The PoIFN complex is an ideal model for investigating IFN evolution during speciation and domestication [20]. Compared to domestic pigs, minipigs are less domesticated and experience diverse viral pressures. Bama minipigs are disease-resistant and genetically stable [11,12]. Thus, we cloned 17 subtypes of PoIFN-α genes in Bama minipigs in an attempt to define the potential mechanism of disease resistance. Similar to the PoIFN-α peptides of domestic pigs, all Bama minipig PoIFN-α subtypes possessed a functional IFabd domain, which is a type I IFN signature [21], numerous attachment positions for type I IFNAR subunits and one

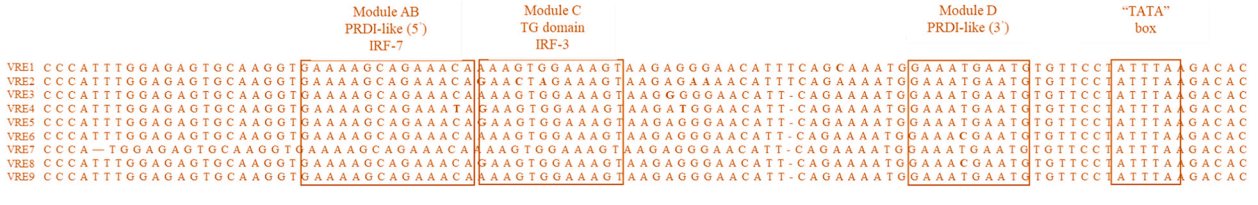

Fig. 1. Sequence alignment of Bama minipig PoIFN-α promoters. The promoter regions were amplified from gDNA using sequence-specific primer pairs. The four promoter modules and TATA box are boxed. Short lines indicate nucleotide deletions.

Table 3
Tissue expression profiles of the 17 Bama minipig PoIFN-α subtypes. Significant differences are shown as * or **, representing $p < 0.05$ and $p < 0.01$, respectively.

Subtype	Skin	Uterus	Heart	Lung	Small intestine	Lymph node	Spleen	Liver	Kidney	Brain
α1	0.00	0.91± 0.043*	0.65± 0.037*	0.31± 0.081*	0.26± 0.027*	3.203± 0.023**	0.423± 0.20*	0.32± 0.023*	0.00	0.00
α2	0.55± 0.173*	1.30± 0.710**	0.91± 0.021*	0.53± 0.025*	0.77± 0.021*	8.11± 2.97**	3.03± 0.95**	0.80± 0.039*	1.97± 0.51**	0.82± 0.040*
α3	0.00	0.34± 0.46*	0.00	0.04± 0.009*	0.00	0.05± 0.009*	0.33± 0.02*	0.00	0.00	0.00
α4	0.00	0.74± 0.046*	0.06± 0.036*	0.33± 0.012*	0.00	0.29± 0.013*	0.00	0.00	0.43± 0.130*	0.00
α5/6	0.71± 0.210*	0.00	0.00	0.00	0.75± 0.123*	2.65 ±1.15**	0.52± 0.039*	0.56± 0.71*	0.93± 0.056*	0.00
α7/11	0.00	0.00	0.00	0.61± 0.040*	0.00	0.00	0.00	0.11± 0.017*	0.26± 0.010*	0.00
α8	0.66± 0.044*	0.82± 0.034*	0.00	0.91± 0.092*	0.66± 0.046*	16.38± 2.990**	5.88± 2.686**	0.61± 0.032*	2.45± 1.583**	0.44± 0.020*
α9	0.00	1.23± 0.984**	0.51± 0.170*	0.24± 0.094*	0.98± 0.391*	11.36± 1.786**	1.48± 0.520**	2.18± 0.762**	0.44± 0.019*	0.00
α10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13± 0.050*	0.43± 0.040*	0.00
α12	0.00	0.75± 0.299*	0.14± 0.103*	0.00	0.93± 0.052*	1.36± 0.324**	1.96± 0.428**	0.04± 0.020*	0.54± 0.042*	0.00
α13/14	0.00	0.00	0.00	0.00	0.00	0.64± 0.373*	0.78± 0.024*	1.19± 0.034**	0.95± 0.052*	0.00
α15	2.47± 1.072**	0.69± 0.033*	0.00	0.00	0.00	3.73± 1.689**	0.57± 0.025*	0.00	0.00	0.00
α16	0.00	0.77± 0.172*	0.00	0.26± 0.101*	0.00	0.90± 0.107*	3.37± 0.133**	0.00	0.00	0.00
α17	0.00	1.03± 0.177**	0.00	0.00	0.00	22.11± 5.584**	0.00	0.62± 0.295*	0.00	0.00

The significant difference is indicated by * or ** which represents difference at $p < 0.05$ or $p < 0.01''$.

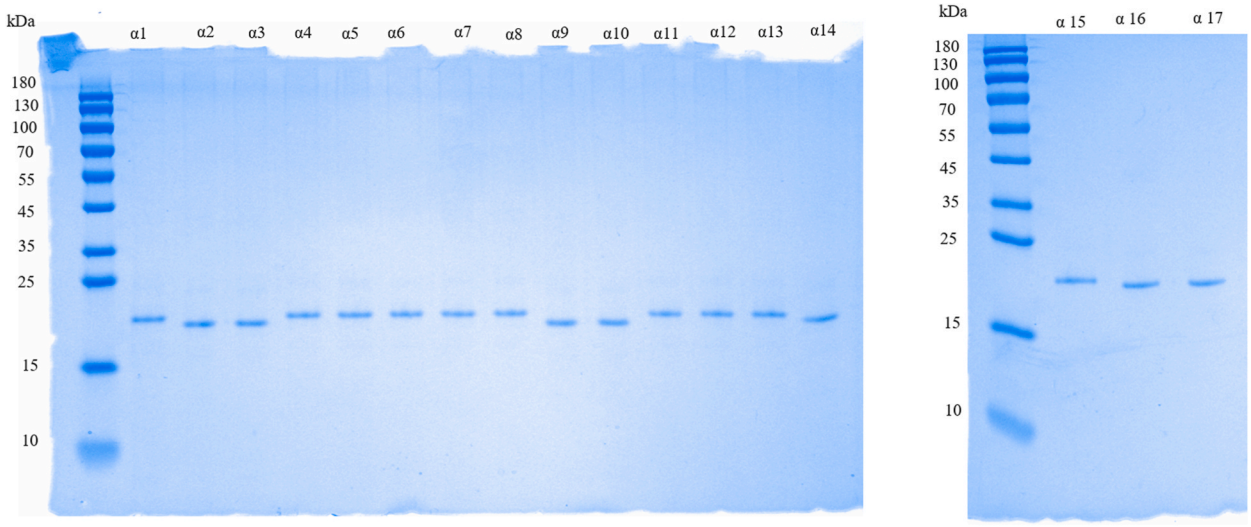

Fig. 2. SDS-PAGE and western blotting analyses of the recombinant proteins of the 17 Bama minipig PoIFN-α subtypes. The recombinant proteins were expressed as His-tagged proteins and purified using Ni-NTA affinity chromatography. The purity was assessed using SDS-PAGE.

putative N-glycosylation site, which are necessary for IFN signaling and protein constancy [5]. Furthermore, all PoIFN-α subtypes had four conserved cysteines, residues that are crucial for the development and structure equilibrium of disulfide bonds [6]. These data suggest striking overall conservation of PoIFN-α between Bama minipigs and domestic pigs. Although they have high sequence homology, the promoter sequences among most type I PoIFN subclasses are relatively different, indicating differential expression of type I PoIFN genes with respect to tissue or cell types and/or different stimuli [5]. Our qPCR analysis revealed considerable differences in the tissue expression of different PoIFN-α subtypes. For example, only PoIFN-α15 was significantly expressed in the skin of Bama minipigs, whereas multiple-subtypes PoIFN-α were significantly expressed in the skin of domestic pigs [5]. In contrast, multiple-subtype PoIFN-α

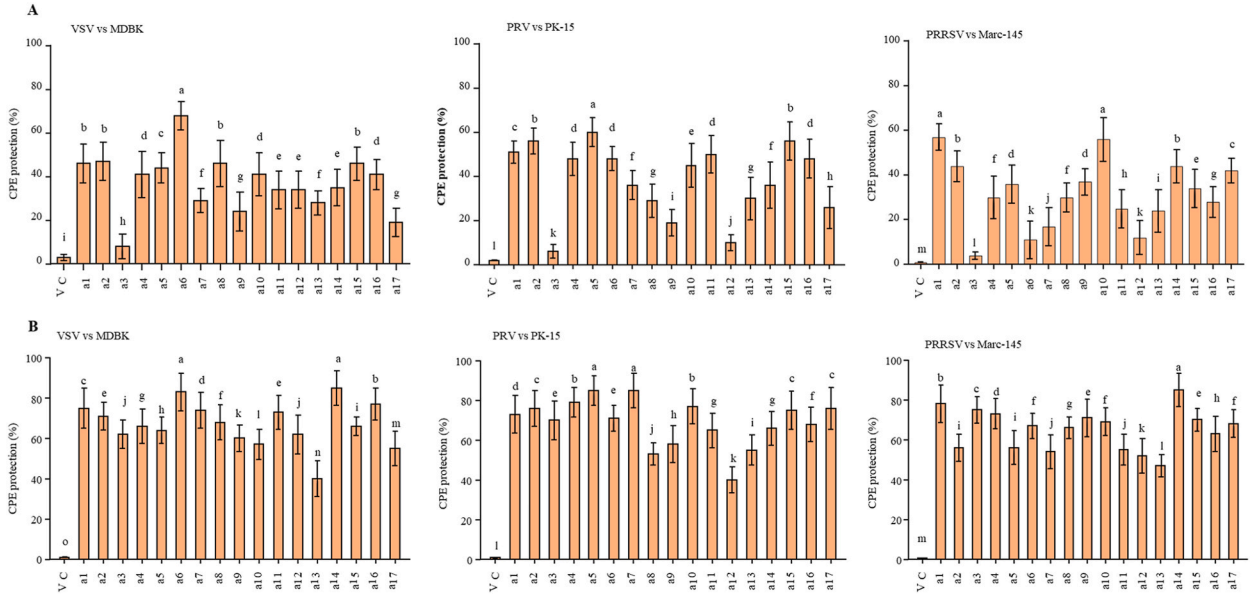

Fig. 3. Comparison of antiviral activities of the 17 Bama minipig PoIFN-α subtypes against three different pig viruses at two different doses. The antiviral activities were detected by CPE inhibition assay using the purified recombinant proteins at a dose of 2 ng/ml (A) or 200 ng/ml (B). Different letters indicate significant differences compared with virus control (VC).

genes were highly expressed in the lymph nodes of Bama minipigs, Whereas only the PoIFN-α12 gene was extensively expressed in the lymph nodes of domestic pigs [5]. To explain such differential gene expression, we cloned PoIFN-α promoters from Bama minipigs. Surprisingly, nine consensus sequences obtained were almost identical, including the four promoter modules and t sequences between. In contras the sequences between modules C and D of domestic pig PoIFN-α promoters are variable [6]. The high level of conservation of the Bama minipigs PoIF-α promoter may be due to long-term selective inbreeding. These experimental data suggest that the differential expression of PoIF-α in Bama minipigs is primarily due to various environmental stimuli, and not by promoter sequence differences. SDS-PAGE analysis showed efficient expression of the 17 PoIF-α subtypes from Bama minipigs in the vector-transfected cells. The molecular weights (20 or 21 kDa) of the 17 recombinant proteins were 2- or 3-kDa heavier than the predicted values (17- or 18 kDa mature peptides + 0.7 kDa His-tag), which could be attributed to protein glycosylation in mammalian cells. Western blotting results detected all purified proteins using an mAb against their C-terminal His-tag. Thus these data indicate the usability of purified proteins for antiviral assays. A previous study showed that most PoIFN-α subtypes in domestic pigs provide nearly full protection against VSV and PRRSV infections in MARC-145 cells and porcine macrophages, with PoIFN-α6 showing the highest antiviral activity against PRRSV, whereas PoIFN-α7 and PoIFN-α11μ (guanosine-deletion mutant of IFN-α7/11) demonstrated low antiviral activities against PRRSV and VSV [5]. However, in the present study, the 17 Bama minipig PoIFN-α subtypes demonstrated dose-dependent and differential antiviral activities against VSV, PRRSV, and PRV. For example, PoIFN-α6 showed the highest antiviral activity against VSV but not against PRV and PRRSV at two different doses. In all evaluations, PoIFN-α7 showed relatively high antiviral activity against the three pig viruses, particularly at the higher dose. An investigation of porcine IFN-complex from Bama Minipigs may further strengthen our knowledge of IFN biology and endorse IFN-based therapy to fight against different viral diseases. Due to the lack of type or subtype-specific antibodies to PoIFN, the differential expression of IFN tissue in miniature pigs was only detected by quantification of the fluorescence signal in qPCR. Therefore, the differential expression of IFN in minipigs requires further investigation and comparison at the protein level.

5. Conclusions

We compared the sequence homology, antiviral activity, and tissue expression patterns of PoIFN-α genes between Bama minipigs and domestic pigs. Although they shared high homology, the tissue expression profiles and antiviral activities considerably differed between species and were not caused by promoter sequence variation. Differences in disease resistance between the two pig species may be due to differential PoIFN-α responses to environmental stimuli rather than PoIFN-α gene variation. Our results provide the basic foundation for the rational use of minipig breeds.

Ethical approval

All animal-related procedures were conducted according to instructions of the Guide for the Care and Use of Laboratory Animals of Yangzhou University and approved by the Medical Experimental Animal Center of Jiangsu Province (No. NSFC2020-dkxy.02,March

27, 2020).

Data availability statement

The coding sequences for the Bama minipig PoIFN-α genes have been submitted in GenBank, and obtained accession numbers are presented in Table 2 and upon reasonable request related data can be obtained from the corresponding author.

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CRediT authorship contribution statement

Aziz Ullah Noor: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Lu Huipeng:** Investigation, Formal analysis, Data curation. **Zhanyu Du:** Resources, Project administration, Funding acquisition. **Song Chengyi:** Resources, Project administration, Funding acquisition, Formal analysis. **Zhou Xiaohui:** Validation, Formal analysis, Data curation. **Liu Xiaoming:** Validation, Formal analysis, Data curation. **Suliman Khan:** Formal analysis, Data curation, Validation. **Huaichang Sun:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis. **Abdelouahab Bellou:** Writing – review & editing, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Azizullah Noor reports financial support was provided by Yangzhou University and Guangdong Provincial People's Hospital. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

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