Identification and Development of African Swine Fever Virus Vaccine Candidates by Reverse Vaccinology

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African swine fever (ASF)

- Highly contagious viral disease with significant economic impact
- Infections often fatal and characterized by fever, hemorrhages, ataxia, and severe depression
- Occurs in several forms ranging from highly lethal to sub-clinical
  - Acute forms: pronounced hemorrhages and death in 3 to 7 DPI
  - Sub-acute and chronic forms: high fever, staggering gait, cough, diarrhea, purple skin, and death in 20-45 DPI
- Affects all age groups, without gender preference
- Caused by ASFV, large enveloped dsDNA Arbovirus ~190 kb genome
African swine fever virus (ASFV)

- Targets and replicates in macrophages
- Natural hosts are warthogs, bush pigs, and giant forest hogs
- *Ornithodoros* ticks are reservoirs and vectors
- Resistant to low temperatures; inactivated by 56°C for 70 mins
- Persist in blood, tissues, secretions, and excretions
- Persist 399 days in Parma hams, 104 days in frozen meat or chilled meat, 30 days in pepperoni & salami (Rev. sci. tech. Off. int. Epiz., 16 (1), 65-78)
- Transmission mainly through oro-nasal route
  - direct: contact b/w sick and healthy animals
  - indirect transmission: biological vectors, swill, fomites including premises, vehicles, implements, and clothes

Bishop et al, 2012
African swine fever (ASF)

- ASF was first identified in Kenya in 1910 as acute haemorrhagic fever with high mortality (up to 100%) in domestic pigs. Outbreaks occurred when domestic pigs came into contact with wild pigs.

- ASF has expanded from its origin in Africa to Southern Europe, the Caribbean, Brazil, Eastern Europe, and Northwest Asia.
ASF Global Distribution

- First case outside of Africa was reported in Portugal, 1957
- Subsequent cases reported in:
ASF in Trans Caucasian Countries (TCC) and Russia

Inset: June 2013 reporting period, outbreaks in Georgia, Nigeria, Chad, Cameroon, and Central African Republic

ASF Upsurge

- Progressive spread likely due:
  - Economic crisis leading to swill feeding
  - Globalization and increased movements and people and products
  - Growth of the pig sector in Africa, with countries more than doubling their populations in less than a decade
- Global pig production growth due to growing demand

Table 1377. Meat Consumption by Type and Country: 2009 and 2010

<table>
<thead>
<tr>
<th>Country</th>
<th>Beef and veal (^1) 2009</th>
<th>Beef and veal (^1) 2010</th>
<th>Pork (^1) 2009</th>
<th>Pork (^1) 2010</th>
<th>Broiler (^2) 2009</th>
<th>Broiler (^2) 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>World</td>
<td>56,668</td>
<td>56,544</td>
<td>100,268</td>
<td>102,953</td>
<td>71,860</td>
<td>75,127</td>
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<tr>
<td>United States</td>
<td>12,239</td>
<td>12,040</td>
<td>21,057</td>
<td>21,271</td>
<td>12,640</td>
<td>13,463</td>
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<tr>
<td>Brazil</td>
<td>7,374</td>
<td>7,592</td>
<td>2,423</td>
<td>2,467</td>
<td>8,032</td>
<td>9,132</td>
</tr>
<tr>
<td>China</td>
<td>5,749</td>
<td>5,589</td>
<td>8,363</td>
<td>8,389</td>
<td>8,692</td>
<td>8,779</td>
</tr>
<tr>
<td>Russia</td>
<td>2,347</td>
<td>2,307</td>
<td>2,442</td>
<td>2,485</td>
<td>3,264</td>
<td>3,344</td>
</tr>
<tr>
<td>Argentina</td>
<td>2,727</td>
<td>2,305</td>
<td>2,187</td>
<td>2,216</td>
<td>2,966</td>
<td>2,923</td>
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<tr>
<td>India</td>
<td>1,905</td>
<td>1,930</td>
<td>1,876</td>
<td>1,881</td>
<td>2,549</td>
<td>2,649</td>
</tr>
<tr>
<td>Mexico</td>
<td>1,971</td>
<td>1,944</td>
<td>1,770</td>
<td>1,774</td>
<td>1,978</td>
<td>2,063</td>
</tr>
<tr>
<td>Pakistan</td>
<td>1,461</td>
<td>1,491</td>
<td>1,480</td>
<td>1,539</td>
<td>1,542</td>
<td>1,660</td>
</tr>
<tr>
<td>Japan</td>
<td>1,211</td>
<td>1,214</td>
<td>1,298</td>
<td>1,358</td>
<td>1,419</td>
<td>1,514</td>
</tr>
<tr>
<td>Other countries</td>
<td>10,406</td>
<td>10,938</td>
<td>7,135</td>
<td>7,750</td>
<td>1,327</td>
<td>1,395</td>
</tr>
</tbody>
</table>

\(^1\) May include meat of other bovines. \(^2\) Excludes chicken paws. \(^3\) Preliminary data. \(^4\) See footnote 4, Table 1332. \(^5\) European Union-27: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Sweden, Iceland, and United Kingdom. \(^6\) Includes buffalo.


See also: <http://fas.usda.gov/crmrnt.asp> and <http://www.census.gov/compendia/statab/2012/tables/12s1377.pdf>
ASF Impact

- Socioeconomic impact:
  - Negative impact on development of pig production sector
  - Constraint to income and food security to small production sector
  - Financial losses due to high mortality, trade restrictions, & control strategies

- Russian Federation:
  - ASF has persisted since 2008 and continues to spread
  - Since 2007 to mid-2012, 600,000 pigs have died/culled
  - Overall losses estimated at 1 billion USD

(www.fao.org/ag/empres.html)
ASF Control

- No drugs for treatment or cure
- No vaccine
- Strict biosecurity is main prevention strategy
- Culling/stamping out is control strategy
- Early detection can expedite control measures and mitigate disease outbreak
ASF Vaccine Development

- Critical countermeasure
- Inactivated virions do not induce protection
- Attenuated vaccine
  - Historically unsuccessful and unsafe
  - Produce adverse reactions, chronic forms, and death

- Good prospects for vaccine development:
  - Pigs surviving infection develop homologous protective immunity
  - Pigs immunized with low virulent and attenuated viruses can be protected against challenge with virulent viruses
  - Cross-protection can be induced
  - Antibody provide partial protection and delay onset of clinical signs
  - Humoral and cellular immunity are required
  - Natural Killer and CD8+ T cells are important components
Desirable Vaccine Product Requirements

- Efficacy: prevents virus amplification; effective for all ages; quick onset of protection, 7 days or less
- Safety: no reversion to virulence for LAVs
- Single dose
- Easy manufacturing, fast scale-up, supply, and distribution
- Robust long term storage
- DIVA compatible; differentiate infected from vaccinated animals
  - Compatible with control and eradication program
- Low cost
Strategy & Considerations of Vaccine Platforms

- Live attenuated virus:
  - Protection has been demonstrated
  - Potential of reversion to virulence and persistence
  - No simplified DIVA strategy

- Subunit vaccines:
  - Limited protection data
  - Require extensive effort for protective antigens identification
  - Potential strengths include safety, easy manufacturing and scale-up, low cost, and DIVA compatible
  - “Must have” to eliminate disease
Our Approach

- Multi-institutional, inter-disciplinary team funded through DHS Broad Agency Announcement (BAA)

- Reverse vaccinology will be used to identify novel *in silico* candidates

- Two protein expression and delivery platforms will be evaluated:
  - Mammalian cell culture
  - Viral-vectored

- Recombinant vaccine candidates will be tested for immunogenicity and safety in pigs

*We anticipate that candidate vaccines will be highly immunogenic and induce strong humoral and cellular responses in pigs*
Conventional Vaccinology

Some microorganisms can’t be cultivated

Antigen selection

Test convalescent sera

Identify components

Test immunogenicity

Purify components

Clone genes

Immunogenicity testing in animal models

VACCINE DEVELOPMENT

Vaccine

5-15 years

Modified from Nature reviews, 2006. 4:932-942
Reverse Vaccinology

RV is a vaccine development strategy that starts with bioinformatics analysis of pathogenic genomes to find potential vaccine candidates. Candidate genes are tested empirically for protective immune responses.

Start from whole genome sequences

Comparative genomics and antigen predictions

*In silico* vaccine candidates

Express recombinant proteins

DNA vaccine preparation

Immunogenicity testing in animal models

VACCINE DEVELOPMENT

Vaccine

Modified from Nature reviews, 2006. 4:932-942
### Key Features of Reverse Vaccinology

<table>
<thead>
<tr>
<th>Conventional vaccinology</th>
<th>Reverse vaccinology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uses the most abundant antigens during disease</td>
<td>Analyses all antigens produced, whether abundant or not</td>
</tr>
<tr>
<td>Organism must be cultivable</td>
<td>Organism does not need to be cultivable</td>
</tr>
<tr>
<td>Animal models required</td>
<td>Animal models required</td>
</tr>
<tr>
<td>High-throughput expression and analysis are not required</td>
<td>High-throughput expression and analysis are important</td>
</tr>
<tr>
<td>Antigen identification is usually time consuming</td>
<td>Antigen identification is rapid</td>
</tr>
<tr>
<td>Only structural proteins are considered</td>
<td>Non-structural proteins are analysed</td>
</tr>
<tr>
<td>Polysaccharides and lipopolysaccharides can be used as antigens</td>
<td>Non-protein antigens, such as polysaccharides and lipopolysaccharides, cannot be identified</td>
</tr>
</tbody>
</table>

*Table modified, with permission, from REF. 6 © (2000) Elsevier Science.*

Nature Reviews Microbiology. 2008: 6:334-335
Reverse Vaccinology Funnel for MenB Vaccine

- 600 potential candidates through selection for surface-exposed or secreted proteins
- 350 proteins successfully expressed in *E. coli*
- 344 proteins purified and used to immunize mice
- 91 novel surface-exposed proteins identified
- WB, ELISA, FACS
- 28 novel proteins have bactericidal activity
- 5 top candidates

3 proteins + OMVs

4CMenB
4CMenB success

- First RV based licensed (2012) vaccine for human
- Compose of 4 components (fHbp, NadA, NHBA, and OMVs) to "increase the spectrum of vaccine coverage, minimizing the possibility of bacterial evasion and the emergence of selection mutants" (Serruto et al, Vaccine. 2012)
- fHbp and NHBA-evasion from complement pathway > pathogen survival
- NNadAmediate adhesion to and entry into cells
- All proteins related to virulence
Our approach overview

In silico analysis by Vaxign and ancillary tools
Screen for transmembrane domains/adhesin probability/present in virulent strains only/ MHC I and MHCII epitopes

Clone and express 14 candidates in mammalian cells
Select 5 ASF hyperimmune serum reactive candidates for large scale-up production
Produce MAbs & PAbs against antigens
Proof of concept (swine immunogenicity and safety) studies

Clone and express 5 candidates in viral vector
Grow, select with ASF hyperimmune serum for large scale production and purify
It is challenging to apply reverse vaccinology without a comprehensive pipeline.

To address this challenge, \textit{Vaxign} was developed.

Dr. Yongqun “Oliver” He
Dr. Allen Xiang
Vaxign: Vaccine Design System

Vaccine target prediction pipeline for reverse vaccinology

- The 1st web-based reverse vaccinology system
- Freely available: http://www.violinet.org/vaxign

Reverse Vaccinology Criteria

1. Transmembrane domains
   ✓ >2 α-helix domains → difficult to isolate
2. Adhesin probability
   ✓ Adhesin is important for pathogen invasion
3. MHC-Epitope binding
   ✓ MHC class I epitope → cell-mediated immunity
   ✓ MHC class II epitope → antibody response
4. Sequence conservation and exclusion
   ✓ Shared genes in pathogens but not in avirulent strains
5. Similarity to host proteins
   ✓ Avoid autoimmunity or immune tolerance
### Complete ASFV Genomes Utilized for RV Comparison and Ranking

<table>
<thead>
<tr>
<th>Accession</th>
<th>Complete genome</th>
<th>Host</th>
<th>Virulence</th>
<th>Length bp</th>
<th>No. ORFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY261360</td>
<td>ASFV Kenya 1950</td>
<td>Domestic pig</td>
<td>high</td>
<td>193886</td>
<td>161</td>
</tr>
<tr>
<td>AY261361</td>
<td>ASFV Malawi Lil-20/1 (1983)</td>
<td>Tick</td>
<td>high</td>
<td>187612</td>
<td>160</td>
</tr>
<tr>
<td>AY261362</td>
<td>ASFV Mkuzi 1979</td>
<td>Tick</td>
<td>unknown</td>
<td>192714</td>
<td>167</td>
</tr>
<tr>
<td>AY261363</td>
<td>ASFV Pretoriuskop/96/4</td>
<td>Tick</td>
<td>high</td>
<td>190324</td>
<td>167</td>
</tr>
<tr>
<td>AY261364</td>
<td>ASFV Tengani 62</td>
<td>Domestic pig</td>
<td>high</td>
<td>185689</td>
<td>162</td>
</tr>
<tr>
<td>AY261365</td>
<td>ASFV Warmbaths</td>
<td>Tick</td>
<td>unknown</td>
<td>190773</td>
<td>167</td>
</tr>
<tr>
<td>AY261366</td>
<td>ASFV Warthog</td>
<td>Warthog</td>
<td>unknown</td>
<td>186528</td>
<td>164</td>
</tr>
<tr>
<td>AM712239</td>
<td>ASFV Benin 97/1 pathogenic isolate</td>
<td>Domestic pig</td>
<td>high</td>
<td>182284</td>
<td>156</td>
</tr>
<tr>
<td>FN557520</td>
<td>ASFV strain E75</td>
<td>Domestic pig</td>
<td>high</td>
<td>181187</td>
<td>166</td>
</tr>
<tr>
<td>FR682468</td>
<td>ASFV Georgia 2007/1</td>
<td>Domestic pig</td>
<td>high</td>
<td>189344</td>
<td>160</td>
</tr>
<tr>
<td>AM712240</td>
<td>ASFV OURT 88/3 (avirulent field isolate)</td>
<td>Tick</td>
<td>low</td>
<td>171719</td>
<td>157</td>
</tr>
<tr>
<td>NC001659</td>
<td>ASFV BA71V strain, tissue culture adapted</td>
<td>tissue culture</td>
<td>avirulent</td>
<td>170101</td>
<td>160</td>
</tr>
</tbody>
</table>
Reverse vaccinology ranking strategy

1. Analysis using the following parameters:
   - Vaxign rank ORFs (1-160) based on each parameter (below)
   - Normalize MHC scores to protein length
   - Include ORFs conserved with swine genome

2. Determine candidates protein localization on viral particle (exposed, internal, etc)
3. Determine ORF conservation among all available genomes
4. Determine gene function by literature search or structural function analysis
5. Send top 30 list to ASF team members and DHS for comments before final selection
6. Select top 14 candidates for protein expression
126 ASFV genes are conserved among all 12 genomes
These genes are considered as ASFV core genes
Functions of the majority of ASFV proteins are unknown

Automated protein structure and function predictions tool (I-TASSER) was developed

### I-TASSER Prediction Verification

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Literature Search Results</th>
<th>I-TASSER Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Function</td>
<td>Protein localization</td>
</tr>
<tr>
<td>1</td>
<td>structural attachment protein involved in morphogenesis</td>
<td>viral membrane</td>
</tr>
<tr>
<td>2</td>
<td>structural protein involved in morphogenesis</td>
<td>viral membrane</td>
</tr>
<tr>
<td>3</td>
<td>structural protein involved in host cell binding and entry</td>
<td>viral membrane</td>
</tr>
<tr>
<td>4</td>
<td>structural protein involved in morphogenesis</td>
<td>viral outer envelope</td>
</tr>
<tr>
<td>5</td>
<td>glycoprotein that interacts w/lectin found on RBC membrane and play a role in the virus budding, attachment to RBC and virus spread</td>
<td>viral membrane</td>
</tr>
<tr>
<td>6</td>
<td>glycoprotein that interacts with CD2v; viral C-type lectin with anti-apoptotic properties; host evasion molecule</td>
<td>viral membrane</td>
</tr>
<tr>
<td>7</td>
<td>structural protein involved in host cell binding and entry</td>
<td>viral capsid</td>
</tr>
</tbody>
</table>

- High Gene Ontology scores (>0.5) correlated with published predicted functions
- Predicted cellular localization and protein binding correlated well (~80%) with published localization
## Protein Expression

<table>
<thead>
<tr>
<th>Candidate #</th>
<th>Expressed in HEK 293</th>
<th>HEK purification capable</th>
<th>Expressed &amp; purified in Baculovirus</th>
<th>Expressed &amp; purified in VV</th>
<th>Recognized by ASF antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td>WIP</td>
<td>Y(B)</td>
</tr>
<tr>
<td>2</td>
<td>Y</td>
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<td>Y</td>
<td></td>
<td>Y(B,H)</td>
</tr>
<tr>
<td>3</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y(B,H)</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y(B,H,V)</td>
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<tr>
<td>5</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
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<tr>
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<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y(B,H)</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>Y</td>
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<td>Y(B,H)</td>
</tr>
<tr>
<td>9</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td></td>
<td>Y(B)</td>
</tr>
<tr>
<td>10</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td></td>
<td>Y(B)</td>
</tr>
<tr>
<td>11</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td>Y</td>
<td>Y(B,V)</td>
</tr>
<tr>
<td>12</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>13</td>
<td>N</td>
<td>NA~</td>
<td>Y</td>
<td>WIP</td>
<td>Y(B,V)</td>
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<tr>
<td>14</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y(B,H,V)</td>
</tr>
</tbody>
</table>

- 7 expressed in HEK; 5 purified
- All 14 expressed in and purified from Baculovirus
- All 5 selected for viral vector expressed; 3 purified
- **12 recognized by ASF antiserum by Western Blot**
Candidate induced antibodies recognize ASFV BA71V (infected Vero cells 24 hr PI)
Successfully expressed and purified candidates may be used to generate reagents for diagnostics
Proof-of-concept studies in pigs

- Immunizations completed
- Evaluate vaccine candidates' immunogenicity in pigs
  - ELISA for antigen specific antibody response
  - ELISPOT assay for the detection of IFN- secreting T-cells
  - [3H]-thymidine for antigen-specific T-cell responses
Proof-of-concept studies in pigs

- Evaluate vaccine candidates' safety
  - Daily monitoring during the first 7 dpi
    - Monitor injection site lesions, animal behavior and weight
  - Selected injection site biopsies will be taken for histo-pathological analysis
Conclusions

- **First application** of reverse vaccinology for ASF vaccine candidate identification

- **Multi-pronged approach** using different recombinant expression delivery systems

- Provide ASF vaccine candidates with **DIVA** capability

- Produce highly valuable **diagnostic reagents** (MAbs and PAbs) for ASFV detection
THANK YOU