Biotechnology -- Impact on Avian Health

AAAP Symposium Program

July 15, 2007

Table of Contents

American Association of Avian Pathologists
2006
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BIOTECNOLOGY – IMPACT ON AVIAN HEALTH
AAAP 50th Anniversary Symposium – July 15, 2007

Sponsoring AAAP Committee: Biotnology committee

The contributions of biotechnology to avian health have been substantial, especially in the areas of diagnostics and vaccine development. The goal of this symposium is to summarize and demonstrate the uses of current technology, acknowledge some of the challenges, and to suggest its future potential.

8.00 – 8.05 a.m.	Welcome – **Calvin Keeler**
Diagnostics	
8:05 – 8:20 a.m.	Overview – How has biotechnology advanced in 15 years, what are some of the challenges, and what does the future hold – **Daral Jackwood**
8:20 – 8:50 a.m.	Biotechnology and its impact on the diagnostic laboratory – **Fred Hoerr**
8:50 – 9:20 a.m.	Sequencing and its diagnostic uses – **Ching Ching Wu**
9:20 – 9:50 a.m	Current and future applications of quantitative PCR – **Mark Jackwood**
9:50 – 10:10 a.m.	BREAK
10:10 – 10:40 a.m.	Real-time quantitative PCR and AI – **Erick Spackman**
10:40 – 11:00 a.m.	Microassays and future diagnostic applications – **Calvin Keeler**
Vaccines	
11:00 – 11:15 a.m.	Overview of developments in the last 15 Years in recombinant vaccines – **Bob Silva**
11:15 – 11:45 a.m.	Recombinant vaccines – challenges, successes, and current products – **Ruud Hein**
11:45 – 12:45 p.m.	LUNCH
12:45 – 1:15 p.m.	BACs and Marek's vaccine developments – **Klaus Osterrider**
1:15 – 1:45 p.m.	The promise of reverse genetics – **Egbert Mundt**
1:45 – 2:15 p.m.	Concept to product – challenges associated with patenting, licensing and marketing recombinant products – **David Shapiro**
2:15 – 2:45 p.m.	Future vaccine developments – RNAi/Immunomodulators – **David Suarez**
2:45 – 3.00 p.m.	Speaker Panel

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We want to thank all of the speakers for their cooperation and contributions.

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Overview – How has biotechnology advanced in 15 years, what are some of the challenges, and what does the future hold.

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The roots of biotechnology date back to 1919 when Karl Ereky first coined the term. Biotechnology evolved from zymotechnology which was the use of fermentation to produce beverages. Defined as any process that produces products from raw materials with the aid of living organisms, biotechnology was initially responsible for the production of chemicals and pharmaceuticals. The discovery that DNA could be cut from one bacterium and placed into another bacterium in 1973 lead to the era of genetic engineering and biotechnology began to flourish. Scientists reported what was possible but the market expectations and benefits of new and improved genetically engineered products were oversold. The full potential of biotechnology was not realized in part due to a basic lack of information in biological systems. The basic information needed to make genetically engineered products a reality has come in part due to biotechnology. Avian health has been positively impacted by genetically engineered vaccines and diagnostics. Some advances are obvious end products while others have been realized through the improvement of existing products. Biotechnology continues to provide technological advances that will benefit avian health. The greatest challenge will be keeping the cost of this technology within reach of the poultry industry.

INTRODUCTION

Fifteen years is a relatively short period for biotechnology. Although there have been many advances attributed to biotechnology in the poultry industry since 1992, the field of biotechnology really began more than 80 years ago (2). The term "biotechnology" is attributed to Karl Ereky, a Hungarian agricultural economist who first introduced it in 1919. In his book *Biotechnologie*, Ereky describes biotechnology as any process that produces products from raw materials with the aid of living organisms. After the first World War, biotechnology was found in German dictionaries and soon was used internationally.

The discovery of the double helix structure of DNA in 1953 by James Watson and Francis Crick is often thought of as the start of biotechnology. However, the use of microorganisms to make foods like cheese, bread and alcoholic beverages started much earlier. In the latter part of the 19th century, the fermentation process or zymotechnology was an important first step for biotechnology. During the 1940's biotechnology was used to make penicillin and in the 1950's steroids were made using fermentation technology.

An offshoot of biotechnology known as genetic engineering was started by two important events. First was the discovery of the DNA double helix structure and then 20 years later in 1973 Cohen and Boyer demonstrated that a section of DNA could be cut from one bacterium and transferred to another. The recombinant DNA technique was born. Between 1953 and 1973, numerous discoveries were needed to make recombinant DNA a reality. DNA polymerase was discovered in 1958 and in 1966, Ochoa and Nirenberg demonstrated that three nucleotides code for each of the 20 amino acids thus cracking the genetic code. Restriction enzymes were discovered in 1971 and a year later Paul Berg cut viral DNA and bacterial DNA with the same restriction enzyme.

The discovery of recombinant DNA by Cohen and Boyer opened the can of worms known as cloning. In 1974, Paul Berg suggested in a paper published in *Science* that there should be a moratorium on cloning experiments until the consequences of this work could be evaluated. A year later, in a famous meeting defined by its location in Asilomar California, scientists debated the implications of cloning and genetic engineering and after a 16-month halt to this research, the National Institutes of Health (NIH) guidelines on recombinant DNA (rDNA) research were established in June 1976.

Technology and corresponding regulations began to move fast in the years following the release of the NIH guidelines. Human growth hormone was produced in bacterial cells in 1977 and in September 1978 a new company called Genentech Inc., was the first to market human insulin produced in bacterial cells (4). A year later, Genentech was producing two kinds of interferon and human growth hormone. The doom and gloom anticipated with the discovery of cloning began to shift to a belief that genetic engineering may improve the environment and the health of humans and animals (2). In the 1980's new discoveries lead to high economic expectations and the benefits of biotechnology and genetic engineering were often exaggerated. By 1988, only five proteins produced using genetic engineering were approved by the U. S. Food and Drug Administration. The power of genetic engineering was not oversold but rather it was stifled by the lack of knowledge of basic biological systems. In the poultry health industry during the 1980s, the lack of nucleotide and protein sequences and a general lack of knowledge regarding immunogenic epitopes on avian pathogens made the application of biotechnology techniques to vaccines and diagnostics difficult if not impossible.

The discovery of the polymerase chain reaction (PCR) in 1985 probably did more to advance the science of biotechnology and genetic engineering than any other discovery (3). Coupled with the sale of the first automated DNA sequencing machine in 1987, scientists now had the tools to rapidly generate the data needed to apply modern genetic engineering to real world problems.

APPLICATION OF BIOTECHNOLOGY AND GENETIC ENGINEERING TO AVIAN HEALTH PROBLEMS.

The first Biotechnology Symposium of the American Association of Avian Pathologists (AAAP) was held on July 23, 1985 in Las Vegas, Nevada in conjunction with the 122nd annual meeting of the American Veterinary Medical Association (1). Since then the AAAP has sponsored two symposiums directly related to biotechnology; one in 1992 on *Improved Diagnosis of Avian Diseases using Molecular Biology* and the other in 2000 on *Molecular Identification and Epidemiology of Avian Pathogens*. One only needs to review the manuscripts published from these symposia to see the impact biotechnology has had on avian health.

The discovery of PCR more than 20 years ago dramatically increased the sensitivity and specificity of diagnostic assays for avian pathogens. Initially the cost of these assays made their routine use uneconomical for the poultry industry. Furthermore, PCR results were unfamiliar and sometimes difficult to interpret. Genetic engineering and automated sequencing has allowed scientists to target genes that are important in pathogenicity and antigenicity so the data that is now generated by PCR based diagnostics is vastly improved. Real-time PCR has reduced the time needed to obtain results and the cost per assay has dropped dramatically. Today, a PCR based diagnostic assay has been developed for nearly every avian pathogen with economic importance to the poultry industry.

Biotechnology has improved other segments of avian disease diagnostics. Many of these improvements have gone unnoticed because they simply make an existing assay better. Examples include the use of genetically engineered proteins in ELISA technology. Expression of proteins with the appropriate epitopes has reduced the cost of producing ELISA antigens and improved the background associated with these assays. These proteins have also made the generation of monoclonal antibodies easier and less expensive.

Genetically engineered vaccines for avian pathogens were predicted shortly after cloning and genetic engineering became the mainstream for many laboratories in the 1980s. They did not become a reality however until the mid 1990s when poxvirus and herpesvirus vectored vaccines were patented. Today recombinant vectored vaccines for Laryngotracheitis, Avian Encephalomyelitis, Mycoplasma, Newcastle Disease, Infectious Bursal Disease Virus and Avian Influenza are available commercially and many others have been developed.

The delay in generating recombinant vectored vaccines that are efficacious for avian pathogens was not due to limitations in genetic engineering but rather it was a lack of basic knowledge regarding the host-parasite relationship. The decision on which genes to clone and insert into the vectors was paramount to the success of these vaccines. The study of protein structure (proteomics) particularly three-dimensional structures has aided in the selection of antigens and epitopes for vaccines and diagnostics. This field of study will continue to provide important information on the basic biology of host-parasite interactions.

Genetic engineering has made it possible to produce safer live-vaccines. The live *Salmonella* and *E. coli* vaccines are safer because they are deletion mutants that can not revert to a more virulent form. The production of these vaccines is dependent on scientists discovering which genes or gene segments are important in virulence. Development of viral-vector vaccines

was in part due to improved safety. The delivery of an expression product (antigen) to the appropriate segment of the bird's immune system is a function of the viral vectors used and thus each vector has its advantages and limitations for a given avian pathogen.

WHAT DOES THE FUTURE HOLD?

Speculation of what the future might hold is obviously just that, speculation. However, based on what is needed in the avian health field and assuming lower costs will occur as new technologies evolve some predictions can be made.

In the diagnostics arena, ELISA technology will continue to benefit from biotechnology. New monoclonal antibody creation techniques like phage display and recombinant (chicken) monoclonal antibodies will provide an opportunity to produce monoclonals that identify unique epitopes that may not be recognized by mice or other production systems. Epitope specific ELISAs and those that identify virulent microorganisms will be developed for diseases that impact trade such as avian influenza, Newcastle disease, *Campylobacter, Salmonella* and *Listeria*. There will also be a market for ELISAs that identify antibodies to specific neutralizing epitopes in diseases where there are multiple antigenic forms of the pathogen like infectious bronchitis virus, avian influenza and infectious bursal disease virus.

PCR based assays, including real-time PCR, will continue to improve and be offered at a lower cost. In the future, PCR will be linked to nucleotide sequencing and microarray technology rather than restriction enzyme digestion because of the reduce cost of these tests and the extra information that can be obtained. On-the-farm or pen-side testing based on PCR technology will likely become a reality for diseases that effect export and inter-state movement of birds.

Because biosensor technology is proving to be versatile and economical, it has the potential to be applied to poultry diagnostics. Chemical sensing of antibodies, antigens, DNA or RNA is possible using biochemical processes linked to semiconductor sensors. The biochemical molecule attaches to a pre-selected target and the energy generated by this process is converted to electrical energy by a transducer. This electrical energy is detected by a semiconductor so the biological binding event can be analyzed using computer technology. Biosensor technology has the potential to make on-the-farm testing for pathogens routine.

In the vaccine arena, advances in the expression of proteins of immunologic importance will be made possible through biotechnology. The current practice of making killed vaccines in mammalian cell culture, eggs and chicks will be replaced by expression of the appropriate protein antigens in plant cells, insects, yeast and other systems. Research into the expression of antigens and epitopes in these alternate systems indicates the proteins are high quality and economical to produce. Plant cells offer a unique expression system because they do not contain any components of animal origin and thus are potentially safer (5). The USDA Center for Veterinary Biologics has already approved a plant cell produced subunit vaccine for Newcastle disease.

As genetic elements that control virulence are identified, more deletion mutant vaccines will be possible. This approach will be more appropriate for pathogens like bacteria, mycoplasma and protozoa that have a large number of genes. The use of bacteriophages to control bacterial pathogens has met with some success and may become a practical solution for the poultry industry in the future.

Recombinant viral vector vaccines will continue to evolve and hopefully become less expensive. Poxvirus and Herpesvirus vectors will be useful long into the future. However, other viral and bacterial vectors will likely be developed for specific applications. Look for immune potentiators to be included in these live-vector vaccines.

WHAT ARE THE CHALLENGES?

The biggest challenges to making biotechnology based products available for the poultry industry are probably economics and regulatory hurdles. Vectored vaccines are commercially available and effective but at a higher cost compared to conventional vaccines. It will be necessary to either lower the cost or justify the higher cost through improved efficacy and/or safety. In the diagnostics area, the cost of PCR based assays has dropped dramatically but they still are not able to compete with low-cost ELISA technology. Ironically, ELISA technology continues to improve due to biotechnology. Regulatory issues for biotechnology based products should be reviewed. The requirements for conventional vaccines and diagnostics may not be practical for genetically engineered products.

The lack of a basic understanding of the biological interactions between parasite and host is still limiting the application of biotechnology's full potential. It will be a challenge to determine what is needed for each vaccine or diagnostic to be successfully implemented. Fortunately, biotechnology will help scientists conduct the basic studies needed to generate this information. Reverse genetics, RNA interference (RNAi) technology, recombinant-antibodies, new expression vectors, microarrays, biosensors and a number of other innovations are available for future studies in immunology, microbiology and molecular biology. Biotechnology has been and will continue to be responsible for significant advances in improving avian health. Funding of this research however, will continue to be a major challenge for scientists.

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Biotechnology and Its Impact on the Diagnostic Laboratory

Frederic J. Hoerr

Molecular detection and characterization of pathogenic bacteria and viruses substantially influenced poultry disease diagnosis and epidemiology in the past 15 years. In 1992, a survey revealed that just six of 89 responding animal/poultry diagnostic laboratories used PCR as a diagnostic test (2). Since then, a molecular definition of highly pathogenic avian influenza became a regulatory standard worldwide. Today, real-time RT-PCR technology forms the national infrastructure for the early detection of highly pathogenic avian influenza and Newcastle disease. Molecular diagnostic technology has moved mainstream to become nearly essential in the rapid and specific diagnosis of infectious diseases of poultry, influencing almost every aspect of the diagnostic laboratory. In 1996, our laboratory completed about 1000 PCR reactions for four agents, which increased to the current level of 8000 reactions for more than 25 agents.

Facilities

The State of Alabama recently dedicated a new full-service animal diagnostic laboratory. The new laboratory, like the one it replaced, is the central reference laboratory in a four-laboratory system, and serves the poultry and livestock industries in Alabama, as well as support for companion animal and wildlife diagnostics. The design of the new diagnostic laboratory brought into focus the requirements for the ever-expanding role of biotechnology in livestock and poultry diagnostics.

During the design phase, key considerations and decisions accommodated biotechnology. The principal architect brought in an experienced and laboratory design specialists who designed the elements of the laboratory from the workbench outward. Not surprisingly, the biotechnology of poultry diagnostics was a driving factor. The initiation of real-time PCR for avian influenza and Newcastle surveillance by the laboratory, which belongs to the National Animal Health Laboratory Network (NAHLN), required moderate but specific obligations on facilities. The following points were important to the design of our new laboratory, and are listed to demonstrate the breadth by which molecular diagnostics permeates the diagnostic laboratory today. Each item was either missing or compromised in our former facility constructed less than 30 years ago.

- Building layout. The delivery dock for supplies and reagents was distanced from the necropsy dock so that the ready flow of critical laboratory supplies (e.g. AI or ND PCR reagents) into the laboratory would minimally impacted by contaminated vehicles and animals during a foreign animal disease event.
- Mechanical considerations. Corridors are relatively positive air pressure to laboratories, minimizing air-borne pathogen circulation in the laboratory. Laboratory air is 100% exhausted. An emergency generator provides continuous power to circuits for critical refrigeration, incubation, and other priority equipment essential to the molecular detection and other laboratory sections.
- Biosafety Level II general operations. Laboratories are distinctly separated from and offices. All offices open to a corridor; none opens into a lab. Lab coats stay in the labs, and each lab has a hand wash sink near the door. Bench tops are cleaned and disinfected daily. An integrated pest management program maintained per CDC recommendations assures minimal contamination potential from insects. Windows cannot be opened.

- Class II biosafety cabinets (BSC) for fresh tissue handling and processing. Many BSCs are exhausted to the exterior; optional for laboratory function but integrated by the engineers with overall HVAC system. Within the BSC, air passing over the specimen exhausts through a HEPA filter, further diminishing airborne agents as sources of contamination of molecular detection procedures. Laboratory air from rooms and BSCs exhausts through Strobic fans that eject a vertical column 75 feet before significant dispersal occurs. As part of our general BSL II operations, all employees receive formal training in BSL II laboratory procedures, including BSC and chemical fume hood operation. Inappropriate activity near a BSC can disturb airflow and promote cross contamination of samples.
- Secure corridors. All halls have card-reader access for employees and approved visitors only. This minimizes laboratory contamination from soiled clothing and footwear, as well as interruptions of laboratory work. Pass-through refrigerators from corridor to laboratories minimize door openings. Specimens are transported through corridors in secondary closed containers that are disinfected or sterilized.
- A molecular detection laboratory physically separate from the virology and bacteriology-mycology laboratories. No direct access occurs from the microbiology labs to the molecular lab except through a clean corridor. The secondary fire egress from molecular laboratory passes through histology, which has no fresh tissue.
- A central molecular detection laboratory with peripheral rooms for special functions. Individual rooms are dedicated rooms for: RNA extraction, DNA extraction, primer storage and preparation, joining primers with specimen (template), thermocyclers, and gel electrophoresis.
- Adequate bench space for expanding services. Rather large bench top equipment for the USDA program for high-throughput PCR for AI/ND RNA extraction and liquid handling was accommodated. This equipment is critical infrastructure for poultry and livestock foreign animal disease surveillance
- Molecular diagnostics and BSL III. Although not certified at this time, we have three rooms and an anteroom certifiable as BSL III attached to virology. These rooms are equipped to support molecular detection equipment and procedures dedicated to contained BSL III operations, including IT and telecommunications for reporting results, as well as video cameras for observation and monitoring safety.
- Other. Rooms with hand pipetting activities have diffused air inlet covers to minimize turbulence. Generous direct and indirect lighting delivered to work surfaces helps ensure a comfortable, safe working environment. Each room has a functional, accurate temperature control.
- Omissions. Due to budget constraints, we deleted a DNA sequencing laboratory module from the original design. We outsource sequencing to commercial/university laboratories.

Personnel

Molecular diagnostics requires educated and experienced personnel for supervision and training, and meticulous, skilled personnel for implementation. A "cookbook" approach to these procedures without a firm understanding of molecular biology is an invitation to serious problems.

The Essential Requirements Laboratory Accreditation of the American Association of Veterinary Laboratory Diagnosticians (AAVLD) (1) lists minimal essential educational requirements for the Head of Molecular Diagnostics as an MS degree in molecular diagnostics, or a BS degree certified Medical Technician with 5 years experience in veterinary molecular diagnostics. The preferred education and training is a DVM and MS, or DVM and PhD, or PhD and 5 years experience in veterinary molecular diagnostics. The preferred education requirement of a molecular laboratory technician is a BS or Medical Technology degree.

The NAHLN has specific training and certification requirements for each foreign animal disease test based on molecular detection, with annual recertification required. For poultry this currently involves certification for real time PCR testing for AI and ND.

Equipment

Improving methodology and technology steadily advance equipment requirements for molecular diagnostics. In general, the new testing platforms provide greater precision and accuracy, and provide the results quicker and in greater numbers that the preceding methods. Key pieces of equipment are expensive ($20,000-50,000) but essential to the mission. These include machines for robotic RNA or DNA extraction and precision liquid handling, various thermocyclers for PCR applications, and sequencing equipment. High quality genomic sequencing of viruses and bacterial pathogens is readily available and routine sequencing is relatively common (3).

Quality Assurance

The QA requirements for the implementation of routinely used molecular tests are increasingly stringent. AAVLD Essential Requirements for Accreditation require validation of all diagnostic tests, including molecular diagnostics, to meet validation principles outlined in the OIE *Manual of Standards for Diagnostic Tests and Vaccines*. Ongoing in-house validation for sensitivity, specificity, accuracy and precision is preferred. Tests are considered validated (1) for use if they "meet ongoing documentation of internal or inter-laboratory performance using known reference standards for the species and /or diagnostic specimens of interest" and meet one or more of the following: 1) endorsement or publication by a reputable organization (e.g. American Association of Avian Pathologists *Isolation and Identification of Avian Pathogens*); 2) publication in a peer-reviewed journal with sufficient documentation to establish diagnostic performance and interpretation of results; or 3) documentation of internal or inter-laboratory comparison to an accepted methodology or protocol".

The Future

This technology will only expand and accelerate. The trend is for quicker definitive results with improved sensitivity and specificity, for higher quantities of specimens, and with an increased amount of information specific to that result.

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Sequencing and Its Diagnostic Use

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Obtaining a pure culture, followed by subtyping, for detection, speciation, and differentiation of pathogenic organisms is considered as well as remains the gold standard for microbiological diagnosis. However, some concerns arise in terms of the requirement of selective culture medium and other special reagents, slow growth of some pathogens, and inability to cultivate some organisms. Since the organisms (viruses, bacteria, fungi, protozoa, and helminthes) are genetically encoded by unique stretches of nucleotide sequences, DNA sequencing or polymerase chain reaction (PCR) in conjunction with DNA sequencing has gradually become the choice of molecular methods as the approach for, supplement to, or confirmation of diagnostic microbiology.

Nucleotide sequence determination nowadays is carried out by using automated "cycle sequencing" reactions based on Sanger's method in which dideoxynucleotides (ddNTPs) are used as the chain terminators to produce a ladder of molecules (DNA fragments at specific bases, either A, C, T, or G) generated by *Taq* polymerase extension of a primer in a thermal cycling protocol. Fluorescent dyes are added to the reactions and a laser within an automated DNA sequencing apparatus is used to analyze the DNA fragments produced. Cycle sequencing reaction requires only very small amount (femtomole quantity) of target for sequencing. Such level of sensitivity makes it possible to obtain specific nucleotide sequences from a clinical isolate or directly from clinical samples.

A more common approach is to amplify a unique genomic nucleotide sequence by PCR using a specific primer pair. The oligonucleotide primers each complementary to one end of the target sequence that is extended towards each other by a thermostable DNA polymerase in repeated reaction cycles consisting of denaturation, primer annealing, polymerization. The strategy often used is to design the primers from the region with the conserved nucleotide sequences among the strains that flank the region with variable sequences among the strains. A very good example in the avian viruses is the analysis of hypervariable regions in the VP2 of infectious bursal disease virus and that in the bacteria the analysis of hypervariable internal transcribed spacer (ITS) regions in between the 16S and 23S ribosomal RNA. Following amplification, the PCR products (amplicons) are sequenced to reveal nucleotide identity. Once the sequence is determined, the nucleotide sequences can be analyzed by a software program (such as DNAstar) to identify restriction sites, open reading frames, start and stop codons, promoter sites, etc., and also can be submitted to a large database (such as GenBank database) to compare and analyze new sequences with all other known sequences for similarity/dissimilarity in the database by using Basic Local Aligment Search Tool (BLAST). Direct comparison of genomic nucleotide sequences of pathogenic strains is the best approach to determine whether two strains are similar or different.

Nucleotide sequencing and sequence analysis have been shown to be valuable in detection and differentiation of variant viral or bacterial strains of known species, identification of non-culturable species, and identification of new species. Specific examples are provided in the

references listed below and will be presented and discussed in the symposium. In conclusion, nucleotide sequencing and sequence analysis allows for the identification of a wide variety of pathogenic organisms in diagnostic microbiology.

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Current and future applications of quantitative PCR

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SUMMARY. Quantitative polymerase chain reaction (qPCR) or quantitative reverse transcriptase-polymerase chain reaction (pRT-PCR) are methods that can be used to determine the amount of pathogen nucleic acid in a clinical sample. Quantitative PCR takes advantage of the liner relationship that exists between the input template and the amount of amplified DNA in a PCR assay. Relative quantification or analysis of input template levels as a difference from controls, also called semi-quantitative, methods include limiting dilution, and co-amplification of an internal reference standard. Truly quantitative PCR methods are competitive PCR and real-time PCR. Competitive PCR uses an internal reference standard that competes with the unknown template, whereas real-time PCR uses kinetic quantification to determine the amount of starting template in a reaction. Kinetic quantification by real-time PCR calculates the amplification efficiency independently for each reaction tube eliminating the need for internal controls and assumptions regarding amplification efficiency. Thus, real-time PCR has become the industry standard for quantitative nucleic acid analysis. Although qPCR detects the amount of nucleic acid in a clinical sample, and not the number of viable organisms, with the proper controls, qPCR data can be correlated with viral or bacterial titer *in vitro* and *in vivo,* with a reasonable level of accuracy.

Abbreviations: Ct= cycle threshold, qPCR= quantitative polymerase chain reaction, RT-PCR= reverse transcriptase-polymerase chain reaction.

The polymerase chain reaction (PCR) and reverse transcriptase-PCR have been used in diagnostic tests to quickly detect and identify disease agents directly from clinical samples. However, for some disease agents, pathogenicity depends on the number of organisms present making it important to quantify the level of infection in the host. Quantitative polymerase chain reaction (qPCR) or quantitative reverse transcriptase-polymerase chain reaction (pRT-PCR) are methods that can be used to determine the amount of pathogen nucleic acid in a clinical sample.

Quantitative PCR takes advantage of the liner relationship that exists between the input template and the amount of amplified DNA in a PCR assay. The linear relationship is defined by the formula:

$$\mathbf{Y= X(1+E)^n}$$

Where Y= the concentration of amplified DNA, X= the concentration of input nucleic acid, E= the efficiency of the reaction, and n= the number of amplification cycles (2). Theoretically, if the efficiency of the reaction is 100% then the amount of amplified DNA should double after each cycle. However, the efficiency of a PCR reaction is almost never 100% and it can vary greatly between reactions. Consequently some qPCR methods measure relative quantification or input template levels as a difference from controls. Also called semi-quantitative, these methods fail to measure or hold E constant and include **limiting dilution**, and **co-amplification** of an internal reference standard (1).

Limiting dilution. Limiting dilution methods are conducted by initially setting up a standard curve with known quantities of input template. Next, an unknown quantity of template is amplified and the amount of amplified product is plotted on the standard curve to extrapolate the amount of initial unknown template. Since the efficiency of PCR reactions vary from tube to tube, this method yields semi-quantitative data.

Co-amplification. Co-amplification methods use an internal reference standard in the PCR reaction in an attempt to keep the variables influencing the efficiency of amplifying the unknown and reference standard constant. The reference standard template is usually different from the unknown template, uses different primers, and the amplicon is often smaller of larger than the unknown amplified product so it can be differentiated by agarose gel electrophoresis. However, PCR template amplification efficiency can very with template length and the nature of the sequence. In addition, the thermodynamics of primer binding can affect the amount of amplified product. Thus, co-amplification methods that utilize a separate internal reference standard also result in semi-quantitative data.

There are two truly quantitative PCR methods; **competitive PCR** and **real-time PCR** (1, 2, 3).

Competitive PCR. In competitive PCR, the reference template shares the same primer sites as the unknown template, and is amplified in the same reaction tube. Thus, the reference template competes with the unknown template for primers, polymerase enzyme, and nucleotides, essentially holding thermodynamics and efficiency (E in the above equation) of the reaction constant. Competitive PCR is conducted by making serial dilutions of a known amount of reference standard template then adding that to a constant amount of unknown template for the amplification reaction. The amplified products of the reference template and the unknown template must be different (usually slightly larger or smaller) so they can be differentiated on an agarose gel. The relative intensities of the amplified products on the gel are measured by densitometric methods, and the ratio between the known and unknown amplified products reflects the difference between the initial amounts of the two templates. Thus, the initial amount of unknown template can be calculated (see Fig. 1).

There are some elements that must be considered when conducting competitive PCR. First, the amount of reference standard must bracket the expected amount of

A

B

#	IDV	%	AREA	AVG
1n	15466	8.0	703	22
2n	7854	4.1	462	17
3n	15300	7.9	612	25
4n	9180	4.8	510	18
5n	13440	7.0	560	24
6n	9520	4.9	560	17
7n	11760	6.1	490	24
8n	9990	5.2	555	18
9n	10878	5.6	518	21
10n	11466	5.9	546	21
11n	9139	4.7	481	19
12n	11655	6.0	555	21
13n	13986	7.2	666	21
14n	14040	7.3	702	20
15n	14700	7.6	700	21
16n	14637	7.6	861	17

Figure 1. (A) Agarose gel detection of competitive PCR products showing serial dilutions of known competitor template (upper bands) and a constant amount of unknown template (lower bands). (B) densitometric analysis of competitive PCR products. The area for each band is calculated and the larger size of the competitor template is corrected for incorporation of EtBr. A regression curve is calculated and the ratio where competitor and unknown template =1 is used to extrapolate the initial amount of unknown template in the sample (figure, complements of Dr. G. Zavala, University of Georgia, Athens, GA).

unknown input template because slightly different amounts of input template can result in extremely large differences in amplified product. This occurs because all PCR reactions have a lag phase, exponential phase and a plateau phase. And, the liner relationship that exists between template and amplified product only occurs during the exponential phase of the PCR reaction (see Fig. 2). Also, significant differences in length between the reference standard and unknown amplified products should be avoided because they will have different efficiencies of amplification, affecting the amount of final amplified product. In addition, the length of the amplified product must be considered when conducting densitometric analysis of the amplified product, since the same amount of a longer amplified product will be brighter on an EtBr stained agarose gel stained than a shorter amplified product.

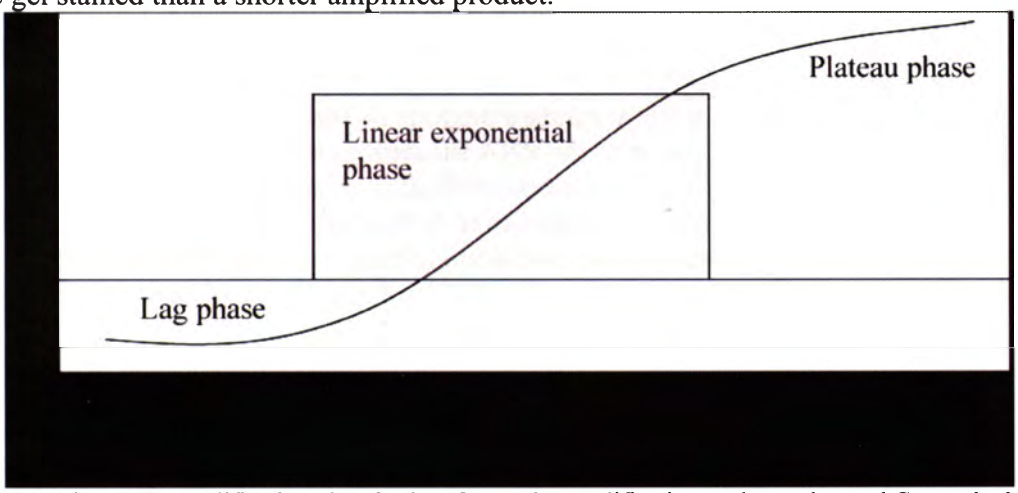

Figure 2. Real-time PCR amplification plot. Cycle refers to the amplification cycle number and Ct= cycle threshold.

Real-time PCR. Real-time PCR uses kinetic quantification to determine the amount of starting template in a reaction. Real-time is a typical PCR reaction that includes a fluorescent dye, that labels double stranded PCR amplified product, or a fluorescently tagged probe that is used to detect the amplified product as it is being made. Fluorescence is measured using a spectrophotometer, which is integrated into the thermocycler. Amplification kinetics is determined by detecting the amount of amplified product after each cycle during the linear log phase of the reaction (see Fig. 2). The data is fed into a computer, which plots the amplified product from a series of cycles on a logarithmic scale to calculate the average efficiency of the reaction. The initial template concentration can then be determined using the above formula. The main advantage of kinetic quantification by real-time PCR is that the amplification efficiency is independently calculated for each reaction tube, which eliminates the need for internal controls and assumptions regarding amplification efficiency.

Real-time PCR product can be detected by a nonspecific fluorescing dye such as SYBR green I or by a labeled probe. SYBR green I dye produces a fluorescent signal when it intercalates into double stranded DNA (dsDNA). As the dsDNA product in the PCR reaction increases, so does the intensity of the fluorescent signal. However, SYBR green I is nonspecific,

detecting any amplified product, whereas labeled probes are specific for the amplified target sequence. There are many types of probes for detection of PCR product, but the TaqMan® (Applied Biosystems, Foster City, CA) probes are most widely used and will be discussed here. TaqMan® probes are typically 18 to 22 bp in length and carry a fluorescent tag (reporter) on the 5' end, and a quencher dye on the 3' end. While the fluorescent tag and quencher dye are attached to the probe, the quencher dye "quenches" the signal and little or no fluorescence it detected. During the annealing step in the PCR cycle, the TaqMan® probe hybridizes with its complementary sequence on the PCR product. When the Taq polymerase replicates the DNA, the 5' exonuclease activity of the polymerase removes the probe from the template, destroying it in the process and releasing the fluorescent tag from the quencher dye. As a result, the fluorescent signal in the reaction increases. Generally the increase in fluorescence for a reaction is reported as a cycle threshold (Ct) value (see Fig. 2). Cycle threshold is the cycle number where fluorescence in the reaction crosses a minimum value considered to be positive. An inverse relationship exists between Ct value and the amount of stating template in the reaction. The Ct value in Figure 2 is approximately 17.

Real-time RT-PCR can also be quantitative but a number of additional variables must be considered. Most important are the RNA extraction methods and the efficiency of the reverse transcriptase to copy the RNA template into DNA, which can vary from tube to tube. Consequently an internal reference RNA standard ought to be incorporated into the experimental design. However, precise quantification of the RNA standard as well as variation in RNA extraction methods can still affect the absolute quantification of unknown template RNA.

The most significant development in real-time PCR is automation that integrates template extraction, purification, and amplification. Some manufacturers claim a test can be run from clinical specimen to quantified product in 30 minutes. One fully automated real-time PCR apparatus combines 96- or 384-well plate technology for high throughput qPCR.

It is important to remember that qPCR detects the amount of nucleic acid in a clinical sample, and not the number of viable organisms. Thus, qPCR data should always be interpreted with this understanding. However, with the proper controls, it can be correlated with viral or bacterial titer *in vitro* and *in vivo,* with a reasonable level of accuracy.

Future applications of qPCR include evaluating microarray data, and measuring gene expression profiles in pharmocokinetic studies. It is also being used in mechanistic and diagnostic cancer studies, functional genomic studies on the immune response and screening for mutations involved in genetic diseases.

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Real-time quantitative PCR and AI

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Microarrays and future diagnostic applications

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Although DNA microarrays (sometimes referred to as DNA chips) have become key tools in human preclinical and clinical research, to date they have had a smaller impact in the area of diagnostics and animal applications. Whereas previous generations of scientists studied a single gene or a single organism, this technology permits the simultaneous study of multiple genes based on either their sequence or expression. This technology provides an additional tool in their arsenal for developing improved methods of disease detection and vaccine development.

The concept of immobilizing probe molecules in order to detect targets can be traced back to the 1960s and the development of the immunoassay. DNA microarray technology evolved from the use of the Southern blot, developed in the 1970s. Traditionally, DNA microarrays are composed of treated glass slides (the size of a regular microscope slide) upon which are immobilized hundreds or thousands of gene probes. These gene probes can be derived from PCR products or they can be synthetic oligonucleotides. Proper design of the microarray is critical for obtaining statistically relevant data. In use, the microarray is simultaneously exposed (hybridized) to target samples, traditionally fluorescently labeled cDNA products derived from mRNA. The fluorescent image of the microarray slide which is generated after washing and scanning results in the simultaneous measurement of the expression of thousands of genes, as measured by the amount of mRNA present in the sample at that point in time. Because of the scale of information generated, data analysis and management are critical. Only with the proper management and analysis of this information can the potential of microarray technology be realized.

Diagnostic applications of microarrays initially focused on the detection of inherited human disease traits. However, microarray techniques have also been successfully developed for use in diagnostic microbiology. In a classic paper, Wang *et al.* (14) described a microarray capable of identifying over 140 respiratory viruses. Their technology was validated using clinical specimens from the respiratory tract of human subjects. Wilson *et al.* (16) developed a Multi-Pathogen Identification (MPID) microarray that identified 18 pathogenic prokaryotes, eukaryotes, and viruses. These researchers amplified unique regions of DNA from each microorganism, and then used the microarray to detect the poresence or absence of pathogen-specific DNA sequences with a detection limit as low as 10 femptograms of pathogenic DNA.

There are several published reports of influenza diagnostic microarrays. Initial efforts identified strains of equine influenza and copuld differentiates HA1 from HA3 and NA1 from NA2 (12) while other studies differentiated HA1, HA3, HA5 and NA1 from NA2 of type B human influenza strains (5, 7). The "FluChip" (13), the "Mchip" (4, 10) and the CombiMatrix Influenza A detection system all reflect the effort being given to microarray-based detection technologies for influenza which in some instances can identify all 16 HA subtypes.

Our laboratory has also developed a microarray designed to identify type A avian influenza, and to differentiate between hemagglutinin and neuraminidase subtypes. This cDNA-based microarray contains PCR products specific for the HA1, HA5, HA7, and HA9 subtypes, as well as the NA1, NA2, and NA3 subtypes. The slides also contain a pan-influenza probe, based on the matrix (M) gene sequence. Each element on the array is spotted in duplicate in each of four subarrays, yielding eight spots representing each element on the array.

More recent applications of the microarray allow scientists to readily identify pathogenic microorganisms by performing rapid large-scale sequence analyses. Developing a form of "microbial forensics", Read *et al.* (11) pioneered the use of a microarray to determine the origin of an anthrax infection. More recently, commercial enterprises such as Affymetrix (Santa Clara, CA) have developed pathogen-specific sequencing arrays. By sequencing up to 300 kb of a genome within 48 hours, these arrays can rapidly identify sequence variations between different bacterial or viral strains. This array-based "resequencing" technology has been used not only to identify genetic variations of influenza viruses (15) but has been used to develop a method to detect multiple viral and bacterial respiratory pathogens (8, 9).

There are other significant potential applications of microarray technologies to poultry health. Gene-expression profiles of infected animals can provide a "signature" or "diagnostic marker" for infection by specific agents (2). This approach has already been used successfully in a bovine model, where Johne's disease in cattle is associated with the enhanced expression of a number of genes, including IL-5 (3), although it has not been used for poultry. We also envision expanding this approach to flock monitoring. In the future it may be possible to inform a company veterinarian about the immune status of his flocks. It might also be possible to determine if they have been exposed to specific infectious agents or are particularly susceptible to specific pathogens. Microarrays have already been used in production animal models to determine immunological correlates for protection. By characterizing the bovine immune response against experimental challenge with *Mycobacterium bovis,* researchers have correlated the IFN-gamma response and IL-4 mRNA expression with the severity of disease and have thus been provided with a measure for protection (1).

Microarrays also have great potential in the area of vaccine development. By examining the transcriptional activity of all the genes of a pathogenic microorganism under *in vivo* conditions, rarely expressed but potentially important genes can be identified (6). Increasingly, vaccine manufacturers are including immune enhancing compounds in their formulations. Microarrays can be an attractive tool to use to generate the efficacy data required for licensing and marketing.

The flexibility and high-throughput capability of microarray technology offers tremendous opportunities for infectious disease research. These new tools will allow microbiologists to develop a better understanding of host-pathogen interactions and stimulate the development of novel approaches to infectious disease diagnosis, treatment, and prevention.

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Overview of developments in the last 10-15 years in recombinant vaccines

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This introductory talk will describe the various types of recombinant DNA vaccines that have been developed for the poultry industry. The talk will not discuss the efficacy of specific recombinant DNA vaccines. Instead, I will focus on describing how various recombinant vaccines are made and some advantages and disadvantages of each.

Conventional live vaccines are usually obtained by either isolating a naturally attenuated organism that is immunologically related to the pathogen or by attenuating a pathogen by some process such as repeated cell culturing. In general, live attenuated vaccines are more efficacious than killed or subunit vaccines. However, live vaccines for poultry suffer from several shortcomings. First, development of live vaccines is a balancing act where the viral vaccines must be attenuated to a degree where the vaccine is safe yet still be immunogenic and capable of inducing a protective immune response. In addition, viral pathogens often evolve to pathogens of greater virulence, necessitating the development of ever newer and more protective conventional vaccines. One way to circumvent these problems is to use molecular biology to engineer novel vaccines. With our current level of molecular techniques, many different approaches are available and some of these approaches will be described in further detail.

Plasmid vaccines. A simple plasmid that contains a strong promoter driving a gene derived from a pathogen is one of the simplest constructs. By using only one gene from a pathogen there will be no risk that the construct will produce disease. Plasmid-based vaccines can also be easily delivered to the chicken embryo (7). Potential plasmid-based vaccines were developed for several poultry pathogens, including avian infectious bronchitis virus (4), infectious bursal disease virus (3, 6), avian influenza viruses (5) and Newcastle disease virus (10) among others. However, many plasmid constructs expressing one gene often do not generate a very protective response. Problems of expressing adequate amounts of the immunogenic protein have also limited the use of plasmid-based vaccines.

An alternative to conventional live vaccines and plasmids is to use molecular techniques to attenuate the pathogen and use the attenuated product as a live vaccine. Presumably, as the pathogen evolves to evade the molecularly modified vaccine, additional molecular modifications can be used to rapidly generate the next generation of effective vaccines.

Cosmid and BAC derived vaccines. The technology of utilizing overlapping cosmid clones and bacterial artificial chromosomes (BAC) have simplified the process of molecularly attenuating viral pathogens. In one example, the Marek's disease virus (MDV) genome was cloned into five overlapping cosmid clones (9). Each of the five cosmid clones can be readily mutated and infectious MDV is re-generated when all five cosmids are co-transfected into susceptible chicken embryo fibroblasts. Recently, overlapping cosmid clones of an MDV were used to delete the MDV vIL-8 gene. The mildly virulent deletion virus protected susceptible maternal antibody-positive chickens at a higher level than the commonly used Rispens vaccine (2).

Viruses cloned as BACs also allow easy manipulation of large viral genomes. MDV has also been cloned as a BAC (11). Once cloned as a BAC, the MDV genome can easily be manipulated using the two-step Red-mediated recombination procedure, as described by Klaus Osterrieder's group (13). Recently, MDV and turkey herpesvirus (HVT) BACs were shown to be potential vaccines (1, 12).

Reverse genetics. RNA viruses are more difficult to engineer than DNA viruses. RNA viruses cannot be directly cloned as cosmids, BACs or plasmids. However, they can be reversed engineered from DNA copies. Full length cDNA copies can be cloned into plasmids and fully infectious RNA viruses can be rescued from the cDNA clones. Using this approach, Peeters et al. generated a recombinant Newcastle disease virus vaccine (8).

I have presented a brief overview of a few of the molecular techniques that are currently being used to manufacture the next generation of poultry vaccines. Specific examples of these techniques and the products they have generated will be covered by the following speakers.

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Abstract: Recombinant Vaccines-Challenges and Successes

In this presentation, the major challenges, failures, and recent successes using DNA recombinant technology in the development of poultry recombinant or genetically modified organism (GMO) will be discussed. The discussion will primarily focus on the viral recombinant/vector type of vaccines. However, the importance of reverse genetic vaccines (e.g. Avian Influenza), gene deleted bacterial vaccines (e.g. Salmonella and E. coli), and the possible future DNA, chimeric and subunit types of poultry vaccines can not be ruled out.

Viral recombinant /vector vaccines are constructed by inserting the gene(s) encoding for the protective protein(s) from donor virus(es) into the viral genome of the vector virus. In poultry, DNA viruses like the Fowl pox (FP) or Turkey Herpes virus (HVT) have been commonly used as vector viruses. In the future, Adenoviruses and RNA viruses, such as NDV, may be used as vectors.

About 25 years ago, it was predicted that poultry recombinant (vector) vaccines would be introduced at a rapid pace and would replace several of the classical vaccines. However, it has only been in the last 5 years that we have been able to introduce the first recombinant vector vaccines based on FP. And only recently, have recombinants based on HVT as a vector been introduced.

The ideal poultry vaccine should: be safe, efficacious, have an extended duration of protection, be convenient to administer, and easy to produce. An ideal recombinant vaccine should also provide an economic benefit for the poultry producer over the conventional vaccines.

The challenges biological companies face in the development of recombinant vaccines are: extensive, time consuming, and costly research and development, including failures; time consuming and costly licensing procedures; and patent issues. These are the main reasons for the increased vaccine costs. Therefore, a recombinant vaccine should clearly show advantages over the classical type of vaccines.

Bacterial artificial chromosomes (BACs) and the development of novel vaccines against Marek's disease

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Summary

Cloning of an entire genome of Marek's disease virus (MDV) as an infectious bacterial artificial chromosome (BAC) clone was first reported seven years ago (Schumacher et al., 2000). Since then, a number of wild-type and vaccine strains were established as mini-F based plasmids in *Escherichia coli*, and mutagenesis of various open reading frames and other genetic structures have advanced our understanding of disease development and progression, particularly with regard to tumor formation and metastasis. In contrast, our knowledge of gene products required for early lytic virus replication and especially animal-to-animal transmission remains fragmentary. The establishment of versatile and fast mutagenesis procedures in *E. coli* has allowed us to not only investigate in detail individual open reading frames, it will certainly also enhance our ability to engineer and test novel, more efficacious mono- or multivalent vaccines. The latter will probably concentrate on other viral diseases of the domestic chicken, against which vaccination protocols are ongoing, such as infectious bursal disease virus, Newcastle disease virus, and possibly influenza virus.

Significance of Marek's disease and properties of the causative agent.

Since the first description of the disease in 1907 when Jozef Marek reported on a polyneuritis in chickens [20], MD has changed its clinical picture, and in the 1950's a major increase in the incidence of visceral lymphomas was reported [4]. Ten years later, the etiological agent of MD was identified as a herpesvirus and termed MDV [7]. MDV is classified as an alphaherpesvirus; together with its close relatives, gallid herpesvirus 3 (GaHV-3; formerly referred to as MDV serotype 2, MDV-2) and meleagrid herpesvirus (MeHV-1; also referred to as turkey herpesvirus, HVT), MDV forms the genus *Mardivirus* within the *Alphaherpesviridae* subfamily. Only MDV has been shown to cause clinical disease and tumors in chickens or turkeys [5]. Four pathotypes are currently recognized: mild (mMDV, e.g., CU-2), virulent (vMDV, e.g., JM16), very virulent (vvMDV, e.g., RB-1B), and vv+ MDV (e.g., RK-1). The genomic organization of all three members of the *Mardivirus* genus is very similar, consisting of a unique long (U_L) and a unique short (U_S) region. The U_L and U_S regions are bracketed by inverted repeat regions, which are termed terminal and internal repeats-long (TR_L and IR_L) and terminal and internal repeats-short (TR_S and IR_S), respectively [10]. Complete sequence analyses, however, have shown marked genetic differences between the three viruses in terms of GC content and sequence similarity, especially in the inverted repeat regions [1,17,23,35].

Infectious herpesvirus genomes – the BAC era.

MDV mutagenesis and generation of recombinant viruses used to be a tedious procedure, although the principle and the methodology were adopted from other herpesviruses. Briefly, mutagenesis relied on homologous recombination in chicken cells of wild-type MDV with

cloned sequences harboring flanks that would result in the introduction of a marker, usually green fluorescent protein (GFP) or □-galactosidase, in lieu of the gene to be studied. In the case of MDV, the method was championed by Morgan, Parcells and co-workers as well as Hirai and colleagues in the early 1990's [6,24,25,30]. As in other herpesviruses, but potentiated by the highly cell-associated nature of MDV, the downside of the methodology has been that recombinant viruses have to be purified to homogeneity, a process that 1) puts enormous evolutionary pressure for "survival" on the recombinant virus and therefore can easily result in the introduction of compensatory mutations elsewhere in the genome, and 2) would take up to 30 passages in cultured cells to arrive at a homogenous, recombinant virus population [25,26].

In 1997, a breakthrough in herpesvirus mutagenesis was reported by Messerle and co-workers, when they achieved cloning of the then largest known herpesvirus, human cytomegalovirus (HCMV), as in infectious clone in *E. coli* [21]. The resulting HCMV BAC changed the field entirely, because much of the drawbacks that we know for MDV, such as slow growth in cell culture and fast attenuation through serial passage in cultured cells, could be overcome by using the newly developed methodology. Once introduced in *E. coli* as an extrachromosomal DNA, the viral genome could stably be maintained in bacteria, free of evolutionary pressure, and – equally important – was amenable to the respectable toolbox for genetic manipulation strategies in prokaryotes [9,18,34,39,40].

Only three years after the first description of a herpesvirus BAC, establishment of an MDV BAC was reported, which represented an attenuated version of the vv+ strain 584A [31,37]. At the same time, a second approach for cloning MDV DNA in its entirety was developed by Reddy and colleagues who cloned the genome of vvMDV strain Md5 as a set of four overlapping cosmid clones [29]. Therefore, analysis of the roles of genes or genetic structures in MDV virulence using BAC mutagenesis and testing of reconstituted, recombinant viruses was impossible until a BAC from vv MDV strain RB-1B was developed [28]. The original version of the RB-1B BAC (pRB-1B), however, contains a genome that, upon reconstitution in chicken cells, will result in viruses that are unable to spread horizontally from infected to contact birds [15]. Recent studies tackling this problem have shown that some genes are rendered non-functional in pRB-1B by the insertion of single nucleotides and thereby causing frame-shift mutations, among them UL44 encoding gC and UL13 encoding the unique-long serine-threonine protein kinase (Spatz et al., unpublished data). Repair of the mutation in UL13 alone did not result in virus that was able to spread horizontally (Blondeau et al., manuscript in press). In contrast, simultaneous repair of gC, UL13 and US2, the gene deleted for insertion of mini-F vector sequences did result in MDV that can be transmitted to contact chickens (Jarosinski et al., manuscript submitted).

The use of BACs or BAC-derived MDV as MD vaccines

With the establishment of the first infectious MDV clone it was apparent that the technology could possibly be used for the generation of a new class of vaccines. There are three most commonly cited problems with MD vaccines and vaccination. First, the cold (liquid N_2) chain must be maintained from the production site to the end user. Second, MD vaccines have relatively poor standards with respect to biosafety and homogeneity between batches in that primary chicken cells are used for production. Third and probably most importantly, newly emerging and ever more virulent and aggressive MDV have constantly outsmarted vaccines after a number of years ever since the inception of comprehensive, world-wide vaccination in the late 1960's and early 1970's [16,22,36,37]. Although a clear increase in virulence of MDV strains and changes in induced disease patterns as well as disease progression are caused by newly

emerging MDV [2,3,11,13,14,38], the alterations in those strains at a genomic level are only poorly understood. Therefore, efforts have chiefly concentrated on the rational design and the engineering of novel vaccine candidates. The appealing feature of BACs is that they can be used for mutant virus generation, including the expression of foreign genes in an MDV background, and as such provide a versatile and easy-to-manipulate platform for (vectored) vaccines [16]. On the other hand, it has been demonstrated with infectious cloned DNA of two different vaccine strains, Rispens-CVI988 and 584p80C, that MDV BACs could be used as a modified live virus vaccine in the disguise of a DNA vaccine where application of cloned, infectious viral DNA will result in reconstitution of the vaccine virus *in vivo* [27,32,33].

With regard to the generation of novel vaccines, a number of laboratories have concentrated on attenuating wild-type strains with the hope to arrive at a preparation that will still have good replicative abilities but be apathogenic for the target animal, the chicken. Here, Meq-negative viruses that are based on the Md5 cosmid clones have yielded promising results, and other candidates include mutants devoid of R-LORF4, pp38, UL36 or vIL-8 [8,12,15,19,26]. To this date, none of these vaccines has been proven to be superior to the vaccine gold standard, CVI988-Rispens, that is now frequently used. Rispens vaccine breaks have, however, been reported and we must not let our eyes off the ball and concentrate on the conception and testing of novel vaccines or other pro- and metaphylactic measures. While vaccine engineering is rational to a certain extent, all new candidates need to be tested in vaccination/ challenge studies, first under laboratory conditions, then in the field. While the road ahead of us certainly is not going to be a high-speed thruway, we are well endowed with an arsenal of techniques to face the challenges of certainly bumpy passageways to the sustained control of one of the most important and devastating infectious diseases of poultry.

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The Promise of Reverse Genetics of IBDV

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Summary

Only by basic research of an infectious agent its biology can be understood and new ways how to fight the disease which it causes can be discovered. Molecular manipulation of virus genomes can serve as a means to create or improve vaccines against diseases in human and animal. The discovery of reverse transcriptase enabled scientists to apply the techniques of recombinant DNA technology to RNA viruses and to establish reverse genetics systems. Reverse genetics systems (RGS) now serve as the tools to on the one hand investigate the biology of RNA viruses and on the other hand generate tailor-made recombinant viruses to serve as vaccines. Employing the RGS of infectious bursal disease virus (IBDV) functions of viral proteins, regulatory elements during replication, and markers for attenuation have been discovered. This knowledge helped to establish attenuated vaccine candidates and vaccines which provide a broad spectrum of protection against antigenic different IBDV isolates. There is still a tremendous lack of information about the replication of IBDV but the RGS will help researchers to build more efficient and thus low cost vaccines and improve diagnostics in the future.

Generation of recombinant RNA and its targeted manipulation by molecular techniques are still a challenge to researchers, which has several reasons. Two crucial methods used in molecular manipulation of nucleic acids are based on the chemical nature of DNA and are not applicable in the manipulation of RNA, molecular cloning and PCR. Only the discovery of reverse transcriptase (1, 25) opened up the field of recombinant DNA technology for RNA viruses. Another difficulty arising from the function of most RNA molecules in the cell is that they are not designed to be stable over an extended time, but their existence has to be regulated very strictly. Therefore, RNA degrading enzymes are abundant, they are extremely stable and destroy RNA molecules under a wide range of reaction conditions. Working with RNA requires taking strict precautions to inactivate RNAses present in cells or bacteria, sticking to every surface and even resisting autoclaving. Besides the nature of the RNA molecules, it is also the nature of the majority of RNA virus genomes, that poses additional challenge. In contrast to most DNA viruses, the genomes of which consist of one molecule of DNA, the genomes of many RNA viruses are segmented and thus complicate any manipulation.

Recombinant DNA technology has undergone tremendous progress within the past two decades and has reached a high standard. Its application already influences our daily life, mainly in food production and advanced medical products. Examples are the generation of transgenic plants and animals, the targeted breeding of plants and animals, the generation of innovative pharmaceuticals including vaccines, and the diagnosis and treatment of human diseases.

Four major discoveries enabled the rapid development of recombinant DNA technology to present day standards: (i) the discovery of restriction endonucleases by Smith and Wilcox (22), the first in vitro generated recombinant nucleic acid (7), enzymatic DNA sequencing by the chain termination method (21), and the development of the polymerase chain reaction (20). The application of the mentioned methods as well as molecular cloning in bacteria, yeast or vertebrate or invertebrate cells is confined to DNA. Transcription of RNA into complementary DNA (cDNA) using reverse transcriptases is thus a prerequisite for the application of this technology in the world of RNA viruses. The discovery of reverse transcriptases by Baltimore (1) and Temin and Mizutani (25) belongs to the milestones in the world of recombinant technologies. Only with the availability of these enzymes it became possible to transcribe viral RNA genomes into viral cDNA and establish reverse genetics systems (RGS) for RNA viruses.

What are reverse genetic systems? For the establishment of these systems three prerequisites need to be fullfilled: i) establishment of the full length sequences of the particular virus ii) generation of full length cDNA clones under a certain promoter iii) finding susceptible cells which can be transfected with a high efficiency. For most of the animal RNA viruses RGS have been established within the last three decades. The first RGS was developed for a bacterial RNA virus (Q□virus, 24). Three years later the first RGS for an animal RNA virus (poliovirus) was described by Racaniello and Baltimore (19). Since then RGS have been established for almost all animal virus families.

The RGS for the *Birnaviridae* for its member infectious bursal disease virus (IBDV) has been established by Mundt and Vakharia (17). One prerequisite for this RGS was the determination of the exact 5' and 3' ends for both of the two genome segments. This was published by Mundt et al (16). The Birnavirus family is characterized by its bisegmented double-stranded RNA genome which is encapsidated within a single-shelled icosahedral particle of 65 to 70 nm diameter. The structure of the viral genomic RNA gave the name (Birna) for the family. IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (9). The larger segment A encodes a polyprotein of approximately 110 kDa which is autoproteolytically cleaved by the viral protease VP4 (4) to form the viral proteins (VP) VP2, VP3, and VP4 and four structural peptides derived from the VP2 precursor, pVP2 (8). A second open reading frame (ORF) preceding and partially overlapping the polyprotein gene (3, 23) encodes VP5, which has been detected in both IBDV-infected chicken embryo cells (CEC) and in bursal cells of IBDV- infected chickens (13). The shorter segment B encodes a single protein VP1, which represents the RNA dependent RNA polymerase (27).

The RGS for IBDV opened up the genetic manipulation of a virus, which is a common threat for the poultry industry in the US and world wide since the husbandry of poultry has changed during the last thirty years from backyard flock to industrial standards with several thousands of birds in one self-contained building, several of which are located in complexes. Due to the husbandry poultry is more susceptible to diseases in general and viruses in special. The development of vaccines to protect the birds from diseases now follows high standards but there are indices that viruses counteract and reemerge in antigenic variants or new, more virulent strains. There was an increase of morbidity as exhibited through increased processing plant condemnations, higher feed conversions, and depressed average daily weight gains. Many conditions during husbandry are considered to be immunosuppressive to chickens. In the focus of this discussion are IBDV and infectious chicken anemia virus due to their ability to cause immunosuppression. In North America commonly isolated variant IBDV doesn't cause clinical disease and therefore will not be detected as the primary cause of disease or mortality in the flock. This stays in contrast to the

presence of classical as well as very virulent IBDV, which are causing clinical disease and direct losses.

The RGS for Birnavirus has been used for research on several topics which are important for a better understanding of the nature of the virus. The first application of the RGS was the generation of an IBDV not able to express the nonstructural protein VP5, IBDV-VP5minus (15). This experiment showed that VP5 was not necessary for the replication of the virus. The finding that the VP5minus mutant showed delayed growth in cell culture but finally reached the same titer as the wild type virus makes the mutant an interesting candidate for a marker vaccine. Moreover, in infection experiments in SPF chickens the virus was strongly attenuated (28) as shown by the absence of any lesions in the bursa of Fabricius (BF). Both properties, marker approach and attenuation, were investigated in additional infection experiments in chicken (Mundt and van Loon, not published). Chicken vaccinated via the eye-drop route were not sufficiently protected against challenge with the IBDV strain F52/70. In addition, chicken vaccinated with wild type IBDV D78 developed an insufficient antibody response against VP5 (Mundt and van Loon, not published). This led to the conclusion that an IBDV VP5minus virus was not applicable as a vaccine candidate using the mass application in chicken flocks. One of the next topics which were analyzed was the question why certain IBDV isolates are able to infect cell cultures whereas most isolates of the field are unable to infect cell cultures. Lim at al. (10) showed by using the reverse genetics approach that two amino acids (279, 284) were responsible for the ability of IBDV to infect cell cultures. Mundt (12) showed by his investigations that amino acid 254 and 284 are important for the ability of IBDV to infect cell cultures. Using this approach van Loon et al. (26) adapted the very virulent (vv) IBDV strain UK661 to cell culture and showed that adaptation to cell culture resulted in a complete attenuation of the virus as shown by infection experiments in chicken and the resultant virus was able to protect the chicken in challenge experiments. The finding of the amino acids responsible for infection of cell cultures will allow the adaptation of field isolates to cell culture for propagation of vaccine candidates in the future. In addition, it has been shown that VP2 (5) as well as VP1 are able to influence virulence in chicken (11). In contrast, Boot et al. (2) showed that one single IBDV protein is not responsible for a certain virulence phenotype (here very virulent) indicating that virulence of IBDV is determined by more than one element of the virus. This showed clearly that virulence of IBDV can be influenced by a single protein (VP1, VP2, or VP5) but is also balanced by other viral elements. The knowledge about the function of certain viral components can be used for the attenuation of IBDV and thus for the development of vaccines. One important step for vaccine development is the ability of the virus to infect cell cultures. Using the above mentioned information a vaccine candidate was developed which showed the ability to grow in cell culture to high titers (18). This is important since the production of vaccines for the poultry industry has to be cost efficient. In addition, by additional manipulation of the cDNA an IBDV was rescued, which showed the ability to induce protection against both, the classical IBDV as well as the E/Del subtype of IBDV. Based on this characteristic the generated virus was called broad spectrum IBDV vaccine. This finding was the starting point for the question which amino acids are responsible for the different antigenicities of the different IBDV subtype strains. In a concise analysis of amino acid sequences followed by site directed mutagenesis experiments amino acids responsible for the differences in antigenicity were determined (data not published). It was observed that different combinations of amino acids resulted in the same reaction pattern. In addition it was found that amino acids located 100 amino acids apart influenced one epitope. The rationale behind this finding could be explained by the

crystal structure of VP2, which was published recently (6). For this approach the reverse genetics was applied indicating that diagnostic approaches might be possible in the future.

In summary with the development of the reverse genetics of IBDV it became possible to analyze the function of specific viral elements in a more directed way. This approach is basic research and is hypothesis driven. Based on the obtained results a possible application can be deduced which will probably result in improved vaccines or diagnostics.

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Concept to Product - Challenges of Development, Patenting, Licensing, and Marketing of Recombinant Poultry Health Products

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Introduction

As the Central Dogma of Molecular Biology (DNA to RNA to Protein) and the many complex processes surrounding it (replication, transcription, translation, etc.) have become better understood, the ever increasing promise of biotechnological revolution has continued almost unabated. Yet, in some disciplines, poultry vaccines as an unfortunate example, this promise has gone conspicuously unfulfilled. For those of us (this author included) who believe that technology and a free market can combine to solve most serious problems, this is surely a disappointment. While advances in molecular biology have provided improved solutions in some areas of veterinary medicine, the arena of poultry health products is not one of them. Perhaps we should not be so surprised. It would not be the first time in human history where technological advances have been neutralized or minimized by non-technological developments. We have airplanes which can carry large numbers of people at 600 mph, however, for trips less than 400 miles, the door to door average speed they allow is little better than twice that of a passenger car. Email provides virtually free and almost instantaneous communication. Despite this almost magical capability, we complain constantly about the quantity and quality of the emails we send each other, along with evil thoughts for the devilish, digital boxes that contain them. In both cases, there are reasonable explanations which were also unexpected. Airlines operate in a tortuous regulatory environment combined with market pressures to maximize profits with full airplanes. This results in limited routes, large hubs, and incomprehensible fee structures. Additionally, few people could have predicted the added security and shoe removal adventures which are now a routine part of air travel. One of the biggest problems with email, SPAM, might have been better anticipated using the "Tragedy of the Commons" (1, 2, 3) concept. Nonetheless, it threatens to negate a tremendous communications tool.

Poultry diagnostics have advanced tremendously through the application of biotechnology. Several of today's other presentations will present clear evidence supporting this. With regard to poultry health products, however, either biotechnology has failed us or we have failed to exploit biotechnology cost-effectively. The first category III recombinant vector poultry vaccine was licensed in the USA in 1994 (4). This was a Fowl Pox virus genetically engineered to express Newcastle virus genes. The first herpesvirus recombinant vector vaccines were licensed in 1999 (5, 6). These were HVT viruses vectoring a variety of genes for Newcastle and Marek's Disease. Currently there are 11 FPV and herpesvirus vector poultry vaccines licensed in the USA for commercial poultry (7, 8). (The licenses for the first two herpesvirus recombinants were terminated without prejudice in 2006 (9).) These biologics include vaccines for Marek's Disease, Newcastle Disease, Fowl Pox, Avian Influenza, Laryngotracheitis, *Mycoplasma gallisepticum*, and Gumboro Disease. Despite this wide selection, none of these are the commercial vaccine of choice for their respective diseases. Their total receipts make up only a tiny fraction of the global poultry vaccine sales. In fact, despite the tremendous increase in our

knowledge of poultry disease at the molecular level, the best-selling products for most major diseases of poultry (e.g. coccidiosis, ND, IB, IBD, and MD) have changed little in the past several decades.

The reasons for this technological failure are numerous, including mistaken strategies, incorrect product configuration, mis-application of technology, inadequate understanding of market needs, regulatory obstacles, underestimation of intellectual property costs and values, and the lack of rationality resulting from large amounts of biotechnology venture capital.

"If the only tool you have is a hammer, you tend to see every problem as a nail" (10)
Poultry vaccine companies, large and small, past and present, with their rapid development of reasonably effective and safe vaccines for the most common viral diseases of commercial poultry, deserve major credit for the early rapid growth of the modern poultry industry. Their ability to apply basic virology (isolation, characterization, serial passage, efficient propagation) to rapid commercialization of vaccines and early successes with such organisms as MDV, NDV, IBV, and IBDV created a straightforward, successful business model for subsequent viral vaccine targets. However, not all poultry pathogens yield to this strategy. Many non-viral organisms such as *E. coli*, *Eimeria spp.*, *Clostridium spp.*, *Hemophilus gallinarum*, *Pasteurella multocida*, and *Salmonella spp.* are not good vaccine candidates by most immunological standards. Even many viral pathogens, e.g. ILTV or AIV, are not promising candidates for immunization for a variety of reasons. The best or improved solutions to many poultry diseases will not be vaccines. Likewise, many of these same solutions will not involve biotechnology. Since most all early poultry health experts had either strong pathology or microbiology backgrounds, the same MLV or inactivated vaccine strategies were mistakenly used for diseases which will be prevented by other means. Adding molecular biology will not likely change this. While not as elegant, sometimes just putting a mild disinfectant in the chiller water may be a better way to reduce salmonella levels than using phages or genetically engineered vaccines.

Specificity versus Breadth
Molecular biology by it's name alone, implies a greater specificity. We now talk knowledgeably about specific epitopes, post-translational cleavage, open reading frames, insertion sites, and subunit vaccines. Why do we think that using ever tinier, more specific parts of an organism will help the bird's immune system produce better immunity, when we've known for decades how important breadth of immunity to a wide variety of antigens is? In a nutshell, this is why molecular biology has offered such tremendous gains in diagnostics (where specificity is prized) and underperformed in vaccinology.

Project Mentality / Regulatory Pipeline
Most biologics companies pursue advanced biologics products on a project or specific product basis. A recombinant viral construct is formulated, often with only minimal market considerations. Sometimes the final configuration more accurately reflects expediency from a molecular biology standpoint rather than the realities of farm virology. Even if a more thorough consideration of alternative recombinant configurations is proposed, such experimentation is usually stymied by intellectual property restrictions or the current regulatory framework. The decision to pursue the licensing and manufacture of a construct is decided on a go/no-go basis as several decision points. While the licensing and manufacturing cost estimates may be accurate, the market potential is usually based on numerous assumptions about the future product's

performance. Historically, these have been overly optimistic. Conversely, some potentially fruitful markets for unsolved disease problems are ignored because the existing markets are small. The vaccine companies, despite having a large number molecular options, are unable to adequately exploit the flexibility and speed offered by biotechnology. The current regulatory system requires them to "guess" the proper configuration very early in the process.

The current USDA regulatory pathway is long and difficult for a recombinant poultry vaccine. Due to the extremely high volume/low margin character of today's poultry biologics business, this process can only be justified for the major poultry diseases. In most cases, there are no opportunities for large scale field testing of novel biologics until near the end of the regulatory journey. It is virtually impossible to simulate the efficacy and safety pressures a vaccine encounters in a commercial operation in any laboratory situation. Biotechnology offers speed and flexibility. Rigid product development programs and increasingly complex regulatory pathways offer neither. Licensing recombinant vaccines in multiple countries is significantly more challenging than licensing conventional vaccines globally. This adds to cost and increases the commercial risk.

Product Configuration

Although most biologics companies are familiar with field poultry diseases and are often comprised of former live production employees, many product development decisions do not reflect this. As mentioned above, some product configurations, despite their scientific elegance, appear to be designed with little consideration of the commercial situation. A pox vector, while wonderfully versatile as viewed by a molecular biologist, is doomed by administration limitations. A perfect, reactionless ND vaccine is nice but until the thornier IB and ILT problems are solved, enthusiasm will be limited. Pathogens exhibiting antigenic diversity such as IBD are not likely to be defeated by narrowing the number of antigens delivered. In the case of vector vaccines, we have focused a lot of effort on the various vectors; locating, perfecting, and patenting insertion sites without equal attention to finding the perfect cargo.

Failure to Communicate

Unfortunately, producers of any genetically modified products often operate in a defensive mode in the hope that their product line will not be banned outright. Public concerns about such products help to drive the increasingly stringent regulatory requirements. While reasonable safety issues must be addressed, a fear of genetic modification may slowly remove one of the best tools we have to combat organisms which continue to do their own genetic engineering unencumbered by government, media, or public pressure (e.g. Avian Influenza Virus).

Intellectual Property / Venture Capital

In my many discussions with molecular biologists regarding vaccine development, I cannot think of a single instance where a scientist made unwarranted promises or exaggerated the potential of a biotechnology. Biotechnology has been over-bought much more than it has been over-sold. The business constraints of confidentiality and publicly traded companies often encourage the dilution or simplification of a complex scientific message by the time it gets to the boardroom. Often, the technical limitations and assumptions do not make the trip along with the potential benefits.

The desire and pursuit of patents for poultry vaccines has been unproductive and perhaps counterproductive. The global poultry health market is quite small compared to most other commercial branches where biotechnology is applied. The legal expenses of any adversarial situation arising between two corporate vaccine developers could quickly exceed any possible profit. Very few companies are earning significant income from poultry vaccine patents, while many patents may have a blocking effect on research. Since some patents may apply to biological products in multiple species, poultry products often have a lower development priority since the potential profits are lower than for other livestock or companion animal species. The desire of patent holders not to "contaminate" their technology with outside information often leads to product development in a vacuum. This lack of input results in unprofitable product configurations.

Solutions

Though the tone of this presentation has been decidedly negative, biotechnology still has tremendous potential to improve poultry health and poultry health products. Following are some suggestions:

1. Regulatory: Simplify and modernize the regulatory pathway to reflect the capabilities of biotechnology. Allow modification to existing products at the molecular level and additions to combination products without each new construct having to start anew. Allow field trials earlier in vaccine development. Focus mostly on safety and purity as regulatory issues. Allow efficacy to be decided in the marketplace and be flexible with regard to potency and combination products.

2. Product Development:

A. Vaccine companies should develop future products in close cooperation with live production companies even if it means paying their scientists and strategists to work inside a customer's company. This will bring home the realities of the market.

B. Don't just look at the current market size. Consider potential markets and evaluate not only the development cost versus potential market but also the realistic probability that a particular technology can solve a particular problem. Don't make future sales spreadsheets and forget the assumptions.

C. Educate the decision-makers better as to the limitations of biotechnology. This will lead to better use of research funds. The early vaccine company decision-makers had an excellent understanding of poultry disease, as well as conventional vaccinology, immunology, and microbiology. Today, poultry production realities and biotechnology are farther apart than they were three decades ago. It is a greater challenge to be conversant in both.

3. Intellectual Property: Either forget about patents and focus on superior products or enter into aggressive cross-licensing agreements with other vaccine developers so that fuller use of biotechnology can get to the poultry marketplace.

4. Communication: Continue to energetically apply biotechnology in those areas where it has a proven record (e.g. diagnostics). This validates the discipline and may help it to gain more acceptance publicly.

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Future of Vaccines for the Poultry Industry

David L. Suarez

Introduction

We continue to improve our understanding of avian immunology and are gaining new technological tools that have potential to be used for the immunization of domestic animals. With all these advances we still have to balance the protection that we receive from treatment (i.e vaccination) versus the cost to administer the treatment. The poultry industry is an extremely difficult sector of animal agriculture to vaccinate for because of the low value for individuals birds, the short production lives for most birds, and the costs to administer vaccines. The broiler industry provides the most extreme example with an extremely small margin per bird, extremely short production lives, and the difficulty of vaccinating large numbers of birds once placed in the house.

It is necessary to first define the goals of the vaccination program. This is commonly divided into three categories including; 1) is the vaccination programs to prevent or reduce clinical disease only; 2) will the vaccination program provide clinical protection and also reduce virus shedding as part of an eradication program. If used as part of an eradication program, vaccination is just part of an extensive program that would include animal movement controls, surveillance, and education; 3) is the vaccination program to reduce or eliminate pathogens that are of human health concerns, for example, Campylobacter in chickens or *E. coli* O157 in cattle. The approaches for vaccination can differ greatly between the different methods. For example if control of clinical disease is the primary factor, then for a disease like avian influenza virus, the production of serum antibodies can prevent systemic disease but still allow high levels of virus replication on mucosal surfaces that allows easy transmission to other birds. If the goal is eradication, then reduction of pathogen shedding must also be considered, which would include the induction of influenza specific secretory IgA, interferons, or other measures on mucosal surfaces. One additional variable is if the vaccination program is implemented on a continual basis or on an emergency basis. Particularly for foreign animal diseases, vaccination is not routinely practiced and is only considered after an outbreak has occurred. This could include ring vaccination or other approaches.

With this introduction, the ideal future vaccines would be available at low cost, be administered by mass administration methods, have no or limited vaccine reactions, allow differentiation of vaccinated from infected animals (DIVA), provide rapid onset of immunity even in the face of maternal or preexisting immunity, provide long-term protection, work in a variety of poultry species when needed, and provide broad cross protection for antigenically variable targets. Current vaccines are far from these ideal goals, and the most optimistic predictions of future vaccines won't live up to all of these expectations. However, current and future vaccine technology can provide significant improvements over available vaccines. This paper will outline some of the areas that I feel will make the greatest impact in the future.

Live Vaccines

Live vaccines are likely to continue to be important for the poultry industry because they provide the best immunity by stimulating both arms of the immune system. Additional live vaccines can often be administered by mass vaccination methods that allow administration by natural routes of exposure. Live vaccines have historically had issues of reversion to virulence and vaccine reactions. For example infectious laryngotracheitis vaccines if allowed to cause rolling infections can increase in virulence and cause severe disease in chickens. Another

example of vaccine reactions is with Newcastle disease vaccines, where vaccine reactions, particularly with more aggressive vaccines like LaSota can often cause respiratory disease, particularly when the birds are infected with other disease agents. A balance often is needed to have a vaccine that provides a strong immune response, but with limited vaccine reactions. The poultry veterinarian must use his clinical judgment to determine the optimal vaccine strategy for the birds in his care. However several emerging technologies may improve the situation.

Replication incompetent vaccines

The first class of vaccines I would like to consider are viruses or vectored vaccines that are replication incompetent. These vaccines are missing a key viral protein that does not allow the viruses to complete the viral life cycle, but the viral particles can still infect like a live vaccine. This allows for both cellular and humoral immunity to be stimulated, but provides the safety of a killed vaccine. No commercial vaccines are available using this technology, but these types of vaccines have been tested and shown to provide protection in chickens with several different systems. This has included the use of a human adenovirus vector (5, 16) and Venezuelan equine encephalitis (VEE) vector(13). In both systems the viruses are grown in special cell culture lines that allows the complementation of the missing viral proteins. The use of a viral vectored vaccine, canarypox virus in mammals, that has aborted replication in non-target hosts has already been developed commercially. This canarypox vector is genetically engineered to express proteins of the different target viruses that can produce a protective immune response. However canarypox virus can infect but it can't complete it's replication cycle in a mammalian host, and therefore is considered to be an extremely safe vaccine (14). This type of vaccine appears to have great potential in the future, but major obstacles to use remain. The primary obstacle is that because the viruses don't replicate, it requires the administration of a higher level of viral particles to achieve protection. This large amount of viral particles may result in an increased cost per vaccination that is higher than the poultry industry is willing to pay. The second obstacle is the delivery mechanism.

DNA Vaccines

The use of bacterial eukaryotic expression plasmid for expressing viral genes has many of the same characteristics as the replication incompetent viruses in that it can stimulate both arms of the immune system, and has a high margin of safety because it is replication restricted. This type of vaccine, although proven to work in poultry for several different pathogens(3, 4, 12), suffers from high cost due to the inefficient uptake of the DNA based on current methods of vaccine administration. DNA vaccines currently are useful tools for research and diagnostic development(8), but until the cost of the vaccine and administration and greatly reduced, it does not appear to have a future for poultry vaccines.

Adjuvants with inactivated antigens

The used of killed adjuvanted vaccines are commonly used because of the high levels of antibody and duration of immunity that can result. The use of modern adjuvants, particularly oil emulsion vaccines have allowed relatively small amounts of antigen to be used which has allowed low cost for the vaccines. This class of vaccines have typically used inactivated whole virus or bacteria as antigen and it has required parenteral administration. These vaccines, although often effective in controlling disease, have serious limitations because of the difficulty for mass vaccination, need to parenteral vaccinate, issues of long withdrawal times, and occasional issues of vaccine not being properly inactivated. However, recent technological

innovations may provide new life to this type of vaccine. First several new expression systems are available that can produce large amounts of protein for subunit vaccines that provide advantages over existing whole virus vaccines. In particular subunit vaccines can allow for DIVA vaccination for diseases like avian influenza where vaccination can be measured by hemagglutination inhibition titers and infected birds can still respond to the AGID or other nucleoprotein based diagnostics for type A influenza. These new subunit expression systems, including plant cell culture and baculovirus expression, can provide cost effective expression of proteins at a reasonable cost. A plant cell culture expressed vaccine for Newcastle disease vaccine has already been licensed for use in the U.S. The second major issue with the subunit vaccines are the need for parenteral administration. However, several mucosal adjuvants have been described that potentially allows oral or respiratory exposure to generate a protective immune response. This includes the use of cholera toxin to adjuvant both for Newcastle disease virus and coccidial infections(15, 17). A commercial vaccine using killed antigens with a mucosal adjuvant are currently being used in Mexico for both infectious bronchitis and avian influenza virus. The available or newer generations of mucosal adjuvants will likely provide a way for mass vaccination using subunit vaccines.

Immunomodulators

The last area of promise is to apply our improved understanding of the avian immune system with immunomodulators to improve or alter the immune response after vaccination. This can include enhancing the innate immune response to make birds more resistant to infection, like with an improved interferon response. Alternatively, you can induce or apply cytokines to improve the immune response. Lastly, the immune response can be altered to select for either a predominantly cell mediated or humoral immune response. All of these approaches rely on our understanding of the host pathogen interaction.

The innate immune response is the first line of defense against pathogens, and the host has a wide range of potential responses to pathogens. The different triggering mechanism for the innate response are still being determined, but toll like receptors and the presence of double stranded RNA are known to play roles. One recent example is an adenovirus vectored vaccine for foot and mouth disease that also expressed porcine alpha interferon. The increased levels of interferon provided early protection from virulent challenge until the adaptive immune response became active(2). This provided more rapid protection that is extremely valuable in emergency vaccination programs during an outbreak.

The use of cytokines to improve the immune response has been attempted with DNA vaccines with mixed results. With Eimeria the inclusion of several different cytokines improved the protective response to vaccine(9), but with infectious bursal disease no improvement was observed (7). Many examples in other species suggest that the inclusion of specific cytokines can improve the immune response, but we are still just learning to manipulate the avian immune response. The application of cytokines can shape the immune response to either T_h1 response or a T_h2 response, by directing differentiation of CD4 T helper cells to express different cytokines. The T_h1 cells drive the immune response more towards a cell mediated immune response and the T_h2 cells produce more of a humoral response. The outcome of disease for some infectious agents is determined by whether a more T_h1 or T_h2 cytokine response dominates. For some chronic diseases like Leishmania in mice, the Th1 cellular immune response can resolve infection where the T_h2 response exacerbates disease(10). In poultry viruses some diseases like Marek's disease are controlled primarily through cellular immunity, and the virus has multiple counter measures to evade the host immune response(1). With a better understanding of the

host/pathogen interaction, the immune response can be more efficiently targeted for control. This will likely include the expression of different cytokines to achieve this goal.

Other immunomodulators are likely to be included in the vaccines of the future. They may act as non-specific immune enhancers where the function is not completely understood, or they may be included because of known specific action. For example, a recent class of specific immunomodulators are the CpG motifs that are commonly found in bacteria that triggers an innate immune response from toll like receptors. The CpG motif has been shown to provide significant improvements in immune response with a number of different vaccines, but the response in poultry has been variable(6, 11). The future of this class of immunomodulators for poultry is unclear as both cost and protection have to be considered.

The future for vaccination in poultry remains bright as we increase our understanding of the host immune response and as new technology becomes available. The new technology will often have more regulatory scrutiny to assure safety, particularly with vectored vaccines which are genetically modified organisms. Several vectored vaccines have already been licensed and more are likely to be licensed in the future, but the increased cost for licensure increases the cost associated with the vaccines. The marketplace eventually makes the decision if a new vaccine provides enough additional benefit to balance the often higher cost of vaccines.

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