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


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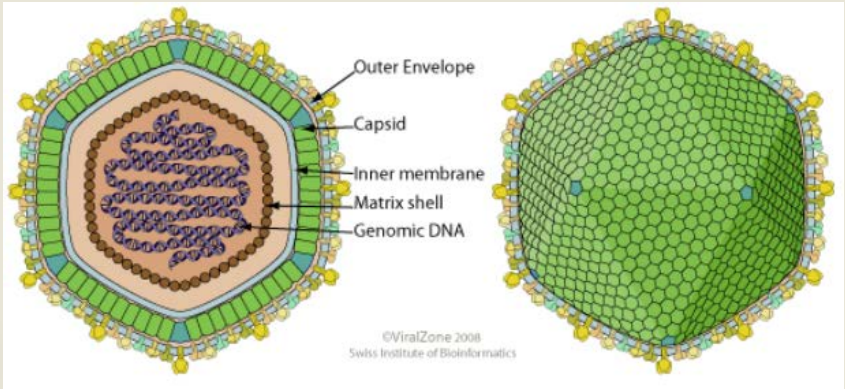
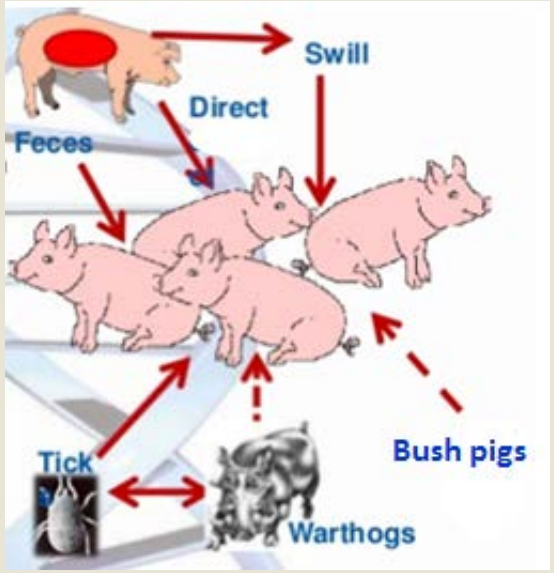
# African swine fever (ASF)

- 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



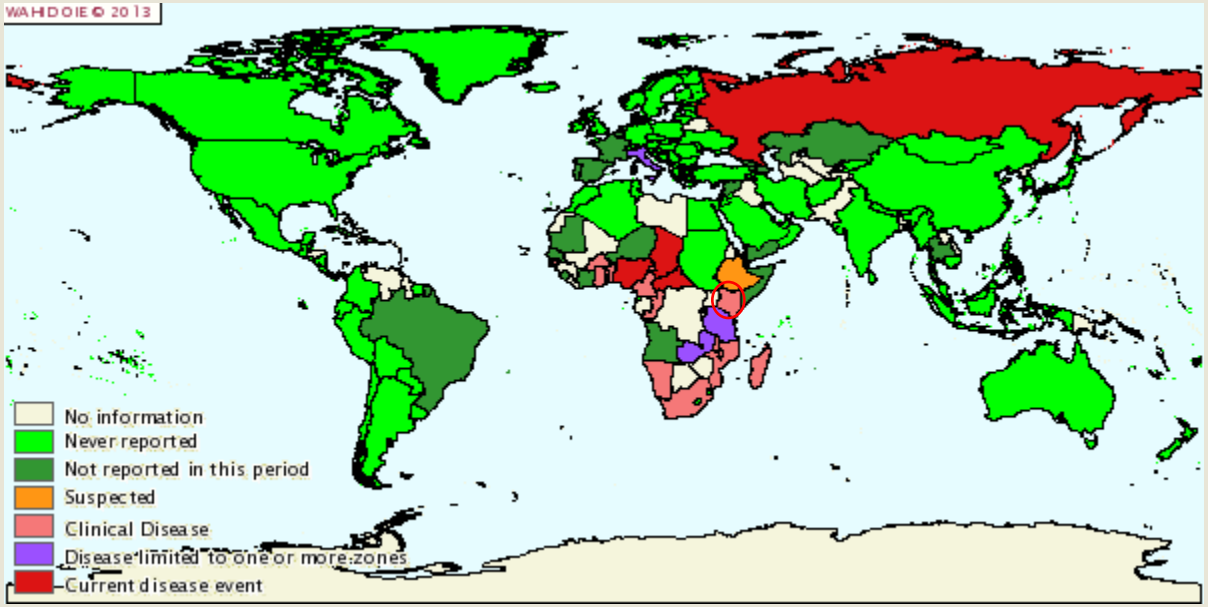

Bishop et al, 2012

# 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

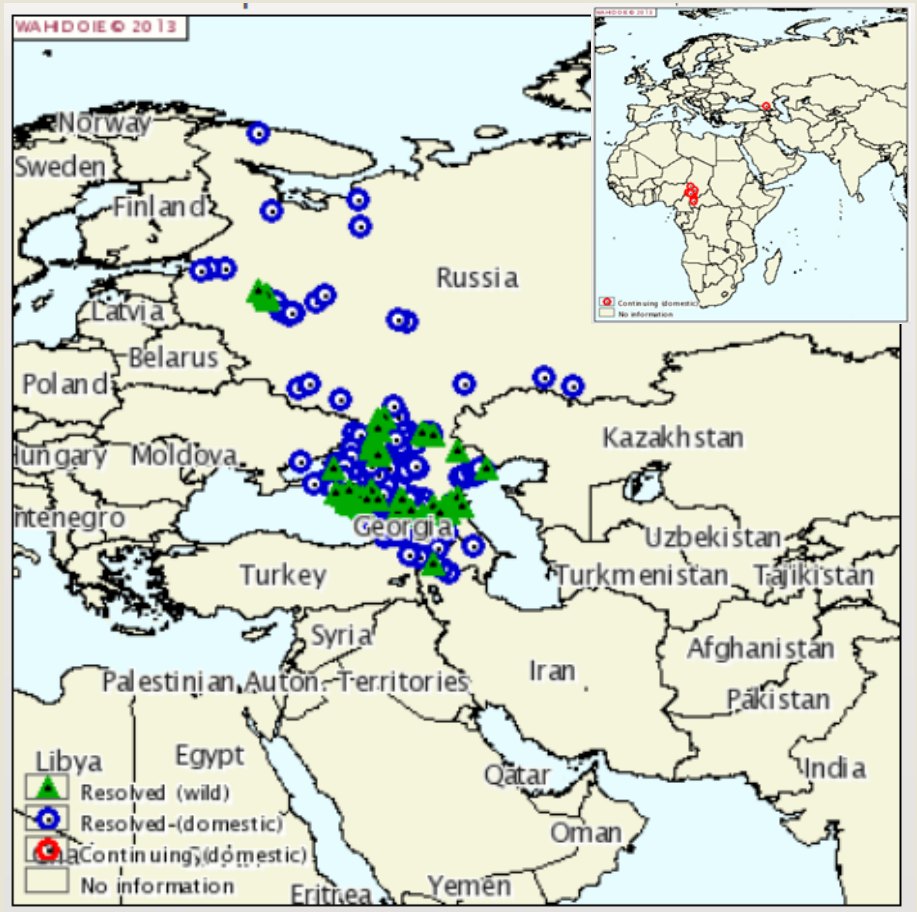



www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap: Jan-June 2012 Reporting period

- First case outside of Africa was reported in Portugal, 1957
- Subsequent cases reported in:

  Spain, 1960-95; France, 1964/67/77; Italy, 1967/80; Malta, 1978-79;
  Sardinia, 1978-present; Belgium, 1985; Netherland, 1986
  Cuba, 1971/78-80; Dom. Rep., 1978-81; Haiti, 1979-84
  Brazil, 1978-81

# ASF in Trans Caucasian Countries (TCC) and Russia




www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap: Jan 2007-June 2013 Reporting period

**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**

[In thousands of metric tons (56,668 represents 56,668,000). Carcass weight basis for beef, veal, and pork. Broiler (chicken, 16-week-old) weight based on ready-to-cook equivalent]

| Country | Beef and veal [1] | | Country | Pork | | Country | Broiler [2] | |
|---|---|---|---|---|---|---|---|---|
| | 2009 | 2010 [3] | | 2009 | 2010 [3] | | 2009 | 2010 [3] |
| **World** | **56,668** | **56,544** | **World** | **100,268** | **102,953** | **World** | **71,860** | **75,127** |
| **United States** | **12,239** | **12,040** | China [4] | 48,823 | 51,097 | **United States** | **12,940** | **13,463** |
| EU–27 [5] | 8,262 | 8,185 | EU-27 [5] | 21,057 | 21,271 | China [4] | 12,210 | 12,457 |
| Brazil | 7,374 | 7,592 | **United States** | **9,013** | **8,653** | Brazil | 8,032 | 9,132 |
| China [4] | 5,749 | 5,589 | Russia | 2,688 | 2,773 | EU-27 [5] | 8,692 | 8,779 |
| Russia | 2,347 | 2,307 | Brazil | 2,423 | 2,577 | Mexico | 3,264 | 3,344 |
| Argentina | 2,727 | 2,305 | Japan | 2,467 | 2,485 | Russia | 2,966 | 2,923 |
| India [6] | 1,905 | 1,930 | Vietnam | 1,876 | 1,881 | India | 2,549 | 2,649 |
| Mexico | 1,971 | 1,944 | Mexico | 1,770 | 1,774 | Japan | 1,978 | 2,063 |
| Pakistan | 1,461 | 1,491 | Korea, South | 1,480 | 1,539 | Iran | 1,542 | 1,660 |
| Japan | 1,211 | 1,224 | Philippines | 1,298 | 1,358 | South Africa | 1,443 | 1,514 |
| Canada | 1,016 | 999 | Ukraine | 713 | 795 | Argentina | 1,327 | 1,395 |
| Other countries | 10,406 | 10,938 | Other countries | 6,660 | 6,750 | Other countries | 14,917 | 15,748 |

[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, Slovakia, Slovenia, Spain, Sweden, and United Kingdom. [6] Includes buffalo.

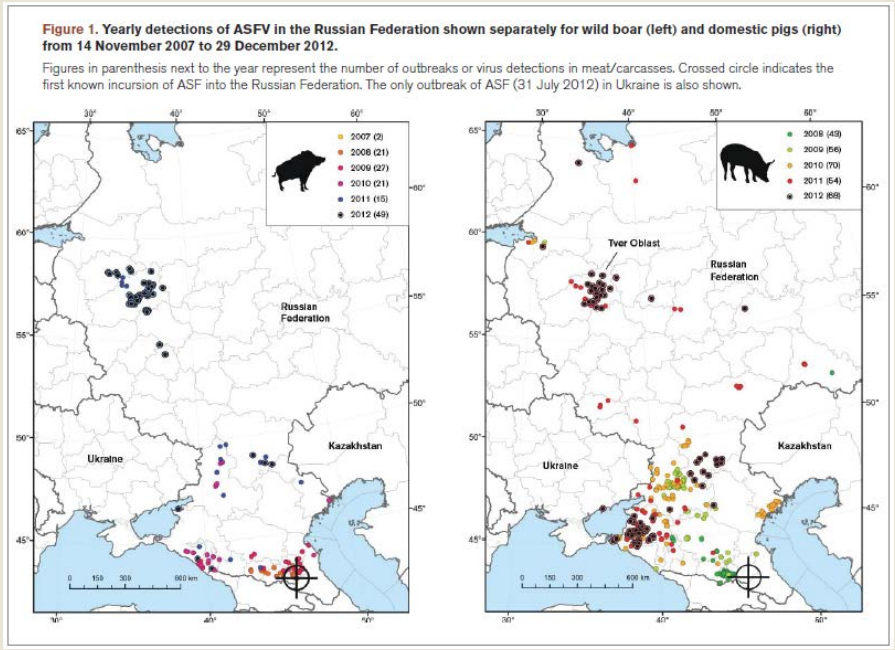
Source: U.S. Department of Agriculture, Foreign Agricultural Service, *Livestock and Poultry: World Markets and Trade*, annual. See also <http://www.fas.usda.gov/currwmt.asp>.

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

Carcasses of pigs prepared to be destroyed by burning as a part of disease control measures at an ASF outbreak site in the Russian Federation. © *Andrey Gogin*

# 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

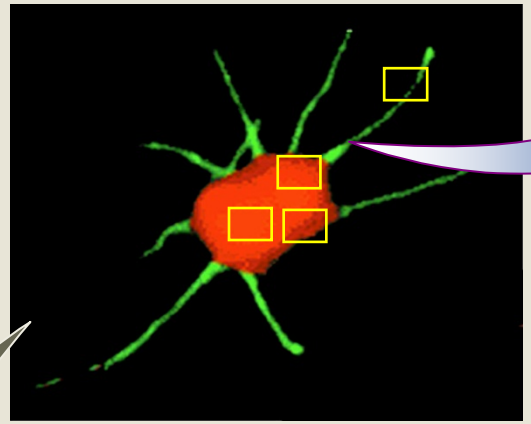
# Our Approach

- 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

Test immunogenicity

Purify components

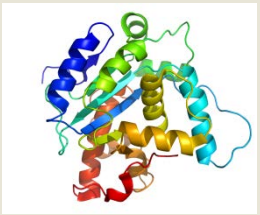
Clone genes

Identify components

5-15 years

Immunogenicity testing in animal models

VACCINE DEVELOPMENT



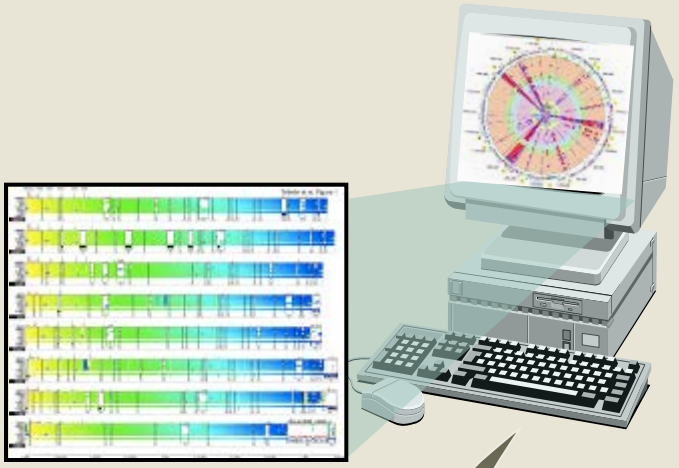
Vaccine



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**

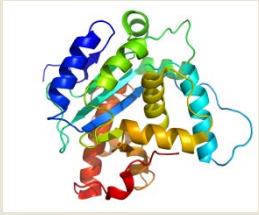
**1-2 years**

**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

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

*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*

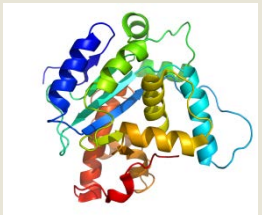
344 proteins purified and used to immunize mice

91 novel surface-exposed proteins identified WB, ELISA, FACS

28 novel proteins have bactericidal activity

5 top candidates

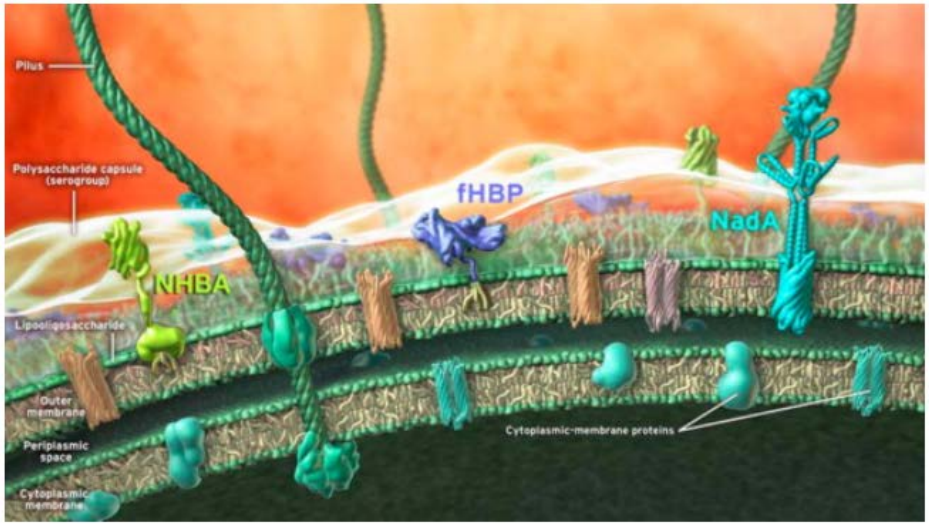
3 proteins +OMVs



4CMenB

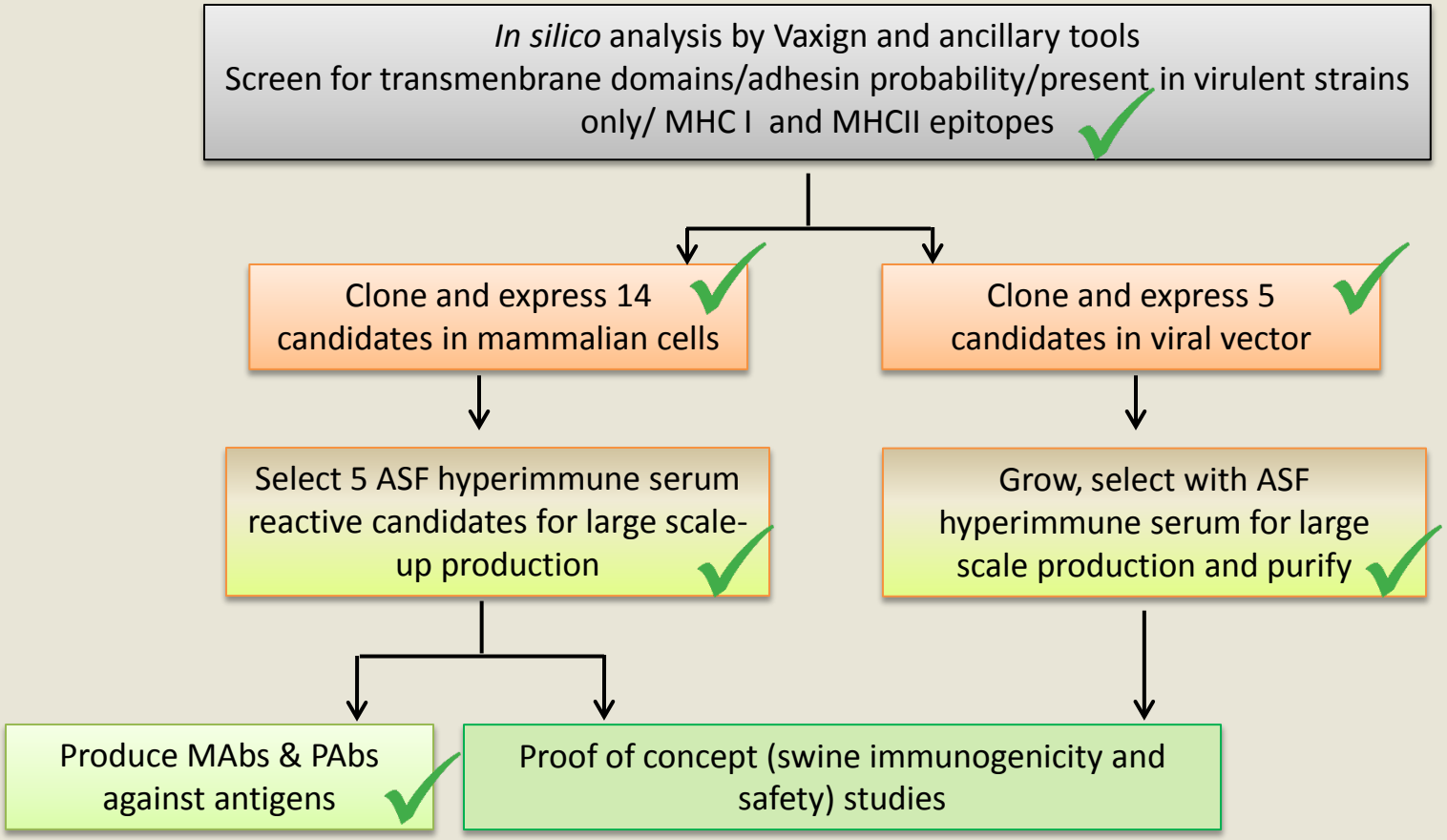
# 4CMenB success

- 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
- NNadA-mediate adhesion to and entry into cells
- All proteins related to virulence




Serruto et al, Vaccine. 2012

# Our approach overview



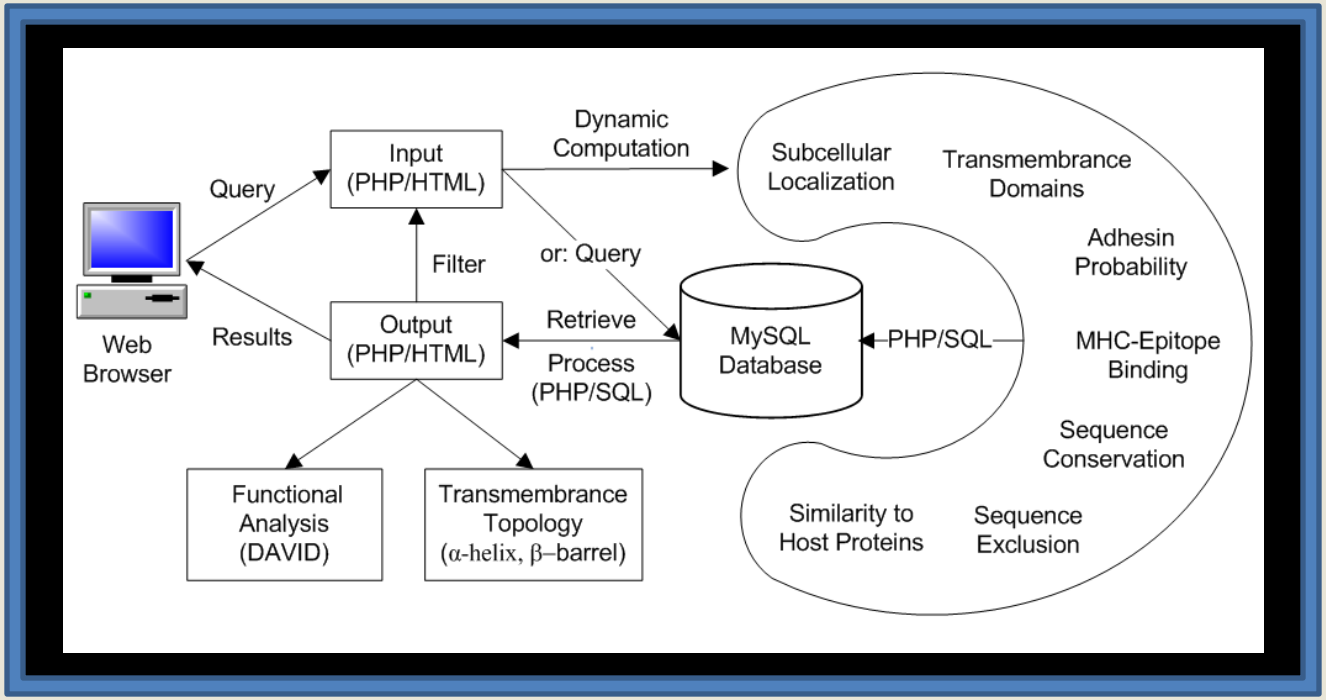
**It is challenging to apply reverse vaccinology without a comprehensive pipeline**

**→ To address this challenge, 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

**He Y, Xiang Z, Mobley HLT**. Vaxign: the first web-based vaccine design program for reverse vaccinology and an application for vaccine development. Journal of Biomedicine and Biotechnology. 2010, Article ID 297505.

# Reverse Vaccinology Criteria

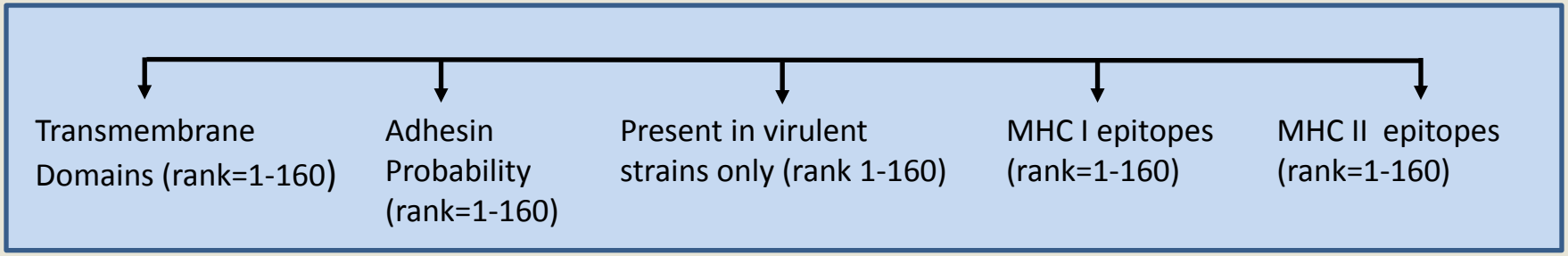
1. Transmembrane domains
   - ✓ >2 $\alpha$–helix domains → difficult to isolate
2. Adhesin probability
   - ✓ Adhesin is important for pathogen invasion
3. MHC-Epitope binding
   - ✓ MHC class I epitope → cell-mediated immunity
   - ✓ MHC class II epitope → antibody response
4. Sequence conservation 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

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

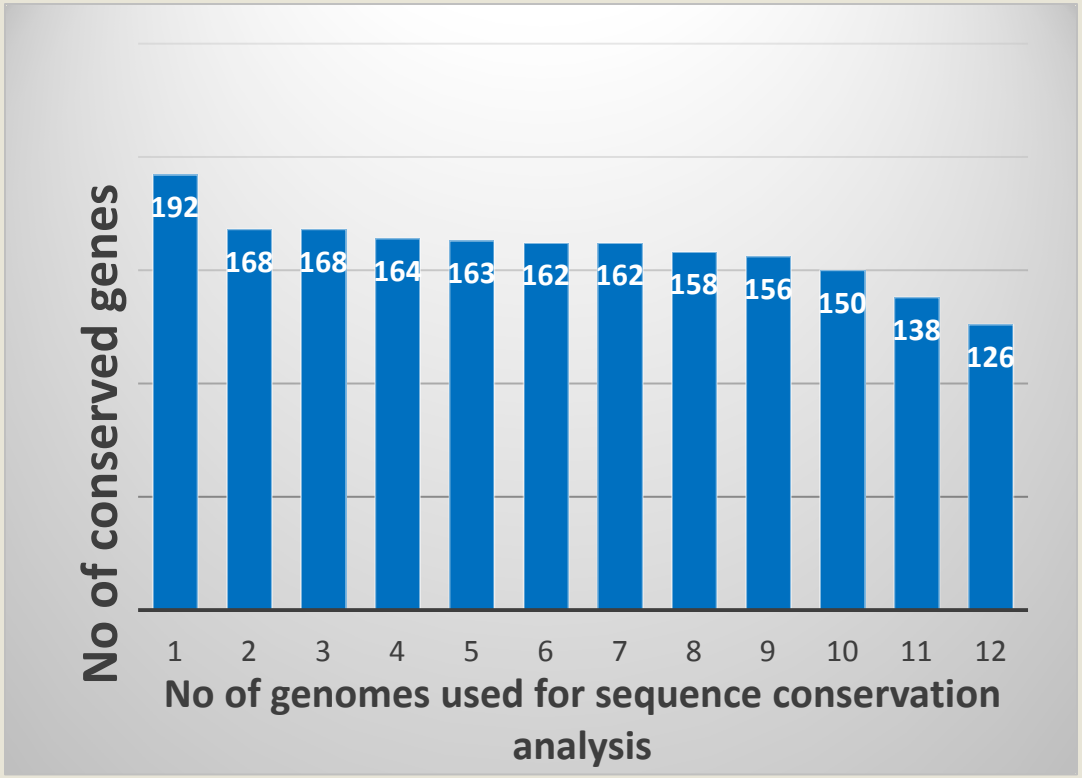
# Reverse vaccinology ranking strategy

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

# ASFV Genome Analysis

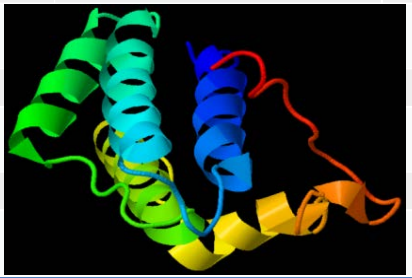



- 126 ASFV genes are conserved among all 12 genomes
- These genes are considered as ASFV core genes

# ASFV 3D structure-based functional analysis

**Consensus Prediction of Gene Ontology Terms**

| Molecular Function | | | Biological Process | | | Cellular Component | | |
|---|---|---|---|---|---|---|---|---|
| GO ID | GO Term | GO-Score | GO ID | GO Term | GO-Score | GO ID | GO Term | GO-Score |
| GO:0016972 | thiol oxidase activity | 0.56 | GO:0055114 | oxidation-reduction process | 0.59 | GO:0005786 | signal recognition particle, endoplasmic reticulum targeting | 0.17 |
| GO:0008312 | 7S RNA binding | 0.17 | GO:0006614 | SRP-dependent cotranslational protein targeting to membrane | 0.17 | GO:0005886 | plasma membrane | 0.07 |
| GO:0005525 | GTP binding | 0.17 | GO:0008299 | isoprenoid biosynthetic process | 0.07 | GO:0016021 | integral to membrane | 0.07 |
| GO:0017111 | nucleoside-triphosphatase activity | 0.15 | GO:0006807 | nitrogen compound metabolic process | 0.05 | GO:0005739 | mitochondrion | 0.03 |
| GO:0046872 | metal ion binding | 0.15 | GO:0009405 | pathogenesis | 0.04 | GO:0045263 | proton-transporting ATP synthase complex, coupling factor F(o) | 0.03 |
| GO:0000287 | magnesium ion binding | 0.11 | GO:0015696 | ammonium transport | 0.04 | | | |
| GO:0016829 | lyase activity | 0.11 | GO:0015670 | carbon dioxide transport | 0.04 | | | |
| GO:0005515 | protein binding | 0.07 | GO:0072488 | ammonium transmembrane transport | 0.04 | | | |
| GO:0016740 | transferase activity | 0.06 | GO:0055085 | transmembrane transport | 0.04 | | | |
| GO:0016301 | kinase activity | 0.04 | GO:0008652 | cellular amino acid biosynthetic process | 0.03 | | | |



- Functions of the majority of ASFV proteins are unknown
- Automated protein structure and function predictions tool (I-TASSER)was developed

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Xiang, et al. Pan-genome structure prediction and function annotation of African swine fever virus. (Abstract) ASM, 2012.



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# I-TASSER Prediction Verification

| Proteins | Literature Search Results | | I-TASSER Results | | |
|---|---|---|---|---|---|
| | Function | Protein localization | Molecular Function | Biological Process | Cellular Component |
| 1 | structural attachment protein involved in morphogenesis | viral membrane | protein binding | antigen processing & presentation | membrane |
| 2 | structural protein involved in morphogenesis | viral membrane | metal ion binding | metabolic process | cytoplasm |
| 3 | structural protein involved in host cell binding and entry | viral membrane | catalytic activity | metabolic process | cell part |
| 4 | structural protein involved in morphogenesis | viral outer envelope | protein binding | signal transduction | membrane |
| 5 | glycoprotein that interacts w/lectin found on RBC membrane and play a role in the virus budding, attachment to RBC and virus spread | viral membrane | protein binding | metabolic process/cell adhesin | membrane |
| 6 | glycoprotein that interacts with CD2v; viral C-type lectin with anti-apoptotic properties; host evasion molecule | viral membrane | protein binding | cell proliferation/adhesin | extracellular space and membrane |
| 7 | structural protein involved in host cell binding and entry | viral capsid | protein binding/catalytic activity | metabolic process | viral capsid |

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

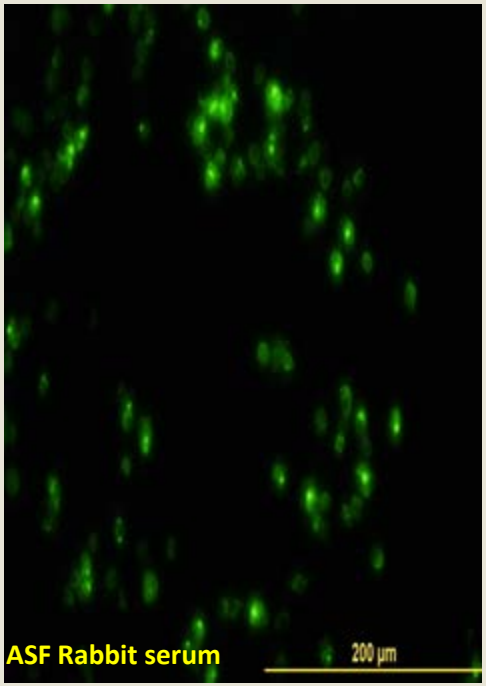
# Protein Expression

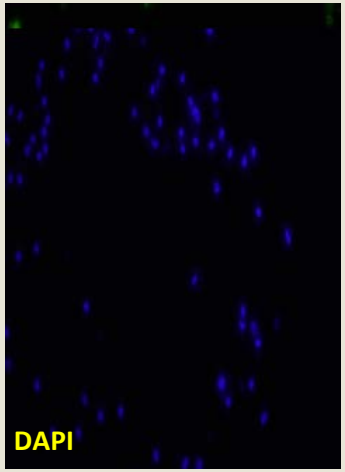
| Candidate # | Expressed in HEK 293 | HEK purification capable | Expressed & purified in Baculovirus | Expressed& purified in VV | Recognized by ASFV antiserum |
|---|---|---|---|---|---|
| 1 | N | NA~ | Y | WIP | Y(B) |
| 2 | Y | N | Y | | Y(B&H) |
| 3 | Y | Y | Y | | Y(B&H) |
| 4 | Y | Y | Y | Y | Y(B,H,&V) |
| 5 | Y | N | Y | | Y(B&H) |
| 6 | Y | Y | Y | | Y(B&H) |
| 7 | N | NA~ | Y | | N |
| 8 | Y | Y | Y | | Y(B&H) |
| 9 | N | NA~ | Y | | Y(B) |
| 10 | N | NA~ | Y | | Y(B) |
| 11 | N | NA~ | Y | Y | Y(B&V) |
| 12 | N | NA~ | Y | | N |
| 13 | N | NA~ | Y | WIP | Y(B&V) |
| 14 | Y | Y | Y | Y | Y(B,H,&V) |

| | |
|---|---|
| N/NA~ | No/Not Applicable |
| Y | Yes |
| WIP | Work In Progress |

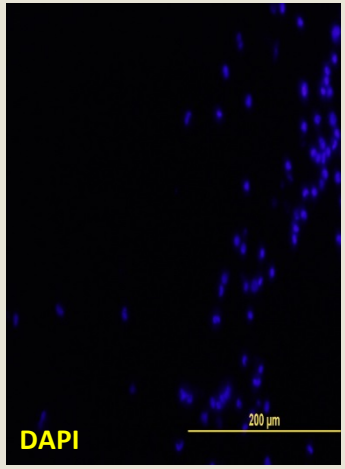
- 7 expressed in HEK; 5 purified
- All 14 expressed in and purified from Baculovirus
- All 5 selected for viral vector expressed; 3 purified
- **12 recognized by ASF antiserum by Western Blot**

# Antibodies production








- 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

**Dr. John Neilan**
**Dr. David Brake**
**Dr. Tom Burrage**

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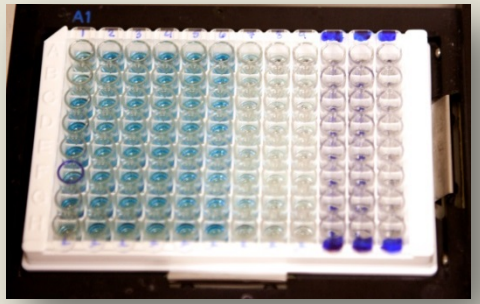
# Proof-of-concept studies in pigs

- 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