

General Interest

High Hydrostatic Pressure for Development of Vaccines

ADRIENNE E. H. SHEARER* AND KALMIA E. KNIEL

*Department of Animal and Food Sciences, University of Delaware, 044 Townsend Hall, 531 South College Avenue,
Newark, Delaware 19716-2150, USA*

MS 08-574: Received 19 November 2008/Accepted 23 February 2009

ABSTRACT

Disease management in the food industry is complex and includes use of good hygienic practices, antimicrobials, and immunization. Vaccines are available against many, but not all, disease agents affecting animals reared for human food. Fewer vaccines are currently licensed and widely available for human foodborne pathogens. Increased resistance to antimicrobials provides additional impetus to develop new vaccines. In addition to the need for new vaccines, new methods of vaccine production are desired. Some current methods of vaccine production can involve use of hazardous chemicals, provide inconsistent results, or present risk to vaccine recipients with certain allergies. The efficacy of high hydrostatic pressure (HHP) for inactivation of a variety of foodborne pathogenic microorganisms has been well established, and some of these microorganisms have been demonstrated to retain immunogenic properties, suggesting HHP may have application for the development of vaccines. Studies on the effect of HHP on infectivity and immunogenicity of various viruses, a protozoan parasite, and one bacterial species are presented. Control of several of these pathogens is important for animal health and economic stability in several sectors of the food industry. The research to date on the potential for vaccine development by HHP is presented.

VACCINES AND THE FOOD INDUSTRY

The food industry is vulnerable to the harmful impact of disease agents from the health of livestock to that of the human consumer. There are an unfortunate number of examples of disease outbreaks among livestock, which have resulted in reduced productivity, emergency treatment, mortality, depopulation, and trade restrictions, amounting to economic losses measured in billions of U.S. dollars (34, 55). Human illnesses attributed to foodborne disease are estimated in the millions in the United States alone (53).

Management of disease-causing microorganisms is a complex challenge that must be addressed throughout production, processing, distribution, and preparation of food. Vaccination is one preventative measure that can protect livestock, and in some cases, the human consumer, from microbial diseases associated with foods. Vaccination of livestock may be preferable to the use of antimicrobials, due to the development of resistance (81), and prevention of disease is generally less stressful for the animals (57) and less costly for the producer than management after infection. Internationally, the 2004 value of veterinary vaccines market was US\$3.1 billion, of which the majority was dedicated to livestock (55). In the United States, some recommended vaccination schedules aim to prevent as many as 10 different diseases in livestock (21, 27, 54, 64, 82). Additional vaccinations may be warranted in cases in which other diseases are diagnosed in sufficient proximity to present undue risk. In addition to direct immunization of food

animals, the practice of vaccinating wildlife to prevent the spread of disease to livestock has been performed, such as for rabies and brucellosis prevention (77, 84). Irrespective of the current availability and use of vaccines, research on the development of new and more efficacious vaccines remains a priority of the Animal Health National Program of the U.S. Department of Agriculture, Agricultural Research Service (76).

Among human disease agents for which food can serve as a transmission vector are rotavirus and hepatitis A virus (HAV), vaccines against which are readily available in the United States. Rotavirus is the most common cause of severe diarrhea among children, leading to an estimated 55,000 hospitalizations annually for children in the United States, and to more than 600,000 deaths of children annually worldwide (10). Currently, two oral vaccines against rotavirus are licensed for use in the United States (78). An inactivated HAV vaccine was licensed by the U.S. Food and Drug Administration in 1995 and 1996 for persons older than 2 years of age, and in 2005, an HAV vaccine became available for younger children. HAV vaccination of children is currently recommended for children by the Advisory Committee on Immunization Practices, American Academy of Pediatrics, and American Academy of Family Physicians (12). Vaccination of adults against HAV is recommended for individuals at high risk for exposure to HAV, including clinical workers and persons traveling abroad. Debates continue on whether widespread HAV vaccination of food handlers is appropriate, and who should cover the cost for such an effort.

Among other human disease agents potentially trans-

* Author for correspondence. Tel: 302-831-0115; Fax: 302-831-2822;
E-mail: ashearer@udel.edu.

missible through foods, vaccines are available on a very limited basis, or research is still in progress. In the United States, an investigational human vaccine against botulinum toxin produced by *Clostridium botulinum* is available (3) as is a vaccine to protect against anthrax caused by *Bacillus anthracis* (9); however, these are administered only to people considered at risk for occupational exposure, such as laboratory workers and military personnel. Research continues for vaccine development against these and other foodborne diseases, with efforts directed at vaccination of humans directly, or vaccination of food animals recognized as reservoirs for human disease agents, such as vaccination of cattle for control of the human pathogen *Escherichia coli* O157:H7 (52, 60, 61) and poultry for control of salmonellae (4). Candidate vaccines have reached preclinical or clinical trial phase against a number of foodborne diseases including cholera, enterotoxigenic *E. coli*, shigellosis, and typhoid fever (83).

VACCINE TECHNOLOGY

Current vaccines and new vaccines under investigation are predominantly of one of four types: inactivated whole cells (or viral particles); live, attenuated cells or particles; toxoids; and immunological subunits. Each has advantages and disadvantages.

Inactivated whole cells are prepared by chemical or heat treatment (65) and are generally considered safer than live, attenuated vaccines because there is no opportunity for replication or reversion to a virulent state (44); however, inactivated agents may not be as potent as live, attenuated preparations and may require booster injections or the addition of adjuvants to help improve the rate, duration, or strength of the immune response (2, 44).

Live, attenuated preparations generally induce strong and long-lasting immunity; however, a risk of reversion to virulence exists (44). Several approaches are used to develop live, attenuated vaccines, including passage of a wild-type strain in other hosts and/or cell culture until there is loss of pathogenicity without loss of immunogenicity, use of agents that are natural pathogens in other hosts but not that of the host to be immunized, use of naturally avirulent strains, and selective deletion or inactivation of certain genes (19). Toxoids are chemically or thermally detoxified toxins used to immunize against intoxications such as those caused by tetanus and diphtheria organisms (19). Subunit (or component) vaccines comprise individual components of the disease agent such as purified antigens and those derived by recombinant technologies. The design of subunit vaccines requires knowledge of the specific component, product, and/or its genetic precursor that causes the pathology. Antigenic polysaccharides or proteins can be isolated and purified from pathogenic organisms grown in the laboratory. Recombinant strategies are the most recently employed for the preparation of designed vaccines. Genes coding for a specific antigen can be incorporated into and expressed by bacteria, yeast, insects, or mammalian cells, and the antigen is subsequently purified. The same technique can be used for the expression of antigens by avirulent vectors including bacteria and plants. The use of

plants or other forms of oral vaccine delivery is appealing because it theoretically may improve mucosal immune response. Subunit vaccines may also help circumvent the need to generate large numbers of a disease agent for whole or attenuated vaccines, particularly for those agents that must be propagated in the natural host rather than in the laboratory.

Vaccines can also be designed to differentiate animals that have been naturally infected from those that have been vaccinated, termed marker, or differentiating infected from vaccinated animals (DIVA) vaccines (79). Vaccines with antigen gene insertions, deletions, or alterations will induce a different antibody response profile in the vaccinated individual, responses detectable by serological tests (46). Vaccinated animals that cannot be distinguished from naturally infected animals may be ineligible for international trade, and DIVA vaccines may be an approach to overcome this issue (80). DIVA vaccines may also serve as important tools to assess disease eradication programs (46).

In addition to the vaccine itself, other ingredients used for the preparation, stabilization, and preservation of vaccines may be present in some vaccine preparations. These may include antimicrobials to kill or prevent growth of contaminating microorganisms, egg protein if the agent has been attenuated by passage through egg embryos, formaldehyde for toxoid preparations and for use to eliminate contaminating viruses and bacteria, and monosodium glutamate used to stabilize vaccines from environmental pressures (11). Additionally, these preparations may include adjuvants such as aluminum salts, mineral oil, liposomes, and virosomes to enhance the delivery and immunogenicity of nonreplicating vaccines (46). Some of the aforementioned methods and/or reagents can be hazardous to handle or present disposal and environmental concerns. Certain vaccines are not recommended for some individuals with sensitivities to even trace amounts of certain ingredients.

The ideal vaccine has been described as having the following characteristics: strong, broad, and lasting immunogenicity in all individuals; no side effects; ease of delivery; stability; and low cost (19, 44, 46). Marker vaccines to differentiate natural immunity from induced immunity are desired, as the disruption of trade relations may be a deterrent to vaccination (34, 46). Finally, the preparation technique should ideally be nonhazardous and environmentally friendly. HHP is being explored as a new method of vaccine development with theoretical potential to deliver immune-inducing, inactivated, whole disease agents by means less hazardous than chemical treatments.

HHP PROCESSING

HHP is an emerging food processing technology with multiple and varied potential applications. HHP has been shown to induce the formation of gels (43), aid starch gelatinization (20), affect phase transitions (41), kill some insect eggs (7), affect enzymatic reactions (1), affect allergenicity of certain food components (15, 28, 30, 37, 39), and affect bacterial toxins (49). HHP can also be used as a process aid for certain shellfish products (8, 16). However, the utility of HHP for the inactivation of pathogenic and spoilage

microorganisms has received the majority of research attention, and its success for this purpose has been commercialized for a number of different food products (16, 31). The efficacy of HHP on various microorganisms and the conditions that affect HHP efficacy have been reviewed (47, 58).

During treatment, pressure is distributed uniformly and instantaneously, independent of product shape and size (58, 75). Processing can be done in batch or semicontinuous fashion in commercially available units of varying sizes. The application of pressure leads to an increase in temperature, known as adiabatic heat; however, the application of pressure can reduce the temperatures necessary to inactivate some organisms as compared with temperatures required when heat is applied as the sole treatment. One major appeal of HHP is its minimal effect on the freshlike properties of foods as compared with thermal processing (75).

Pressure has served as an important tool to study macromolecular assemblies (70), with applications for food production as well as pharmaceuticals. The predominant effects of HHP are reportedly on the tertiary and quaternary organization of polymers rather than on covalent interactions (75). The mechanism of microbial inactivation by HHP has been attributed to the potential disruption of multiple structures and functions, such as bacterial membrane permeability, replication and transcription, partial unfolding of viral protein coat (33, 50, 58), and parasite infective structures (66). Depending on what structures or functions in microorganisms are disrupted by HHP, it is theoretically possible to achieve inactivation of a pathogen, without altering properties that elicit an immune response in its host. Indeed, research with a number of pathogens provides support for this possibility.

HHP AND VACCINE RESEARCH

Immunogenicity of HHP-treated viral agents. The exploration of HHP for vaccine development dates back to at least 1956, with inactivation of poliomyelitis virus (Lansing strain) with some retention of immunogenicity (5). During this period, vaccines against polio were being developed by conventional means, and a global eradication effort ensued years later. Interest in the potential use of HHP for vaccine development appeared to be revived in the early 1990s, with new research studies published by Silva and colleagues (69). More than a dozen different disease agents have been evaluated for pressure susceptibility and subsequent retention of immunogenicity (Table 1).

The majority of HHP vaccine development research effort has been dedicated to viruses. Several of these studies have focused on diseases of livestock, including bovine rotavirus (63), foot-and-mouth disease virus (FMDV) (36), infectious bursal disease virus (IBDV) of poultry (72, 73), vesicular stomatitis virus (VSV) (18, 69), and rift valley fever virus (59). While vaccines exist for many of these viral diseases, limitations reported include incomplete chemical inactivation, changes in immunogenic properties (36), variable efficacy, and the challenges in staying current amidst viral mutability (24, 72). HHP development of vaccines against human viral diseases has also been explored,

such as coxsackievirus group B (CVB) (14), Mayaro virus (22), and yellow fever virus (25). While these may not be associated with handling or consumption of food, there is much to be learned from these studies on HHP effects on viral particles. Collectively, the viruses studied (Table 1) represent seven different RNA virus families, both enveloped and nonenveloped, and with different capsid structures (icosahedral and helical).

HHP inactivation of many of the aforementioned viruses is achieved at pressures somewhat at the low end of the capabilities of commercial equipment used for food processing. These include pressures in the range of 140 to 310 MPa; however, the treatment times have ranged from 30 min to 8 h (Table 1) for inactivation of 4-log (69) to 7-log (22, 36, 67) titers. In several cases (IBDV (72, 73), FMDV (36), CVB (14), and yellow fever virus (25)), the temperatures utilized are at or below 16°C, or required the addition of another agent such as urea for the inactivation of FMDV (36). Pressure susceptibility of the viruses was evaluated by cell culture infectivity assays (22, 25, 36), mouse assay (14), or the natural host (72).

The majority of these pressure-inactivated virus particles were screened for immunogenicity by their ability to elicit neutralizing antibodies in animal models. Pressure-treated bovine rotavirus (63), CVB (14), FMDV (36), Mayaro virus (22), and simian rotavirus (62, 63) were demonstrated to elicit neutralizing serum antibodies as well as the native, untreated virus particles. For example, in one study, pressure-treated (250 MPa at -15°C, with 1 M urea) FMDV (1 µg) was mixed with Freund's incomplete adjuvant and administered weekly to rabbits in three inoculations. The rabbits were bled on the fourth week and the sera collected. Dilutions of heat-inactivated sera were mixed with 10² PFU FMDV for 1 h, and virus-sera mixtures were then evaluated for infectivity in baby hamster kidney cells by plaque assay. The results obtained with pressure-treated FMDV were compared to that of the native, non-pressure-treated FMDV and found to be comparable (36). VSV immunogenicity was similarly determined by the production of neutralizing antibodies in rabbits, and the researchers further noted that the rabbits exhibited no side effects when administered pressure-treated VSV (69).

Three studies with pressure-treated viruses evaluated the retention of immunogenicity by challenge studies in animal models (14, 25) or the natural host (72, 73). Animals were inoculated with pressure-treated virus particles and subsequently challenged with non-pressure-treated virus to determine protective effects of such vaccination. Mice vaccinated with three inoculations of pressure-treated yellow fever virus survived challenge at least 21 days with a lethal dose of untreated virus (25), and 67% of mice vaccinated with pressure-treated CVB survived challenge with a normally fatal infectious dose of CVB (14). Tian and collaborators (72, 73) evaluated IBDV in chickens, the natural host. Pressure-treated IBDV (230 MPa at 0°C for 2 h) was not infective to chickens, and chickens subsequently challenged with infectious IBDV exhibited no disease symptoms.

A number of techniques have been utilized to evaluate

TABLE 1. High-pressure effects on pathogenicity and immunoreactivity

Pathogen	Host	Symptoms	Management	Treatment	Findings	Reference(s)
Bovine rotavirus	Cattle	Gastroenteritis	Vaccination	250 MPa, 1 h	10 ⁵ decrease in infectivity; elicited neutralizing antibodies as well as native particle	63
CVB	Human	Flulike		230 MPa, -16°C	Induced CBV-specific serum antibody as low as 1:1,500; 67% mice vaccinated by HHP virus survived challenge	14
FMDV	Cloven-hoofed animals	Mouth and foot blisters, weight loss, decreased milk production	Vaccination	200 MPa, 1 M urea, -15°C	7-log reduction infectivity in cell culture; elicited neutralizing antibodies in rabbits as well as native particle; capsid integrity appeared maintained	36
IBDV	Chicken	Immunosuppression	Vaccination	180 MPa, 0°C, 3 h; or 230 MPa, 0°C, 2 h	No infectivity (5-log reduction in 50% tissue culture infective dose in chicken fibroblast or chickens); no disease symptoms in chickens upon challenge; serum antibody against native IBDV detected 7 days after immunization by HHP IBDV; altered morphology, protein dissociation proposed	72, 73
Mayaro virus	Human (zoonotic, mosquito-borne)	Fever, pain, gastrointestinal disruption		250 MPa, 8 h	Seven-fold decrease in titer (PFU/ml); elicited neutralizing antibodies in rabbits as native particle; conformation of glycoproteins, fusion-active state	22
Poliomyelitis	Human	Paralysis	Vaccination	980 MPa	Immunogenicity partially retained	5
Rift Valley fever virus	Livestock (zoonotic)	Abortion	Vaccination	250 MPa, 30 min, 25°C	4-log titer decrease in a mouse model; envelope deterioration and surface bulges	59
Simian rotavirus	Simians	Gastroenteritis		250 MPa, 30 or 60 min	4- or 6-log decrease in infectivity with 30 or 60 min, respectively; elicited neutralizing antibodies as well as native particle; perturbation of VP4 hemagglutinin protein; discontinuity in outer capsid but RNA not exposed	62, 63
Yellow fever virus	Human	Hemorrhagic fever	Vaccination	310 MPa, 4°C, 3 h	7-log reduction in infectivity in Vero cells, no disease or mortality in mouse assay; immunized mice survived challenge	25
VSV	Insect and mammals			260 MPa, 12 h	4-log reduction infectivity from initial 10 ⁸ PFU/ml; size reduction, irregular shells; capsid proteins irreversibly altered; HHP VSV attaches to host but not internalized	18, 69
<i>Leptospira interrogans</i> ser. hardjo	Cattle	Stillbirth, weak calves, mastitis, lack of milk	Vaccination	200 MPa, 60 min; 160 MPa, 120 min	No mobility or viability (7-log reduction); elevated agglutinating antibody titers in rabbits; outer membrane, axial filament displaced, helical structure altered	67
<i>Eimeria acervulina</i>	Chicken	Intestinal lesions, reduced weight gain	Vaccination (layers), anticoccidial drugs (broilers)	550 MPa, 2 min, 40°C	No disease symptoms in chickens or fecal oocyst shedding (10 ⁵ dose of HHP-treated oocysts); vaccinated and challenged chickens partially protected, reduced lesion severity; sporozoite morphology and recovery affected	66
Hemolysin BL	Humans	Diarrhea		800 MPa, 30 min, 30°C	Slightly reduced cytotoxicity in Vero cells; increased reactivity in enzyme immunoassay	49
<i>Bacillus cereus</i>						
Mammalian tumor cells	Humans	Tumor development and growth	Tumor excision, chemotherapy, radiation	>300 MPa	Induced cell death by necrosis, completely inactivated cells; HHP generates gelatinized mixture of antigen; immunization elicits antibody response; HHP avoids generation of Maillard products and disintegration and lysis of cells	42

the mechanistic effects of HHP on viruses that would be sufficient to disrupt infectivity but not disrupt interactions or structures needed to induce an immune response. The capsid is believed to carry important epitopes, and so a number of studies evaluated capsid integrity of pressure-treated virus particles. Electron microscopy was used to observe pressure effects on the morphologies of VSV (69), rotavirus SA11-4S (62), and IBDV (72, 73). Silva and colleagues (69) reported that the membranes were intact, but bulges were visible on surfaces of pressure-treated VSV. The general morphology of rotavirus SA11-4S was unaltered by pressure (250 MPa for 1 h), but a discontinuity in the outer capsid suggested pressure treatment caused the outer shell to partially detach from the inner shells (62). The integrity of the rotavirus inner shells, however, appeared to be intact, as viral RNA was not more susceptible to RNase as a result of pressure treatment (62). The morphology of pressure-treated IBDV appeared altered and with a broken appearance under electron microscopy (73). Polyacrylamide gel electrophoresis of trypsin-digested IBDV provided evidence for pressure-induced, irreversible changes in capsid proteins of IBDV (72). The similarity between sedimentation patterns of radiolabeled, pressure-treated, and native FMDV suggested capsid integrity was not compromised by pressure treatment (36). Fluorescence microscopy has been utilized to evaluate whether pressure induces conformational changes in viral proteins by probing with bis(8-anilinoanthracene-1-sulfonate) (bis-ANS). A change in fluorescence was observed with FMDV during pressurization; however, the effect was reversed when pressure was released, also suggesting the capsid structure was maintained (36). Changes in fluorescence intensity occurred with pressure-treated VSV (69), and these changes were attributed to dissociation of protein subunits, or dissociation of protein and nucleic acids, which allowed for increased binding sites for the fluorescent probe. Changes in fluorescence with Mayaro virus were attributed to partially irreversible and increased exposure of hydrophobic domains (22). Exposure of hydrophobic regions of surface proteins occurs with some enveloped viruses during fusion of virus with host cell membranes (26). Fluorescence resonance energy transfer studies with pressure-treated Mayaro virus further showed an increased ability for fusion between virus particles and liposomes (22). It has been postulated that HHP induces a stable, fusion-active structural state in some viruses (22, 26, 56, 62, 70) similar to that which occurs when viruses bind to cells, but with simultaneous particle inactivation such that infectious cycles cannot be completed. Studies with pressure-inactivated VSV revealed that VSV can attach to host cell membranes, but is not internalized, a step which may be important for evoking a full immune response (18). Pontes et al. (62) reported that pressure treatment resulted in a loss of hemagglutinating activity of rotavirus due to mild perturbations of the VP4 hemagglutinin protein, a protein believed to be important in a number of virus–host cell interactions including attachment. Pressure-induced changes in virus particles are presumed to be fairly subtle and indicative of specific conformational

changes that disrupt infectivity, but not presentation and recognition of immunogenic properties.

Immunogenicity of HHP-treated nonviral agents.

Fewer studies have been conducted on inactivation and subsequent immunogenicity of nonviral agents. One report by Silva et al. (67) investigated the effect of pressure for inactivation of the bacterium *Leptospira interrogans* ser. *hardjo* and its subsequent immunogenicity. The Hardjoprajitno strain of *L. interrogans* ser. *hardjo* was treated by pressure and evaluated for mobility, viability, structural changes, and immunogenicity. As observed with dark-field microscopy, the leptospire were rendered immobile by 2 kbar (200 MPa) of pressure applied for 60 min or 1.6 kbar (160 MPa) of pressure for 120 min. A lack of growth in media served as an indicator that these treatments also inactivated the leptospire. Intraperitoneal inoculation of rabbits and antibody titer of sera indicated that the pressure-treated leptospire were immunogenic. Electron microscopy revealed that pressure-treated leptospire had displaced outer membranes and axial filaments, and produced loss of helical shape. Studies with binding of the fluorescent probe, bis-ANS, indicated that pressure-treated leptospire showed increased fluorescent intensity, compared with untreated leptospire. The authors suggested that increased binding of bis-ANS is indicative of conformational changes that improve accessibility of the fluorescent probe to hydrophobic regions, and they hypothesized that changes in membrane proteins could explain this observation.

The use of HHP for potential vaccine preparation has been reported with one parasite (66). *Eimeria acervulina* is a poultry protozoan parasite and is closely related to the human parasite *Cyclospora cayentanensis*. *Eimeria* causes coccidiosis, which is characterized by intestinal lesions, compromised weight gain, and mortality in severe cases (51). Vaccines are available, but broilers are often protected by the use of anticoccidial drugs, and resistance to anticoccidials has been reported (13). *E. acervulina* oocysts can be rendered nonpathogenic to chickens with 2-min pressure treatments at 550 MPa at 40°C. Chickens that received a primary oral dose of HHP-treated *E. acervulina* and were then challenged with virulent *E. acervulina* oocysts exhibited disease symptoms less severe, in that lesions of the intestinal tract were dramatically reduced as compared with birds that did not receive a primary inoculation. The protection was not complete, however, as the chickens still shed oocysts and had suppressed weight gain. The mechanism of inactivation is not completely clear, but damage to the internal sporozoite was proposed. HHP-induced damage to the oocyst and sporocyst structures was not detected by the methods employed (microscopy, permeability, excystation, mechanical fragility); however, recovery and morphology of sporozoites from pressure-treated oocysts appeared to be altered after in vitro treatment that simulated the intestinal environment.

Margosch and collaborators (49) conducted a study on the effects of HHP on heat-stable bacterial toxins. While the aim of the study was not to evaluate the potential of HHP for vaccine development, they conducted an experi-

ment that may provide some early insight on the effect of HHP on bacterial toxin cytotoxicity and immunoreactivity. *Bacillus cereus* was treated at 800 MPa for 30 min at 30°C, and the supernatant was tested for reactivity, using an enzyme immunoassay, and cytotoxicity was evaluated with Vero cells. The treatment yielded a slight decrease in cytotoxicity, but an increase in reactivity was seen in the enzyme immunoassay over untreated controls. The authors proposed one possible explanation for this finding was that HHP dissociated the hemolysin BL complex and permitted greater accessibility of the L2 protein component to react with antibodies. This study also demonstrated a lack of correlation between heat stability and barotolerance of bacterial toxins tested.

Animals reared for human food have an abbreviated life span, and consequently, tumor formation is not normally a concern, with the exception of those caused by viral infections such as in the case of Marek's disease of poultry. One study on the effects of pressure on tumor cells demonstrated that pressures above 300 MPa induced tumor cell death and prevented replication (42). Mice were immunized with HHP-treated tumor cells (Raji cell line) and an antibody-antitumor response was found to be comparable to that of mice immunized with live Raji tumor cells. These findings suggest HHP technology may have potential application for development of a cancer vaccine.

As previously described, a considerable number of other viruses (29, 35, 40), bacteria (58), and parasites (45, 71) important for food systems have been shown to be sensitive to HHP, but their immunogenicity after treatment has yet to be evaluated. Pressure susceptibility cannot necessarily be predicted based solely on morphological or other similarities among microbes (29), and there are insufficient data to suggest retention of immunogenic properties occurs uniformly with pressure susceptibility. The use of HHP alone or in combination with other stresses such as UV irradiation and elevated temperatures as a potential strategy for vaccine preparation against simian immunodeficiency virus (38), classical swine fever virus (23), and *B. anthracis* spores (17), respectively, has also been proposed but has not been tested.

HHP-DERIVED VACCINES: POTENTIAL ADVANTAGES AND CONSIDERATIONS

HHP-derived vaccines could have, in theory, many of the advantages of current vaccines with simultaneous reduction of some of their disadvantages. Complete inactivation of a cell or particle by HHP eliminates the concerns of reversion to a virulent state, as with live preparations, so with regard to this issue, the safety of an HHP-derived vaccine should hypothetically be comparable to other inactivated vaccines. However, one of the challenges with development of vaccines, particularly nonlive vaccines, whether inactivated or subunit, is invoking a complete and lasting immune response. For viruses, the whole, intact particle may be necessary for greatest efficacy (69). Pressure appears to inactivate some enveloped viruses such that the full infection cycle is disrupted while the pressure simultaneously induces them into a fusion-active state, a state in

which conformation of the virus surface envelope proteins fuse with host cell membranes (22, 26). The combination of these features suggests that HHP-derived vaccines against certain viral diseases could not only be safe as inactivated vaccines, but also possibly competent as whole live, attenuated preparations. Pressure treatment may even enhance the immune response if antigen exposure is increased by rearrangements in large molecules that would otherwise block antigen accessibility (68). Less work has been done with bacteria or parasites, although a partial immune response observed with pressure-treated *E. acervulina* may suggest that a structure or interaction critical for a full immune response is altered by pressure (66); however, other pressure-treatment conditions, the timing and/or dosing of the pressure-treated protozoa, or addition of adjuvants as possible means to improve the immune response have not yet been explored.

For disease agents inactivated by pressure without the addition of other chemical agents, the concerns of environmental impact and hazards in handling are also reduced. Although HHP equipment may currently have relatively higher maintenance needs, HHP may be considered an environmentally friendly processing method as an alternative for typical thermal treatment of foods because of its potential for reduced waste and energy consumption (74). It is noteworthy that some of the HHP conditions demonstrated to inactivate pathogens with retention of immunogenic properties require extreme pressures or temperatures, or long treatment times, conditions that may be more stressful for the regular care of pressure equipment.

HHP can be applied as a terminal production step in food processing; that is, the product can be packaged and processed in a hermetically sealed, flexible container, preventing subsequent contamination. For food systems, the benefit is in the reduced need for pretreatment of aseptic containers or steps to prevent postprocessing contamination prior to packaging (74), as well as reduced reliance on other preservation strategies. For vaccine preparation and packaging, this feature may reduce reliance on other preservatives used for current vaccines, provided the preparation is adequately pure from contaminants, or it can be verified that any potential contaminants would be destroyed during pressurization. Such potential contaminants may include remnants of cell lines used to propagate viruses, chemicals of biological source used to recover microbes, as well as toxins associated with microorganisms. Additionally, since passage through other hosts to reduce virulence would not be necessary, the risk of potentially allergenic remnants should be reduced. Packages for vaccines prepared by HHP would need to be designed in consideration of pressure stability, transport and storage conditions, technological features readily demonstrated possible with food systems, as well as sized appropriately for dosing (possibly single dosing, depending on the host to be immunized and delivery method), with means to withdraw aseptically the vaccine from the package. However, multidose preparations would be more likely to require formulations more similar to conventionally prepared vaccines to protect from accidental contamination. Because pressure does not typically affect

covalent structures when applied at ambient temperatures, it would not be expected to create new hazards via the breakdown or synthesis of new compounds; however, pressure can reportedly degrade aspartame (6) and enhance some enzyme-catalyzed reactions. The effects of HHP on other compounds have not been exhaustively studied. Thus, toxicology studies for new preparations would be warranted for pressure-derived vaccines as much as for vaccines derived by conventional methods.

Some of the same issues encountered with other methods of vaccine preparation would need to be considered with HHP. As previously mentioned, pressure resistance varies between related populations and even within pure populations. The selection of barotolerant bacteria with repeated pressure treatments has been reported and proposed to result from an accumulation of multiple mutations (32). Additionally, pressure stabilization of microorganisms to heat (48) and pressure-induced sublethal injury with subsequent recovery have been reported (47). Prior stresses can increase barotolerance as well (47). Therefore, HHP conditions previously demonstrated to inactivate pathogens must be verified (considering reversible injury), and stock cultures used to prepare vaccine preparations need to be handled to prevent selection for increased pressure resistance or loss of immunogenic properties. Natural mutability would remain an issue, and vaccines derived by any method would need to be evaluated against variants with potentially new virulence traits. For pathogens that may require some early stages of attachment or infection without propagation for a complete immune response, verification that pressure-treated agents retain these properties would be necessary, or an adjuvant might be needed to enhance an otherwise incomplete response.

Studies to date on pressure-derived vaccines have not addressed the issue of differentiation of natural infection from immunization. This distinction should theoretically be possible as long as any modifications do not affect pressure susceptibility, or are altered by pressure to be undetectable serologically.

FUTURE WORK

The lack of infectivity with retention of immunogenicity of HHP-treated pathogens has been determined primarily in cell culture and by the detection of neutralizing antibodies in other hosts (36, 63, 69), or in some cases, the natural host (66, 72, 73). For those studies that have shown promise in model systems, HHP-derived vaccines will need to be challenged in the natural host, where required. As with vaccines developed by any method, efficacy without detrimental side effects will need to be verified.

Numerous other microorganisms have been shown to be vulnerable to HHP inactivation, but have not yet been evaluated for immunogenic properties after pressure treatment. Additionally, uncharted areas of research in the potential of HHP for vaccine development include vaccines against allergens or toxins.

Although variable, the time to develop a new vaccine with existing technologies is estimated at 8 to 10 years, with an estimated average cost of US\$50 million (55). HHP

technology as a means to prepare vaccines is clearly in its exploratory and developmental infancy. Considerable research is needed to evaluate the potential of HHP technology for vaccine development, but theoretical foundations and the results of several studies suggest this may be an exploration worth undertaking. In doing so, there would be additional gains in a better understanding of the effects of pressure on biological molecules and systems, as well as the immunological character of microbes.

ACKNOWLEDGMENT

The authors gratefully acknowledge Dallas G. Hoover, Ph.D., for review of the manuscript.

REFERENCES

1. Anese, M., M. C. Nicoli, G. Dall'aglio, and C. R. Lerici. 1995. Effect of high pressure treatments on peroxidase and polyphenoloxidase activities. *J. Food Biochem.* 18:285–293.
2. Ansotegui, I. J., E. Errigo, S. Bellegrandi, and R. Paganelli. 1996. Treatment of food allergy with low-dose vaccination. *Monogr. Allergy* 32:241–245.
3. Aron, S. S., R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow, and K. Tonat. 2001. Botulinum toxin as a biological weapon. *JAMA* 285:1059–2081.
4. Barrow, P. A. 2007. *Salmonella* infections: immune and non-immune protection with vaccines. *Avian Pathol.* 36:1–13.
5. Basset, J., P. Lépine, and L. Chaumont. 1956. Effets des hautes pressions sur le virus de la poliomyélite (souche Lansing). *Ann. Inst. Pasteur (Paris)* 90:575–593.
6. Butz, P., A. Fernandes, J. Fister, and B. Tauscher. 1997. Influence of high hydrostatic pressure on aspartame: instability at neutral pH. *J. Agric. Food Chem.* 45:302–303.
7. Butz, P., and B. Tauscher. 1995. Inactivation of fruit fly eggs by high pressure treatment. *J. Food Process Preserv.* 19:161–164.
8. Calci, K. R., G. K. Meade, R. C. Tezloff, and D. H. Kingsley. 2005. High-pressure inactivation of hepatitis A virus within oysters. *Appl. Environ. Microbiol.* 71:339–343.
9. Centers for Disease Control and Prevention. 2000. Use of anthrax vaccine in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morb. Mortal. Wkly. Rep.* 49:1–20.
10. Centers for Disease Control and Prevention. 2007. About rotavirus. Available at: http://www.cdc.gov/rotavirus/about_rotavirus.htm. Accessed 23 June 2008.
11. Centers for Disease Control and Prevention. 2007. Ingredients of vaccines—fact sheet. Available at: <http://www.cdc.gov/vaccines/vac-gen/additives.htm>. Accessed 23 June 2008.
12. Centers for Disease Control and Prevention. 2008. Recommended immunization schedules for persons aged 0–18 years—United States, 2008. *Morb. Mortal. Wkly. Rep.* 57:Q1–Q4.
13. Chapman, H. D. 1993. Resistance to anticoccidial drugs in fowl. *Parasitol. Today* 9:159–162.
14. Chen, Z. M., S. M. Tian, and K. C. Ruan. 2001. A vaccine to Coxsackievirus prepared by high pressure. *Chin. J. Biochem. Biophys.* 33:128–130.
15. Chicón, R., J. Belloque, E. Alonso, and R. Lopez-Fandiño. 2008. Antibody binding and functional properties of whey protein hydrolysates obtained under high pressure. *Food Hydrocolloid.* DOI: 0.1016/j.foodhyd.2008.04.001.
16. Clark, J. P. 2006. High-pressure processing research continues. *Food Technol. (Chicago)* 60:63–65.
17. Cléry-Barraud, C., A. Gaubert, P. Masson, and D. Vidal. 2004. Combined effects of high hydrostatic pressure and temperature for inactivation of *Bacillus anthracis* spores. *Appl. Environ. Microbiol.* 70:635–637.
18. Da Poian, A., A. M. O. Gomes, R. J. N. Oliveira, and J. L. Silva.

1996. Migration of vesicular stomatitis virus glycoprotein to the nucleus of infected cells. *Proc. Natl. Acad. Sci. USA* 93:8268–8273.
19. Dorner, F., and P. N. Barrett. 1999. Vaccine technology: looking to the future. *Ann. Med.* 31:51–60.
20. Douzals, J. P., P. A. Marechal, J. C. Coquille, and P. Gervais. 1996. Microscopic study of starch gelatinization under high hydrostatic pressure. *J. Agric. Food Chem.* 44:1403–1408.
21. Floyd, J. G., Jr. 1996. Vaccinations for the swine herd. Alabama Cooperative Extension System ANR-902. Alabama A & M and Auburn Universities. Available at: <http://www.aces.edu/pubs/docs/A/ANR-0902/ANR-0902.pdf?PHPSESSID=712546876606ea02cdc033d8a197a09c>. Accessed 20 June 2008.
22. Freitas, M. S., A. T. DaPoian, O. M. Barth, M. A. Rebello, J. L. Silva, and L. P. Gaspar. 2006. The fusogenic state of Mayaro virus induced by low pH and by hydrostatic pressure. *Cell Biochem. Biophys.* 44:325–335.
23. Freitas, T. R. P., L. P. Gaspar, L. A. Caldas, J. L. Silva, and M. A. Rebello. 2003. Inactivation of classical swine fever virus: association of hydrostatic pressure and ultraviolet irradiation. *J. Virol. Methods* 108:205–211.
24. Fussell, L. W. 1998. Poultry industry strategies for control of immunosuppressive diseases. *Poultry Sci.* 77:1193–1196.
25. Gaspar, L. P., Y. S. Mendes, A. M. Y. Yamamura, L. F. C. Almeida, E. Caride, R. B. Gonçalves, J. L. Silva, A. C. Oliveira, R. Galler, and M. S. Freire. 2008. Pressure-inactivated yellow fever 17DD virus: implications for vaccine development. *J. Virol. Methods* 150: 57–62.
26. Gaspar, L. P., A. C. B. Silva, A. M. O. Gomes, M. S. Freitas, A. P. D. Ano Bom, W. D. Schwarcz, J. Mestecky, M. J. Novak, D. Foguel, and J. L. Silva. 2002. Hydrostatic pressure induces the fusion-active state of enveloped viruses. *J. Biol. Chem.* 277:8433–8439.
27. Greiner, S. P. 2003. Sheep management schedule. Publication 410-365 of Virginia Cooperative Extension. Available at: <http://www.ext.vt.edu/pubs/sheep/410-365/410-365.pdf>. Accessed 23 June 2008.
28. Grimm, V., M. Scheibenzuber, J. Rakoski, H. Behrendt, G. Bluemelhuber, R. Meyer-Pittroff, and J. Ring. 2002. Ultra-high pressure treatment of foods in the prevention of food allergy. *Allergy* 57(Suppl.):101.
29. Grove, S. F., A. Lee, T. Lewis, C. M. Stewart, H. Chen, and D. G. Hoover. 2006. Inactivation of foodborne viruses of significance by high pressure and other processes. *J. Food Prot.* 69:957–968.
30. Han, G. D., M. Matsuno, Y. Ikeuchi, and A. Suzuki. 2002. Effects of heat and high-pressure treatments on antigenicity of beef extract. *Biosci. Biotechnol. Biochem.* 66:202–205.
31. Harris, C. 2005. The big squeeze. *Meat Process. (N. Am.)* October.
32. Hauben, K. J. A., D. H. Bartlett, C. C. F. Soontjens, K. Cornelis, E. Y. Wuytack, and C. W. Michiels. 1997. *Escherichia coli* mutants resistant to inactivation by high hydrostatic pressure. *Appl. Environ. Microbiol.* 63:945–950.
33. Hoover, D. G., C. Metrick, A. M. Papineau, D. F. Farkas, and D. Knorr. 1989. Biological effects of high hydrostatic pressure on food microorganisms. *Food Technol. (Chicago)* 43:99–107.
34. Horst, H. S., C. J. de Vos, F. H. M. Tomassen, and J. Stelwagen. 1999. The economic evaluation of control and eradication of epidemic livestock diseases. *Rev. Off. Int. Epizoot.* 18:367–379.
35. Isbarn, S., R. Buckow, A. Himmelreich, A. Lehmacher, and V. Heinz. 2007. Inactivation of avian influenza virus by heat and high hydrostatic pressure. *J. Food Prot.* 70:667–673.
36. Ishimaru, D., D. Sá-Carvalho, and J. L. Silva. 2004. Pressure-inactivated FMDV: a potential vaccine. *Vaccine* 22:2334–2339.
37. Jankiewicz, A., W. Baltes, K. W. Bögl, L. I. Dehne, A. Jamin, A. Hoffmann, D. Haustein, and S. Vieths. 1997. Influence of food processing on the immunochemical stability of celery allergens. *J. Sci. Food Agric.* 75:359–370.
38. Jurkiewicz, E., M. Villas-Boas, J. L. Silva, G. Weber, G. Hunsmann, and R. M. Clegg. 1995. Inactivation of simian immunodeficiency virus by hydrostatic pressure. *Proc. Natl. Acad. Sci. USA* 92:6935–6937.
39. Kato, T., E. Katayama, S. Matsubara, Y. Omi, and T. Matsuda. 2000. Release of allergenic proteins from rice grains induced by high hydrostatic pressure. *J. Agric. Food Chem.* 48:3124–3129.
40. Kingsley, D. H., D. G. Hoover, E. Papafragkou, and G. P. Richards. 2002. Inactivation of hepatitis A virus and a calicivirus by high hydrostatic pressure. *J. Food Prot.* 65:1605–1609.
41. Knorr, D. 2002. High pressure processing for preservation, modification and transformation of foods. *High Pressure Res.* 22:595–599.
42. Korn, A., B. Frey, A. Sheriff, U. S. Gaipl, F. Franz, R. Meyer-Pittroff, G. Bluemelhuber, and M. Herrmann. 2004. High hydrostatic pressure inactivated human tumour cells preserve their immunogenicity. *Cell. Mol. Biol.* 50:469–77.
43. Kumeno, K., N. Nakahama, K. Honma, T. Makino, and M. Watanabe. 1993. Production and characterization of a pressure-induced gel from freeze-concentrated milk. *Biosci. Biotechnol. Biochem.* 57: 750–752.
44. Liljeqvist, S., and S. Ståhl. 1999. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *J. Biotechnol.* 73:1–33.
45. Lindsay, D. S., M. V. Collins, D. Holliman, G. J. Flick, and J. P. Dubey. 2006. Effects of high-pressure processing on *Toxoplasma gondii* tissue cysts in ground pork. *J. Parasitol.* 92: 195–196.
46. Lubroth, J., M. M. Rweyemamu, G. Viljoen, A. Diallo, B. Dungu, and W. Amanfu. 2007. Veterinary vaccines and their use in developing countries. *Rev. Off. Int. Epizoot.* 26:179–201.
47. Mañas, P., and R. Pagán. 2005. Microbial inactivation by new technologies of food preservation. *J. Appl. Microbiol.* 98:1387–1399.
48. Margosch, D., M. A. Ehrmann, R. Buckow, V. Heinz, R. F. Vogel, and M. G. Gänzle. 2006. High-pressure-mediated survival of *Clostridium botulinum* and *Bacillus amyloliquefaciens* endospores at high temperature. *Appl. Environ. Microbiol.* 72:3476–3481.
49. Margosch, D., M. Moravek, M. G. Gänzle, E. Märklbauer, R. F. Vogel, and M. A. Ehrmann. 2005. Effect of high pressure and heat on bacterial toxins. *Food Technol. Biotechnol.* 43:211–217.
50. Masson, P., and C. Tonello. 2000. Potential applications of high pressures in pharmaceutical science and medicine. *High Pressure Res.* 19:223–231.
51. McDougald, L. R., and W. M. Reid. 1991. Coccidiosis, p. 780–797. In B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, Jr. (ed.), *Diseases of poultry*, 9th ed. Iowa State University Press, Ames.
52. McNeilly, T. N., S. W. Naylor, A. Mahajan, M. C. Mitchell, S. McAteer, D. Deane, D. G. F. Smith, J. C. Low, D. L. Gally, and J. F. Huntley. 2008. *Escherichia coli* O157:H7 Colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. *Infect. Immunol.* 76:2594–2602.
53. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–636.
54. Meunier, R. A., and M. A. Latour. Commercial egg production and processing. AS-545-W. Available at: <http://ag.ansc.purdue.edu/poultry/publication/commegg/>. Accessed 20 June 2008.
55. O'Brien, D., and S. Zanker. 2007. Animal vaccination and the veterinary pharmaceutical industry. *Rev. Off. Int. Epizoot.* 26:471–477.
56. Oliveira, A. C., A. P. Valente, F. C. L. Almeida, S. M. B. Lima, D. Ishimaru, R. B. Gonçalves, D. Peabody, D. Foguel, and J. L. Silva. 1999. Hydrostatic pressure as a tool to study virus assembly: pressure-inactivation of viruses by formation of fusion intermediate states, p. 497–513. In R. Winter and J. Jonas (ed.), *High pressure molecular science*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
57. Pastoret, P.-P., M. Lombard, A. A. Schudel, J. Plana-Durán, and A. Wennberg. 2007. Introduction: animal vaccination. Part 1: development, production, and use of vaccines. Part 2: scientific, economic, regulatory and socio-ethical issues. *Rev. Off. Int. Epizoot.* 26:17–28.
58. Patterson, M. F. 2005. Microbiology of pressure-treated foods. *J. Appl. Microbiol.* 98:1400–1409.
59. Perche, P. Y., C. Clery, M. Bouloy, M. F. Burckhart, P. Masson, and P. Michel. 1997. Study of inactivation and immunogenicity of rift valley fever virus type clone 13 treated by high hydrostatic pressure. *Am. J. Trop. Med. Hyg.* 57:256–257.

60. Peterson, R. E., T. J. Klopfenstein, R. A. Moxley, G. E. Erickson, S. Hinkley, G. Bretschneider, E. M. Berberov, D. Rogan, and D. R. Smith. 2007. Effect of a vaccine product containing type III secreted proteins on the probability of *Escherichia coli* O157:H7 fecal shedding and mucosal colonization in feedlot cattle. *J. Food Prot.* 70: 2568–2577.
61. Peterson, R. E., T. J. Klopfenstein, R. A. Moxley, G. E. Erickson, S. Hinkley, D. Rogan, and D. R. Smith. 2007. Efficacy of dose regimen and observation of herd immunity from a vaccine against *Escherichia coli* O157:H7 for feedlot cattle. *J. Food Prot.* 70:2561–2567.
62. Pontes, L., Y. Cordeiro, V. Giongo, M. Villas-Boas, A. Barreto, J. R. Araujo, and J. L. Silva. 2001. Pressure-induced formation of inactive triple-shelled rotavirus particles is associated with changes in the spike protein VP4. *J. Mol. Biol.* 30:1171–1179.
63. Pontes, L., L. A. Fornells, V. Giongo, J. R. V. Araujo, A. Sepulveda, M. Villas-Boas, C. F. S. Bonafe, and J. L. Silva. 1997. Pressure inactivation of animal viruses: potential biotechnological applications, p. 91–94. In *High pressure research in the biosciences and biotechnology*. Leuven University Press, Leuven, Belgium.
64. Powell, J., S. Jones, S. Gadberry, and T. Troxel. Beef cattle herd health vaccination schedule. University of Arkansas Cooperative Extension Service. Available at: http://www.uaex.edu/other_Areas/publications/PDF/FSA-3009.pdf. Accessed 20 June 2008.
65. Scarselli, M., M. M. Giuliani, J. Adu-Bobie, M. Pizza, and R. Rappuoli. 2005. The impact of genomics on vaccine design. *Trends Biotechnol.* 23:84–91.
66. Shearer, A. E. H., G. C. Wilkins, M. Jenkins, and K. E. Knier. 2007. Effects of high hydrostatic pressure on *Eimeria acervulina* pathogenicity, immunogenicity and structural integrity. *Innov. Food Sci. Emerg. Technol.* 8:259–268.
67. Silva, C. C. M., V. Giongo, A. J. G. Simpson, E. R. S. Camargos, J. L. Silva, and M. C. Koury. 2001. Effects of hydrostatic pressure on the *Leptospira interrogans*: high immunogenicity of the pressure-inactivated serovar *hardjo*. *Vaccine* 19:1511–1514.
68. Silva, J. L. 1993. Effects of pressure on large multimeric proteins and viruses, p. 561–578. In R. Winter and J. Jonas (ed.), *High pressure chemistry, biochemistry, and materials science*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
69. Silva, J. L., P. Luan, M. Glaser, E. W. Voss, and G. Weber. 1992. Effects of hydrostatic pressure on a membrane-enveloped virus: high immunogenicity of the pressure-inactivated virus. *J. Virol.* 66:2111–2117.
70. Silva, J. L., A. C. Oliveira, A. M. O. Gomes, L. M. T. R. Lima, R. Mohana-Borges, A. B. F. Pacheco, and D. Foguel. 2002. Pressure induces folding intermediates that are crucial for protein-DNA recognition and virus assembly. *Biochim. Biophys. Acta* 1595:250–265.
71. Slifko, T. R., E. Raghubeer, and J. B. Rose. 2000. Effect of high hydrostatic pressure on *Cryptosporidium parvum* infectivity. *J. Food Prot.* 63:1262–1267.
72. Tian, S.-M., J. F. Qian, G. Q. Shao, and K. C. Ruan. 1999. High immunogenicity of the pressure-inactivated virus. *Acta Biochim. Biophys. Sin. (Shanghai)* 31:334–336.
73. Tian, S.-M., K.-C. Ruan, J.-F. Qian, G.-Q. Shao, and C. Balny. 2000. Effects of hydrostatic pressure on the structure and biological activity of infectious bursal disease virus. *Eur. J. Biochem.* 267:4486–4494.
74. Toepfl, S., A. Mathys, V. Heinze, and D. Knorr. 2006. Review: potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. *Food Rev. Int.* 22:405–423.
75. Torres, J. A., and G. Velazquez. 2005. Commercial opportunities and research challenges in the high pressure processing of foods. *J. Food Eng.* 67:95–112.
76. U.S. Department of Agriculture, Agricultural Research Service. 2005. Action Plan: National Program 103—animal health. Available at: http://www.ars.usda.gov/research/programs/programs.htm?np_code=103&docid=15595. Accessed 17 June 2008.
77. U.S. Department of Agriculture, Animal and Plant Health Inspection Service. 2006. Oral rabies vaccination program in the East factsheet. Available at: http://www.aphis.usda.gov/publications/wildlife_damage/content/printable.version/fs_oralrabies_vacc_east_ws.pdf. Accessed 20 June 2008.
78. U.S. Food and Drug Administration. 2008. FDA approves new vaccine to prevent gastroenteritis caused by rotavirus. FDA news release, 3 April 2008. Available at: <http://www.fda.gov/bbs/topics/news/2008/new01814.html>. Accessed 23 June 2008.
79. van Oirschot, J. T. 1999. Diva vaccines that reduce virus transmission. *J. Biotechnol.* 73:195–205.
80. van Oirschot, J. T., M. J. Kaashoek, R. A. M. Rijsewijk, and J. A. Stegeman. 1996. The use of marker vaccines in eradication of herpesviruses. *J. Biotechnol.* 44:75–81.
81. Viswanathan, S. 2008. Poultry workers carry resistant bacteria. *Food Qual.* February/March:12–13.
82. Waldner, D. N., J. Kirkpatrick, and T. W. Lehenbauer. Edited 2007. Recommended vaccination schedules for a comprehensive dairy herd health program. Oklahoma Cooperative Extension Service fact sheet ANSI-4354. Oklahoma State University, Stillwater. Available at: <http://osufacts.okstate.edu>. Accessed 20 June 2008.
83. World Health Organization. 2006. New vaccines against infectious diseases: research and development status. Available at: http://www.who.int/vaccine_research/documents/en/Status_Table.pdf. Accessed 23 June 2008.
84. Wyoming Brucellosis Coordination Team. 2005. Report and recommendations. Presented to Governor Dave Freudenthal, Cheyenne, WY, 11 January 2005.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.