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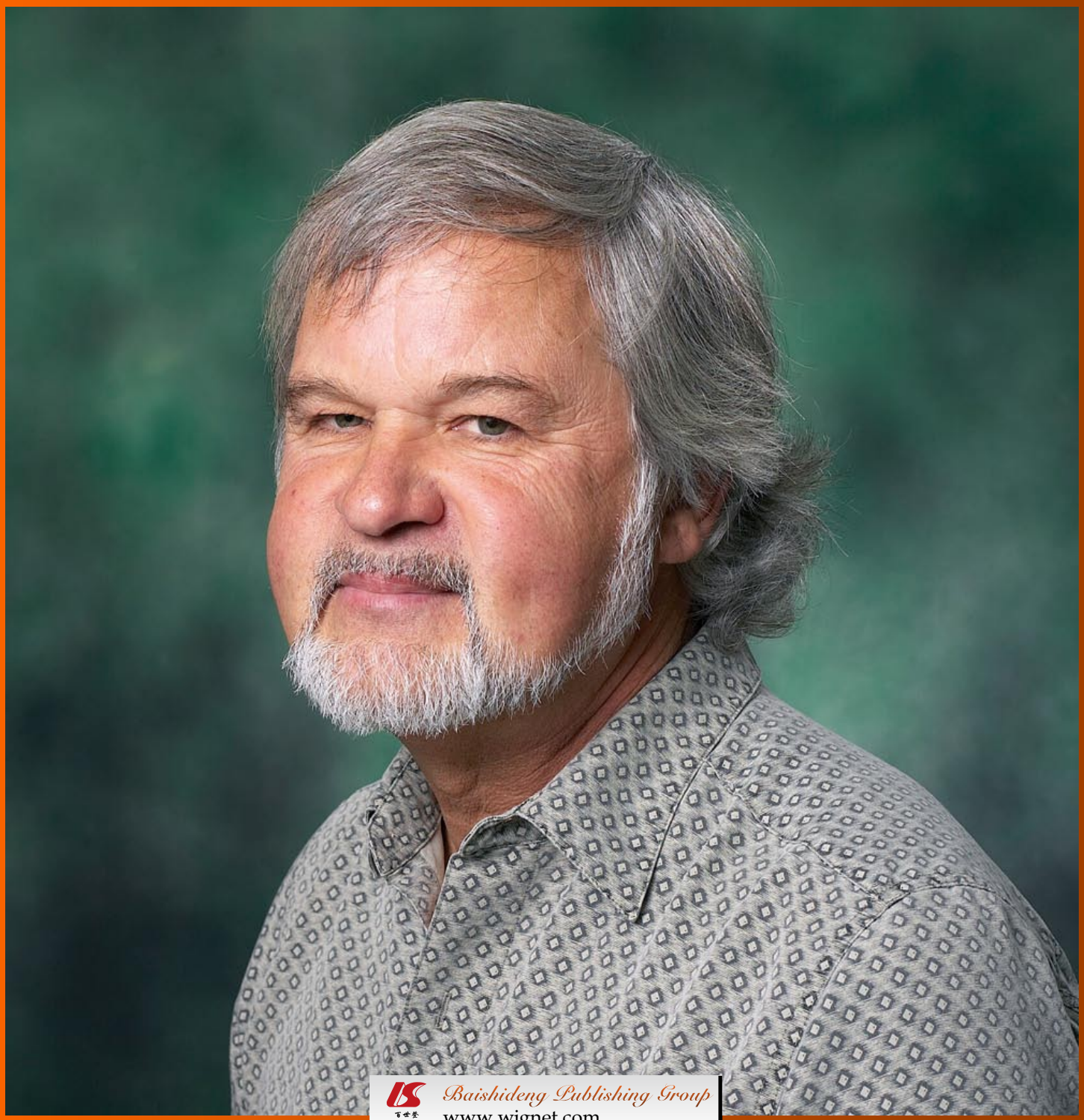

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EDITORIAL

Face up to challenge of virology world

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Figure 1 Editor-in-Chief of *World Journal of Virology*. Xiaoli Lilly Pang, MD, PhD, Associate Professor, 2B4.58 Walter Mackenzie Health Sciences Centre, Provincial Laboratory of Public Health (Microbiology), 8440-112 Street, Edmonton, Alberta T6G 2J2, Canada.

Abstract

Welcome to the *World Journal of Virology* (*WJV*), a new member of the World Journal Series. The World Journal Series was first launched as a peer-reviewed scientific journal covering aspects of research, diagnostics and clinical practice in biomedicine in 1995. *WJV* is an online and open-access peer-reviewed periodical focusing on virology. *WJV* covers a variety of topics in different areas of virology, including advances in basic research, updates in nomenclature, the development of novel diagnostic assays, the epidemiology of viral disorders and, new developments in the clinical management of viral diseases, including new vaccines and antiviral therapeutics. The purpose in launching the *WJV* is to promote knowledge exchange related to the classic human viruses as well as newly emerging viruses and their associated clinical disorders. Continually updating knowledge in a timely manner in this field where information related to the unceasing evolution of viruses is becoming available at a rapid pace is challenging. Thanks to the World-Wide-Web we are able to provide a podium for all authors and readers of *WJV* to address this challenge. I would like to acknowledge the Baishideng publisher, the members of the editorial board, and all contributing authors involved in this inaugural issue of the *WJV*. I sincerely hope all readers, i.e. future contributing authors, will like *WJV* and we look forward to your input in assisting *WJV* to grow and mature.

Key words: Viruses; Viral disorders; Knowledge exchange; Fast-paced communication; Peer-reviewed; Open-access

Peer reviewer: Hsin-Wei Chen, Associate Investigator, Vaccine Research and Development Center, National Health Research Institutes, No. 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan, China

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INTRODUCTION

I am Xiaoli Lilly Pang, MD, PhD, Associate Professor, Department of Laboratory Medicine and Pathology, University of Alberta, and the Provincial Laboratory for Public Health (Microbiology), Alberta, Canada (Figure 1),

and together with Ling Lu, MD, PhD, Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, United States, we will be the co-Editor-in-Chief of the *World Journal of Virology* (*World J Virol*, *WJV*, online ISSN 2220-3249, DOI: 10.5501). We are excited to bring you the inaugural issue of the *WJV*, a bimonthly, peer-reviewed, online, open access journal supported by an editorial board of over 137 specialists in virology research from over 41 countries.

Infectious diseases are a global concern. Virus associated disorders have contributed significantly infectious disease morbidity and mortality in the past and continue to emerge as health threats internationally. From the development of the concept of a virus as a contagious living form by Beijerinck in 1898 to the rapid identification of novel human coronavirus causing outbreaks of a severe acute respiratory syndrome (SARS) by the task forces networking and working collaboratively in 2003, there have been many landmark discoveries in the history of virology for the past 200 years[1]. We anticipate more coming at an unprecedented speed as new research methods are developed and rapid exchange of bioinformation using the world-wide web is now possible[2]. In order to promote new discoveries and knowledge exchange, the *WJV* on which preparation was initiated on November 8, 2010, is launched on February 12, 2012 to facilitate participation in the rapidly evolving and fascinating world of the virology.

Advances on virology have brought many benefits for human and animal health and highlighted the challenges of the future. In this inaugural issue of *WJV*, I would like to address the current need for fast-paced communication and knowledge exchange in virology. One of the remarkable achievements in virology over the past century has been the development and population-based delivery of vaccines against some well-known viruses. Vaccination has eradicated deadly diseases, such as natural smallpox[3]; dramatically lowered the incidence of viral infections such as poliomyelitis[4], prevented transmission and disease progression to the cancer as illustrated by hepatitis B vaccine[5], and has had a significant impact on disease morbidity such as rotavirus associated gastroenteritis in children[6]. Recently, remerging measles outbreaks have raised new uncertainties despite the WHO vaccine campaign against the virus that has been in place for several decades. In 2011, measles outbreaks were reported in 36 of 53 EUR member states; a total of 26 074 measles cases had been reported region-wide[7]. Although incomplete coverage of immunization and underdosing of vaccine are considered to be the major contributing factors, several outbreaks still occurred despite high immunization coverage including administration of double-dose of MMR vaccine[8]. Further studies are required to understand the resurgence of measles across several continents in order to accomplish WHO's new strategic for measles eradication.

Most viruses undergo continuous evolution. A typical example is the genetic drift of norovirus GII.4 strains observed globally. In our recently published study, a unique biannual pattern of norovirus gastroenteritis outbreaks was observed and this trend was clearly associated with emergence of new norovirus GII.4 variants[9]. Each emerging GII.4 genetic variant identified by sequence alteration had high virulence and caused the epidemic outbreaks of gastroenteritis in the susceptible population. The number of outbreaks associated with the newly emerged GII.4 variants increased steadily in the four epidemic years during 8 years of the study. The study provided a basis for predicting when the next norovirus GII.4 variant will possibly appear and what public health measures need to be in place for control of the outbreaks. However, why and where genetic drift in the viral genome of norovirus will occur in such a biannual pattern is largely known. A newly emerged variant of avian influenza virus provided another example of how genetic drift of a virus can allow cross-host infection between birds and humans and caused a deadly disease[10]. Understanding the mechanism of evolution of viruses and the resulting health threats is a key to winning the battle against viruses in future.

Clearly many mysteries and unknowns related to viruses and the diseases they cause remain, regardless of whether they are well-studied viruses or are new emerging viruses, in the fascinating world of virology. Basic, clinical and epidemiologic researches, vaccine and antiviral drug developments, clinical trials of new therapies, education and public awareness, and community and society involvement are encouraged and needed in virology. Resource and information sharing, research networking and collaborative efforts without boards represent new strategies in sciences as well as in virology. A perfect example was a battle against pandemic outbreak of SARS. The time course from diagnosing the first case of SARS in Hong Kong, quarantining outbreak patients in various regions in the world, decoding the full sequences of the novel human coronavirus, took only a few months because of a multinational collaborative effort[11]. Communicating new information and discoveries that result from research and clinical practice in virology becomes essential and achievable in the modern information and digital era. Launch of the *WJV* will join this evolving trend of collaboration and rapid information exchange by adding another fast-track publication in the field of virology.

SCOPE

The open access and peer-reviewing format of *WJV* ensures the articles will be of high quality and of maximal benefits to the authors, readers and society. *WJV* aims to report rapidly new theories, methods and techniques for prevention, diagnosis, treatment, rehabilitation and nursing in the field of virology. *WJV* covers topics concerning arboviral infections, bronchiolitis, central nervous system viral diseases, DNA virus infections, encephalitis, eye infections, fatigue syndrome, hepatitis, meningitis, opportunistic infections, pneumonia, RNA virus infections,

sexually transmitted diseases, skin diseases, slow virus diseases, tumor virus infections, viremia, zoonoses, and virology-related traditional medicine, and integrated Chinese and Western medicine. The journal also publishes original articles and reviews that report the results of virology-related applied and basic research in fields such as immunology, physiopathology, cell biology, pharmacology, medical genetics, and pharmacology of Chinese herbs.

CONTENTS OF PEER REVIEW

In order to guarantee the quality of articles published in the journal, *WJV* usually invites three experts to comment on the submitted papers. The contents of peer review include: (1) whether the contents of the manuscript are of great importance and novelty; (2) whether the experiment is complete and described clearly; (3) whether the discussion and conclusion are justified; (4) whether the citations of references are necessary and reasonable; and (5) whether the presentation and use of tables and figures are correct and complete.

COLUMNS

TheThe columns in the issues of *WJV* will include: (1) Editorial: To introduce and comment on the substantial advance and its importance in the fast-developing areas; (2) Frontier: To review the most representative achievements and comment on the current research status in the important fields, and propose directions for the future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (6) Review: To systemically review the most representative progress and unsolved problems in the major scientific disciplines, comment on the current research status, and make suggestions on the future work; (7) Original Articles: To originally report the innovative and valuable findings in virology; (8) Brief Articles: To briefly report the novel and innovative findings in virology; (9) Case Report: To report a rare or typical case; (10) Letters to the Editor: To discuss and make reply to the contributions published in *WJV*, or to introduce and comment on a controversial issue of general interest; (11) Book Reviews: To introduce and comment on quality monographs of virology; and (12) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on the research in virology. This variety in the formats for communication columns with clear titles and purpose will allow readers to update and absorb important information based on individual need and interest in different fields of virology.

We welcome *WJV* on-line as a new member of the World Series Journals! Fuelled by common interests and a commitment to work to achieve the journal's goals by all authors, readers and editors, I know that the *WJV* will be a useful reference source for research data on virology and clinical practice against virus-associated diseases.

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EDITORIAL

Delivery strategies for novel vaccine formulations

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Abstract

A major challenge in vaccine design is to identify antigen presentation and delivery systems capable of rapidly stimulating both the humoral and cellular components of the immune system to elicit a strong and sustained immunity against different viral isolates. Approaches to achieve this end involve live attenuated and inactivated virions, viral vectors, DNA, and protein subunits. This review reports the state of current antigen delivery, and focuses on two innovative systems recently established at our labs. These systems are the filamentous bacteriophage fd and an icosahedral scaffold formed by the acyltransferase component (E2 protein) of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*.

Key words: Vaccines; Antigen presentation; Antigen delivery systems; Filamentous bacteriophage fd; E2 scaffold

Peer reviewer: Kenji Okuda, MD, PhD, Professor, Yokohama City University School of Medicine, Chouju Medical Institute, 441-8124 Az-ayamanaka, Noyorichou, Toyohasi, Aichi Prefecture, Japan

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LIVE ATTENUATED VIRAL VACCINES

Historically, live attenuated virus vaccines were used to control many viral diseases, such as measles, smallpox, mumps and rubella. These vaccines elicit both humoral and cell-mediated immune memory responses[1-5], however, they are associated with genetic instability and residual virulence[6-7]. For these reasons, current human clinical trials using live attenuated viral vaccines are rare, and research has been limited to non-human primate studies[8] to understand the mechanisms about the protection elicited. The possible causes of pathogenicity may include: (1) reversion to a virulent form of the attenuated virus[9]; (2) recombination of the vaccine strain with wild type, pathogenic virus[10]; (3) ability of viral genomes to persist in tissues or the proviral genomes to integrate into the host genome[11]; and (4) the dysregulation of the immune system by viral proteins[12]. As alternatives to live vectors, a variety of other antigen delivery systems are available for vaccine research.

VIRAL AND BACTERIAL VECTORS

One of the most pursued strategies is based upon the use of a huge range of viral and bacterial vectors, which are defined as non-pathogenic vehicles containing inserted genes from pathogens for their expression and subsequent induction of specific immune responses. Viral vectors such as poxvirus, adenovirus, vesicular stomatitis virus, adenovirus-associated virus (AAV), alphavirus, cytomegalovirus and lentivirus, to name some but not all, have been explored[13]. In addition, vectors have been developed from bacteria such as *Shigella*[14] and *Salmonella*[15], by exploiting their ability to be delivered *via* the oral

route, which is the natural route of infection for a variety of bacteria. Because so many of the factors governing the induction of optimal immunity are incompletely understood (such as the activation of the innate immune system by various components of vectors and their effects upon adaptive immunity), it is hard to know exactly what would make the best vector for any given vaccine. Given the expensive and long development process required for clinical testing, the choice of one vector over another for each vaccine has been largely based upon the key characteristics of the vector and results of preclinical studies rather than comparison of vectors and administration protocols, since regimens, doses, and matrices of prime-boost combinations could lead to innumerable variables. Some of these vectors, in particular adenovectors and poxvectors, including both canarypox and modified vaccinia Ankara, have been employed in clinical trials for an anti-HIV-1 vaccine[16-20], all obtaining unsatisfactory results. More recently, "The STEP trial", which utilized an adenovirus vector encoding HIV proteins, was performed as a proof of concept for prevention or control of HIV infection[21,22]. The trial was stopped when it was clear that the vaccine was not demonstrating efficacy.

One major concern regarding the use of vectors is the immune response against the vector itself,[23,24] especially in persons who have been previously exposed to the virus or a related virus. Other concerns are the disputed risk of integration[25], the limited gene-insert capacity (in AAV and alphavirus vectors[26,27]), the safety issue in neurotropism (such as in the vectors based on herpes virus or vesicular stomatitis virus[28]), and in bacterial vaccine vectors given orally, the transfer of heterologous genes to other bacteria[29].

DNA VACCINES

In addition to the use of vectors, antigens can be delivered by injecting plasmid DNA encoding immunogenic proteins under the control of eukaryotic promoters. DNA vaccination was first described in 1993[30] and accepted as one of the most promising ways of immunization. Overall, available evidence suggests that DNA vaccination is generally well tolerated[31-33], and two veterinary vaccines have been already licensed[34]. The fate of the DNA after injection has been a matter of concern due to the risk of integration of the nucleic acids into the host genome. However, all available data suggest that frequency of integration is below the frequency of spontaneous mutation[35,36]. Notwithstanding these premises, failures and triggering of disease exacerbation instead of protection have also been reported on the use of DNA vaccines. One of the main criticisms for DNA vaccination is their relatively low efficacy, mainly because a large amount of DNA is needed to be injected in order to achieve a strong response[37]. Improvement of the immune response to DNA vaccines has been attempted by aiming at enhanced plasmid uptake by exploring different routes of administration (gene-gun, electroporation), delivery of the antigen in combination with various immunostimulatory cytokines[38], and by using DNA vaccines in combination with adjuvants or cytokines in order to locally recruit different subsets of professional antigen-presenting cells (APCs)[39].

VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) represent another recent important biotechnological advancement. They are composed of one or several recombinantly expressed viral proteins, which spontaneously assemble into supramolecular structures resembling infectious viruses or, in some cases, subviral particles.

VLPs, like viruses, are comprised of one or more proteins arranged geometrically into dense, repetitive arrays. These structures are characteristics of microbial antigens, and the mammalian immune system has evolved to respond vigorously to this arrangement of antigens. Thus, due to their highly repetitive surface, VLPs are able to induce strong B-cell responses by efficiently crosslinking the membrane-associated immunoglobulin molecules that constitute the B-cell receptor. In addition to their ability to stimulate B-cell-mediated immune responses, VLPs have been shown to be highly effective in stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses[40-44]. Currently, VLP-based vaccines are in various stages of development, spanning preclinical evaluation to market. Vaccines for hepatitis B (Recombivax and Energix) and human papillomavirus (Gardasil and Cervarix) have been licensed commercially[41]. Furthermore, VLPs may also be used as a platform for inducing immune responses against antigens of choice. This can be achieved by constructing chimeric VLPs that display heterologous epitopes[45,46]. However, generating chimeric VLPs is largely empirical; it is almost impossible to predict whether individual peptides will be compatible with VLP assembly or whether the insertion will be immunogenic. Another important limitation of this approach is the size and the nature of the epitopes that can be inserted into the VLPs, in particular into their immunodominant regions.

MISCELLANEOUS STRATEGIES

Other promising strategies for vaccine delivery include the use of virosomes (liposomes carrying viral envelope proteins)[47-51], or subunit vaccines expressed in plant[52-56] or insect cells[57-61].

In summary, a variety of vaccine carriers and delivery modalities have been developed in recent years. However, the failure of clinical trials, based on available formulations, indicates that novel vaccine modalities are still very much needed to complement the existing array of options. In this context, we propose two innovative concepts for vaccine design based on the filamentous bacteriophage fd and the E2 protein from the *pyruvate dehydrogenase* (*PDH*) complex of *Bacillus stearothermophilus* (*B.*

stearothermophilus). Both of the delivery systems combine the advantages of safety with the capability to elicit both humoral and cellular antigen-specific immune responses.

NOVEL ANTIGEN DELIVERY SYSTEMS

Filamentous bacteriophage fd system

The system of antigen delivery by bacteriophage is based on modification of the phage display technology. Phage libraries with specific antisera have been screened to identify phage displaying peptides, which correspond to or mimic antigenic epitopes[62]. Immunization with phages displaying these peptides has proven capable of eliciting protective antibodies in animal models of various diseases[63,64]. The filamentous bacteriophage fd (others are M13, f1) is well understood at both the structural and genetic levels[65]. The capsid contains five types of coat protein, four minor (approximately five copies of each, encoded by phage genes Ⅲ, Ⅵ, Ⅶ, and Ⅸ) and one major (approximately 2700 copies, encoded by phage gene Ⅷ). The potential to display a large number of copies on the surface of these filamentous bacteriophages makes them an attractive vehicle for the expression of foreign peptides. There is evidence from immunoassays and nuclear magnetic resonance (NMR) spectroscopy that the exposed peptides have a stable three-dimensional structure closely resembling what they exhibit in the wild-type parent protein[66]. We have shown that the peptides expressed on the pⅧ phage major coat protein can both produce specific antibodies and present T helper epitopes[67]. Moreover, we have observed that the filamentous bacteriophages are taken up and processed by *major histocompatibility complex* (*MHC*) class I and class Ⅱ pathways[68]. In addition, because co-expression of linked helper T-cell and cytotoxic T-cell (CTL) epitopes on the surface of the same APC is a strict requirement for priming a CTL response, we designed hybrid bacteriophages simultaneously displaying helper and/or cytotoxic epitopes on the same virion. They were obtained by infecting bacterial cells, harboring a plasmid encoding a modified gene Ⅷ, with an engineered bacteriophage carrying a second and different copy of a modified gene Ⅷ. Using phage particles coexpressing both helper and CTL peptides, we demonstrated the ability of bacteriophage virions to evoke an antigen specific CTL response both *in vitro and in vivo*[69]. This surprising finding supports the further exploration of this benign bacteriophage as a powerful and versatile antigen-delivery system and makes the fd virions particularly attractive for vaccine design.

The efficacy of filamentous bacteriophage fd antigen delivery system can be further improved by exploiting the possibility of displaying additional peptides or target sequences on one of the minor coat proteins. In particular, targeting APCs can enhance subsequent immune responses and avoid tolerance induction, should this occur in the presence of a co-stimulatory signal. This has been shown, for example, for the DEC-205 receptor, a decalectin involved in the uptake and presentation

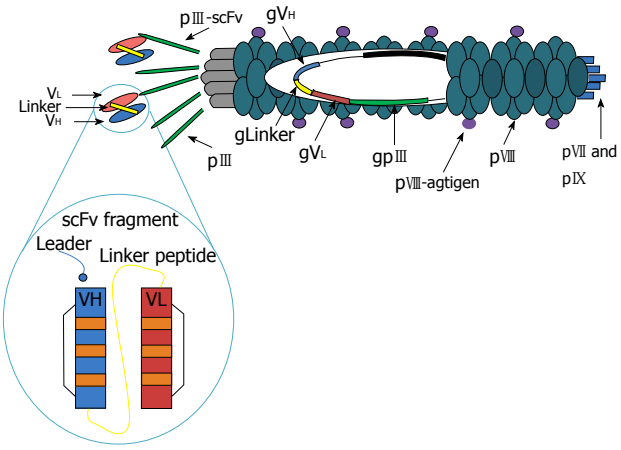

Figure 1 Selective targeting of antigens to dendritic cells. Schematic diagram of the new bacteriophage vector displaying at the N-terminus of the pⅢ gene a single chain antibody fragment (scFv) binding the mouse dendritic cells DEC-205 receptor and an antigenic peptide as N-terminal fusion to the major coat pⅧ protein. The scFv is composed of heavy (VH) and light (VL) chain variable regions of the mouse monoclonal antibody NLDC-145, assembled with a (Gly4Sert)3 linker to yield a 750 bp long single-chain fragment. g: Gene; p: Protein.

by dendritic cells (DCs)[70]. For this purpose, we have engineered two unique restriction sites into the fdAMPLAY388 bacteriophage vector by site-directed mutagenesis, which allows the insertion of peptides/proteins at the N-terminus of the pⅢ gene. Thus, we cloned into the fdAMPLAY vector a single chain antibody fragments (scFvs) from NLDC-145 mouse monoclonal antibody, which is known to bind the mouse dendritic cell-surface molecule DEC-205 (Figure 1). Functional phage particles were purified and Western immunoblotting analysis confirmed the identity of the 90 kDa scFv fusion protein expressed by the recombinant fd. We have recently demonstrated that bacteriophage fd particles displaying the scFv from NLDC-145 mouse monoclonal antibody at the N-terminus of the pⅢ gene were able to induce activation and maturation of mouse DCs, both *in vivo* or *in vitro*, in absence of other stimuli. Furthermore, DCs targeting with fd particles induced a strong antigen specific immune response and, in experiments investigating inhibition of tumor growth, had a protective efficacy similar to adoptive transferred DCs[71]. These results further validate the potential employment of this safe, versatile, and inexpensive delivery system for vaccine formulation.

E2 system

This novel antigen-presenting system is based on the PDH complex of *B. stearothermophilus*[72]. The PDH complex belongs to the family of 2-oxo acid dehydrogenase complexes. They consist of multiple copies of three different enzymes that catalyze the oxidative decarboxilation of 2-oxo acids. In the case of the PDH complex, two of its component enzymes, E1 and E3, assemble over the surface of a large structural scaffold formed by the core enzyme, E2, a dihydrolipoyl acyltransferase. The E2 chain of *B. stearothermophilus* is composed of three independent-

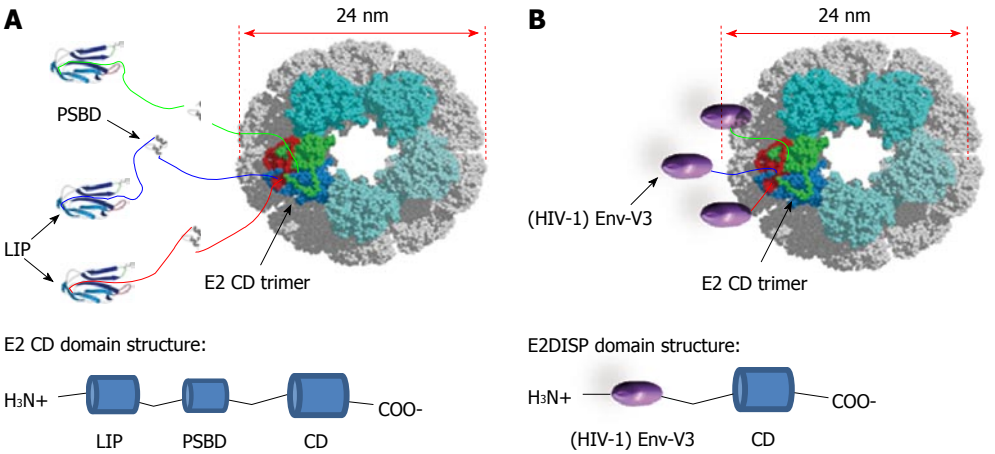

Figure 2 Schematic image of the E2 acetyltransferase display system derived from the *Bacillus stearothermophilus pyruvate dehydrogenase* complex. A: The E2 chain as it occurs in the native *pyruvate dehydrogenase* complex. Twenty trimers of the E2 polypeptide chain form a pentagonal dodecahedron (60-mer) with icosahedral symmetry; B: Representation of the E2 core displaying the HIV-1 Envelope (Env) third hypervariable region V3 on the surface of the scaffold. LIP: Lypoil domain; PSBD: Peripheral subunit-binding domain; CD: Catalytic domain; E2DISP: E2 acetyltransferase display system.

ly folded domains separated by flexible linker regions: a lipoyl domain (LIP) of 9.5 kDa, a peripheral subunit-binding domain (PSBD) of 5.3 kDa and a catalytic (acetyltransferase) core domain (CD) of 28 kDa. The core C-terminal catalytic domain of E2 self-assembles into trimers, which in turn aggregates to generate a 60-chain core with icosahedral symmetry[73]. The 60-mer has a molecular weight of > 1.5 MDa and a diameter of 24 nm as visualized under electron microscope[72]. Surprisingly, E2 can be renatured from denaturing conditions *in vitro* to assemble into the 60-mer virus-like particle, without the need of chaperonins[74]. This finding may be attributed to the heat-stable properties often found in proteins from this thermophilic bacterium. Efficient refolding of E2 into the 60-mer is also possible by replacing the natural peripheral domains with foreign peptides as N-terminal fusions to the CD. The display of exogenous peptides on the E2 scaffold is obtained by using engineered plasmids which allows the insertion of exogenous oligonucleotides at the 5' of the gene coding for the acyltransferase domain of the E2 protein[72]. Thus, a suitably engineered E2 core (E2DISP) can display 60 copies of heterologous polypeptides on the surface of a high molecular mass scaffold[72]. This property can be exploited for vaccine design. Previous work from our laboratory has described that small epitopes (9-15 aa) displayed on the E2 scaffold elicit both cellular and humoral specific immune responses[75,76]. However, displaying larger protein antigens may be more useful, as they contain multiple T cell epitopes as well as antibody determinants.

In theory, there should be no limitation to the size of amino acid sequences displayed on the E2 scaffold. In fact, at the N-terminus of the acyltransferase CD each E2 chain in the *B. stearothermophilus* PDH complex naturally displays 187 amino acid residues in the form of the two folded protein domains (LIP and PSBD domains) and two flexible linkers (Figure 2A). Moreover, the E2 system is naturally designed to present up to 60 copies of the E1 (about 150 kDa) or E3 (about 100 kDa) enzymes noncovalently attached on its surface. Thus, up to 60 large polypeptides can be presented on the E2 scaffold as N-terminal fusions to the acetyltransferase CD. In practice, several proteins, such as the green fluorescent protein (GFP), have been expressed as an N-terminal fusions[72].

These properties of E2 VLPs compare favorably to other types of VLPs, such as the VLPs based on human papillomavirus that can only accept approximately 60 amino acids of foreign antigen[77] or on Hepatitis B surface antigen (HBsAg) that has a limit of approximately 36 amino acids[78]. E2DISP fusion proteins are produced in *E. coli*, and if soluble, the proteins can be purified directly and endotoxin can be removed using standard biochemical techniques. If insoluble, they can be denatured and refolded slowly by step-down dialysis to obtain soluble VLPs. Solubility can also be improved by refolding the fusion proteins in the presence of E2DISP monomers at different molar ratios, which reduces the number of displayed heterologous proteins per VLP. Thus, production of E2 particles is relatively simple and inexpensive, as compared with the mammalian or baculovirus cell culture. In this context we have successfully expressed and refolded a large array of HIV antigens and protein domains. In particular, we have constructed stable VLPs displaying the HIV-1 Gag (p17) protein as an N-terminal fusion to the E2 CD and found that mice immunized with the Gag (p17)-E2 60mer particles mounted a strong and sustained antibody response. Using transgenic mouse model systems, we also demonstrated that CD8+ T cells primed with E2 recombinant particles were able to exert lytic activity[79]. In addition, as schematically represented in Figure 2B, we displayed the HIV-1 Envelope (Env) third hypervariable region (V3), which is known to bind neutralizing antibodies[80] on the E2DISP system. We evaluated the immunogenicity of these purified 60mer VLPs in mice and rabbits, alone and in combination with

an HIV Env glycoprotein (gp160) expression plasmid in an attempt to enhance immunogenicity. Using this system we demonstrated that simultaneous co-immunization with Env(V3)-E2 VLPs and gp160 encoded DNA was more effective than each individual component alone or the DNA-prime/protein-boost immunization regimen in eliciting both neutralizing antibodies in rabbits and CD8+ T cells responses in mice, even in the absence of adjuvants[81].

CONCLUDING REMARKS

Vaccine is one of the most powerful and cost-effective tools of modern medicine. Currently, there is a renaissance in vaccine research due to the fact that healthcare authorities are increasingly recognizing the public health benefit and cost-effectiveness of vaccines. Since vaccines are administered a few times at most, they are also more cost-effective than many other drugs in treating the subsequent diseases. In recent years, several economists have argued convincingly that the cost savings are even more impressive because they go beyond the cost of medical care, and should include income lost due to illness and its sequelae[82]. The revival of interest in vaccines has of course also been underpinned by a rapidly expanding body of knowledge in the fields of immunology and by the impressive biotechnological advancement in recent years. Today, purification of microbial elements, genetic engineering, and improved knowledge of immune protection allow direct creation of attenuated mutants, expression of vaccine proteins in a variety of vectors, purification and even synthesis of microbial antigens, and induction of immune responses through manipulation of DNA, RNA, and proteins. A major aim is to integrate information gathered from classical pre-clinical trials with that derived from different fields of investigation. In this context, several approaches are being pursued to improve rationally designed delivery systems. We have been exploring the advantages of two innovative antigen display systems derived from non-pathogenic prokaryotic organisms, based on the filamentous bacteriophages fd and the acyltransferase component (the E2 protein) from the PDH complex of *B. stearothermophilus*. The fd and E2 antigen delivery systems combine the safety with the capability to trigger both humoral and cellular antigen-specific immune response, even in the absence of adjuvants. Both of these vehicles gain access to APCs, and intersect the human leukocyte antigen class I and Ⅱ intracellular compartments, inducing specific cytotoxic T-lymphocyte responses. Moreover, the E2DISP delivery system offers a unique opportunity to display up to 60 copies of individual or multiple heterologous polypeptides, by preserving their native structure on the surface of a single VLP. These antigen presentation systems could be combined with other well-studied vaccines in an attempt to elicit full-spectrum immune responses.

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OBSERVATION

Emerging and re-emerging viruses: A global challenge illustrated by Chikungunya virus outbreaks

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Abstract

In recent decades, the issue of emerging and re-emerging infectious diseases, especially those related to viruses, has become an increasingly important area of concern in public health. It is of significance to anticipate future epidemics by accumulating knowledge through appropriate research and by monitoring their emergence using indicators from different sources. The objective is to alert and respond effectively in order to reduce the adverse impact on the general populations. Most of the emerging pathogens in humans originate from known zoonosis. These pathogens have been engaged in long-standing and highly successful interactions with their hosts since their origins are exquisitely adapted to host parasitism. They developed strategies aimed at: (1) maximizing invasion rate; (2) selecting host traits that can reduce their impact on host life span and fertility; (3) ensuring timely replication and survival both within host and between hosts; and (4) facilitating reliable transmission to progeny. In this context, *Arboviruses* (or ARthropod-BOrne viruses), will represent with certainty a threat for the coming century. The unprecedented epidemic of Chikungunya virus which occurred between 2005 and 2006 in the French Reunion Island in the Indian Ocean, followed by several outbreaks in other parts of the world, such as India and Southern Europe, has attracted the attention of medical and state authorities about the risks linked to this re-emerging mosquito-borne virus. This is an excellent model to illustrate the issues we are facing today and to improve how to respond tomorrow.

Key words: Health threats; Emerging virus; Arbovirus; Chikungunya virus; Ecosystems; Biosurveillance

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THREATS OF YESTERDAY, TODAY AND TOMORROW

Many infectious diseases severely affect the health of humans, animals and plants. In humans, infectious diseases are responsible for 15 million of 57 million annual deaths in a global population of 6.2 billion people[1]. In the United States, each year, approximately 25% of physician visits are attributable to infectious diseases, with direct and indirect costs estimated at more than $120 billion[2]. Each year, about 2 million people die from acquired immune deficiency syndrome (AIDS) (caused by a *retrovirus*), 1.7 million people die from tuberculosis (caused by a mycobacterium, *Mycobacterium tuberculosis*), and more than 1.6 million people die from diarrheal disease caused by infectious pathogens.

Today, like yesterday, unknown pathogens are emerging in the world, while others, identified long-standing and hitherto regarded as controlled, have their importance increased. Diseases related to these emerging infectious agents can have major consequences in terms of public health, safety, balance of food chains and trade. They have already heavily influenced policy decisions at local, national or international levels while the strategies and specific tools adapted to these scourges are weak or absent, due to lack of integrated knowledge from multidisciplinary research.

Articles that address the emergence or re-emergence of infectious diseases are numerous, but their definition which applies to this concept often varies from one author to another. The definition of emerging infectious diseases (EIDs) in this review adopted by the Emerging Infectious Diseases Interdisciplinary Scientific Program of the French CNRS is as follows: EIDs belong to a nosological entity whose nature is proved infectious, regardless of the pathogen, or only suspected in case of novelty and until the agent is identified. It is understood that the identification of a "new" pathogen is compatible with a previously undisclosed preexisting one. By extension, it is assumed that the EIDs include infectious diseases known endemic showing an obvious resurgence. An EID can affect all types of eukaryotic organisms. The EIDs generally have a high social impact and economic consequences. An EID is obviously unusual; it is surrounded by uncertainty and anxiety, real or perceived, as to its evolutionary potential, its impact on health and the ability of leaders and stakeholders to control the phenomenon. These emerging diseases are therefore: (1) the development of a new disease, a consequence of a new pathogen (in its nature, its mode of transmission, its expression and/or its adaptation to host species); and (2) a disease previously identified but whose manifestations are new (associated with a sudden increase in the incidence, severity or geographical area within a time span of a few weeks/months to one or several decades). This definition therefore includes the resurgence of endemic diseases in more sensitive populations or of less controlled forms, and the emergence of pathogens resistant to treatment.

FACTORS FAVORING THE EMERGENCE OR RE-EMERGENCE OF PATHOGENS

The devastating plague of Athens, Greece, killed 70 000 Athenians in the years 430-426 BC; the Black death, or bubonic/pneumonic plague, killed approximately 34 million Europeans and 16 million Asians in the years 1347-1350; the French pox (French is called here because of a notorious outbreak in the French army), or syphilis, linked crewmen of Colombus' voyages to the Naples outbreak of syphilis in 1494, and subsequently spread the disease to Europe (syphilis killed 5 million Europeans); the smallpox in 1520 was introduced in Central America by Spanish soldiers. It killed most of the Aztec army and 25% of the overall Aztec empire population; the American plague, or Yellow fever, in the years 1793-1798 killed more than 5000 of 50 000 Philadelphians; and the Spanish influenza in 1918-1919 turned out to be the deadliest emerging pandemic since it killed 50-100 million people. All these diseases are among the diseases that have marked the history of infectious diseases. They remain etched in the collective subconsciousness of the people as a threat for the survival of human societies. Sometimes the vector itself is the cause of phobias which are additional to the fear of disease, as is the case for rats [e.g. Black death, Argentine hemorrhagic fever (AHF)] and bats (e.g. Ebola hemorrhagic fever, Marburg hemorrhagic fever)[3,4]. At certain times of history, epidemics were even interpreted as divine punishment with all the irrational behavior; thus, when Black death appeared in Marseille (South of France) in 1347, and spread rapidly in France and Europe, it caused anti-Jewish riot up to massacre of people. Actually, plague remains a public health concern mainly in East Africa and Madagascar[5].

Scientific advances, however, have accomplished major progress in the fight against infectious diseases. The most important achievement was the eradication of smallpox by vaccination[6]. The last case of smallpox was declared in 1977 and, during 1980, the World Health Organization (WHO) announced the eradication of smallpox, a feat of human factors against the hostile factors of the environment. Compared with earlier generations of scientists, we possess an enormous scientific base, and the acquisition rate is high for new information about pathogens. Over the last 5 decades, numerous viruses responsible for new diseases or old diseases whose etiology is recently identified, have been discovered[2,7-10]. To highlight the importance and timeliness of EIDs without focusing only on the AIDS pandemic that killed more than 25 million people over the past 25 years, we should also keep in mind that severe acute respiratory syndrome (SARS), with 10 000 human cases and slightly less than 1000 deaths, had an estimated overall cost of over $100 billion for the global economy; a possible pandemic of bird flu transmission between humans also challenges the government and health authorities in the world. In addition, the zoonosis related to the H5N1 virus has already had devastating effects on certain economies. The epidemic of mad cow disease has caused 182 500 confirmed cases of bovine spongiform encephalopathy, or prion disease, in the UK and the ban on bovine products; the quite recent Chikungunya virus (CHIKV) outbreak in Reunion, with more than 230 000 human cases, had led to more than 180 deaths and also seriously affected the local tourist economy; the yellow mottle virus in rice, the main disease affecting irrigated rice in Africa and Madagascar, can destroy almost an entire harvest and have dramatic consequences on the survival of local populations.

Just to cite some of the major epidemics to take stock of the situation, emergence of new pathogens affecting humans has been found true with the Marburg virus epidemic in 1967, the Lassa virus in 1969, the Rotavirus

Major emerging and re-emerging virus-associated diseases

Syndrome	Aetiological agent	Year of discovery
Diarrhoea	Rotaviruses	1973
	Astroviruses	1975
Respiratory syndrome	Sin Nombre virus (Hantavirus)	1993
	Influenza A/H5N1 virus	1997
	Influenza A/H9N2 virus	1999
	Hendravirus	1995
	SARS-Coronavirus	2003
	Influenza A/H1N1 virus	2009
Encephalitis	Enterovirus -EV71	1997
	Nipahvirus	1998
	West-Nile like Virus	1999
Immunodeficiency	Retroviruses HIV-1 and HIV-2	1983
Cancers	Retrovirus HTLV-1	1980
	Retrovirus HTLV-2	1982
	Herpesvirus HHV8 (Kaposi KSHV)	1994
Hepatitis	Hepatitis C virus (HCV)	1989
	HEV	1990
	HFV	1991
	HGV	1993
Haemorrhagic fever	Ebola virus (Filovirus)	1977
	Hantaan virus	1977
	Guanarito virus	1991
	Sabia virus (Arenavirus)	1994
Anemia	Parvovirus B19	1975
Exanthema	Herpesvirus-HHV6	1988
	Herpesvirus-HHV7	1990
Arthralgia	env-mutant Chikungunya virus	2005

Figure 1 Major emerging and re-emerging virus-associated diseases in human. The etiological agents of infectious diseases identified since 1973, as newly discovered viruses of public health importance. It includes a few arboviruses that are transmitted to humans by arthropod vectors, upon biting. Among known arboviruses, some are transmitted by mosquitoes (Bunyaviridae: La Crosse encephalitis, California encephalitis, Rift Valley fever; Flaviviridae: Dengue fever, Yellow fever, Japanese encephalitis, West Nile; Togaviridae: Eastern equine encephalomyelitis, Western equine encephalomyelitis, Venezuelan equine encephalomyelitis; Ross River fever, O'Nyong-nyong fever, Chikungunya), others by ticks (Bunyaviridae: Crimean-Congo hemorrhagic fever; Flaviviridae: Tick-borne encephalitis; Omsk hemorrhagic fever; Reoviridae: Colorado tick fever). Most of the emerging pathogens in humans originate from zoonosis.

in 1973, the Ebola virus in 1976, the Hantaan virus in 1977, the influenza A/H5N1 virus in 1997, the SARS-Coronavirus in 2003, the influenza A/H1N1 virus in 2009. Pathogens identified as the causative agents of a disease can include the human T-cell lymphotropic virus 1 (HTLV- Ⅰ) in 1981, the HTLV-Ⅱ in 1982, the human immunodeficiency virus (HIV) in 1983, the hepatitis C virus in 1988, and the human herpes virus-8 (HHV-8 or Kaposi sarcoma-associated Herpesvirus KSHV) in 1995 (Figure 1).

These cases may illustrate the diversity of situations encountered, such as (1) new diseases due entirely to a new virus as in the case of AIDS; (2) previously unrecognized diseases that are identified through quantitative or qualitative changes and due to improved diagnostic methods, such as CHIKV-caused disease that can be confused with Dengue (DEN) cases[11]; (3) diseases that appear in a new region, such as the epidemic West Nile virus introduced to the USA in 1999; or (4) diseases which existed previously in animals and are transmitted to humans, like avian influenza occurring since 1997. A pathogen such as influenza A/H1N1 virus is the result of a complex (triple) reassortment with portions of the genome that correspond to those of a swine virus, others are related to an avian virus and, finally match a human virus[12].

EMERGENCE LINKED TO CHANGES IN ECOSYSTEMS

An emerging disease may be caused by a disturbance of the ecosystem[13]. The exploitation of nature by man, with consequences such as deforestation and/or practice of hunting, may put men into contact with pathogens. This probably happened for the first human infections with simian immunodeficiency viruses (SIV), which have adapted to a new host, man. The combination of the density of people in ever-growing cities and unsafe sexual practices fostered the transmission of HIV among groups of individuals. Population flow, whether by migration or transportation, in particular the development of air transport, has been a source of rapid spread of HIV in the world. The fact that this virus is a *Lentivirus*[14], whose first symptoms appear only 7 years after the primary infection, has completely hidden epidemic in the face of the world. When it became clear, it was already too late. The spread of a pathogen can be even faster instead of sexual transmission, contamination may occur through aerosols. If we study what happened from November 2002 about the worldwide spread of SARS, a doctor who treated SARS patients in China's Guangdong Province traveled to Hong Kong where he stayed in a hotel. SARS passed to a Hong

Kong resident who visited the hotel, to a businessman guest who traveled to Vietnam, to a flight attendant guest who then flied to Singapore, and to a tourist guest who returned to Toronto, Canada. Then it was only a period of weeks that created a world crisis with more than 8400 infected individuals, leading to more than 900 casualties around the world in 2002-2003[15].

Another example of interest is that following intensive cultivation of corn in Argentina, there has been a very significant proliferation of rats that were at the origin of the AHF associated with Junin virus[16]. Since the first report in Argentina, this *Arenavirus* has caused annual outbreaks with more than 24 000 reported cases by 1993. The transmission of the pathogen resulted from the interaction between the virus, the animal that acted as a reservoir for the virus, and contact with an immunologically naive human population that was exposed to the virus due to environmental change. Among factors that may promote the emergence, it is not uncommon that they may be related to climate changes, leading to the installation of vectors within a new geographic area previously untouched by the disease. It can also result from the adaptation of viruses to vectors by genetic mutations. This last situation is perfectly illustrated by the example of the Chikungunya *Alphavirus* (CHIKV) epidemic in the Indian Ocean islands in 2006 and the first cases in Southern Europe. Also, the combination of different factors such as urbanization and deforestation is expected to have influenced the emergence or re-ermergence of vector-borne diseases such as Yellow fever. Yellow fever, which was originally maintained in forest cycles involving monkeys and canopy-dwelling mosquitoes, was under control in various places of the world such Cuba (1900-1901), Panama (1904), Brazil (1932), Americas (1954-1975), and West Africa (1950-1970), after successful vector-borne disease elimination programs. However, Yellow fever re-emerged in Africa in the 1990s in urbanized areas[13].

All these examples show how the medical and scientific world was wrong when in the late 1970s there was a talk of "the end of infectious diseases". Fifteen years later, the infectious disease specialists needed to make it clear by admitting "the return of infectious diseases" and by proposing the concept of EIDs. The EID could thus be defined as exceeding the average level and retaining attention. There are different situations: (1) the disease caused by a newly identified pathogen did not exist previously (AIDS or SARS); (2) the disease existed before but that was hitherto unknown causative agent (hepatitis C); or, (3) the disease existed before and the causative agent was identified, but appeared for the first time in a geographic area where no cases had been diagnosed previously (West Nile Virus Epidemic in the USA).

ARBOVIRUSES: BEYOND THE THREAT, A GLOBAL PUBLIC HEALTH PROBLEM

Each year, 800 000 people die of malaria and 20 000 of Dengue. While malaria, a human disease caused by a mosquito-borne infectious parasite of the genus *Plasmodium*, is the most important vector-borne disease because of its global distribution, the number of people affected (more than 220 million people) and the large number of casualties (about 800 000 deaths/year), the vector-borne viruses (arboviruses) are clearly the most numerous insect-borne pathogens (Figure 2).

Dengue, a disease provoked by a *Flavivirus*, is at the forefront of arbovirus-induced diseases with more than 80 million infected people a year. Dengue virus (DENV)-infection may be asymptomatic or may induce undifferentiated symptomatic febrile illness with headache, myalgias, arthralgias, rashes (eruptions may be observed on the neck and the face, followed by generalized rash on legs and arms) and leucopenia, most commonly affecting adolescents and adults. DENV-associated disease can also be much more severe (1-2 million people with severe symptoms), and sometime fatal[17], with the dengue hemorrhagic fever (DHF) and dengue shock syndrome (more than 20 000 deaths/year). DHF, which is most common in children of less than 15 years of age but also occurs in adults, is characterized by the acute onset of fever and associated with non-specific constitutional symptoms. There is a hemorrhagic diathesis and a tendency to develop fatal shock. Abnormal homeostasis, plasma leakage and week pulse, are the main patho-physiological changes constantly found. Although very common in the tropics, Dengue has also raged in Europe. The DEN epidemic occurred in Greece in 1927 and 1928 affecting more than 1 million people; this arbovirus outbreak killed 1500 infected persons far from a tropical area[18].

The globalization for arbovirus-induced diseases is a worrying situation because it is also accompanied by the resistance of vectors to insecticides, resistance of pathogens to drugs, unprecedented population growth in developing countries, uncontrolled urbanization in tropical areas where vector-borne diseases occur most frequently. Conditions necessary for arboviruses outbreaks include adequate populations of reservoir hosts (potentially humans in some settings), vector mosquitoes, and appropriate climatic conditions for transmission. The behavior of man on the environment, such as population pressure and agriculture, modifies the ecosystems (e.g. urbanization and deforestation). Anarchic urbanization in developing countries is often accompanied by habits of people who can facilitate the development of vectors. For example, automobile tires discarded in domestic environment where they collect rainwater, make ideal breeding sites for mosquitoes. In these facilities, mosquito larvae can reduce the risk of predation and can also survive in unfavorable climatic conditions. The same consequences can be the result of large water control projects such as irrigation systems and water reservoirs for intensive agricultural conditions.

RESURGENCE: LESSONS FROM CHIKV OUTBREAKS

When arboviruses emerge in endemic regions, they usually generate sporadic infections. However, when infections occur in naive human populations they usually induce

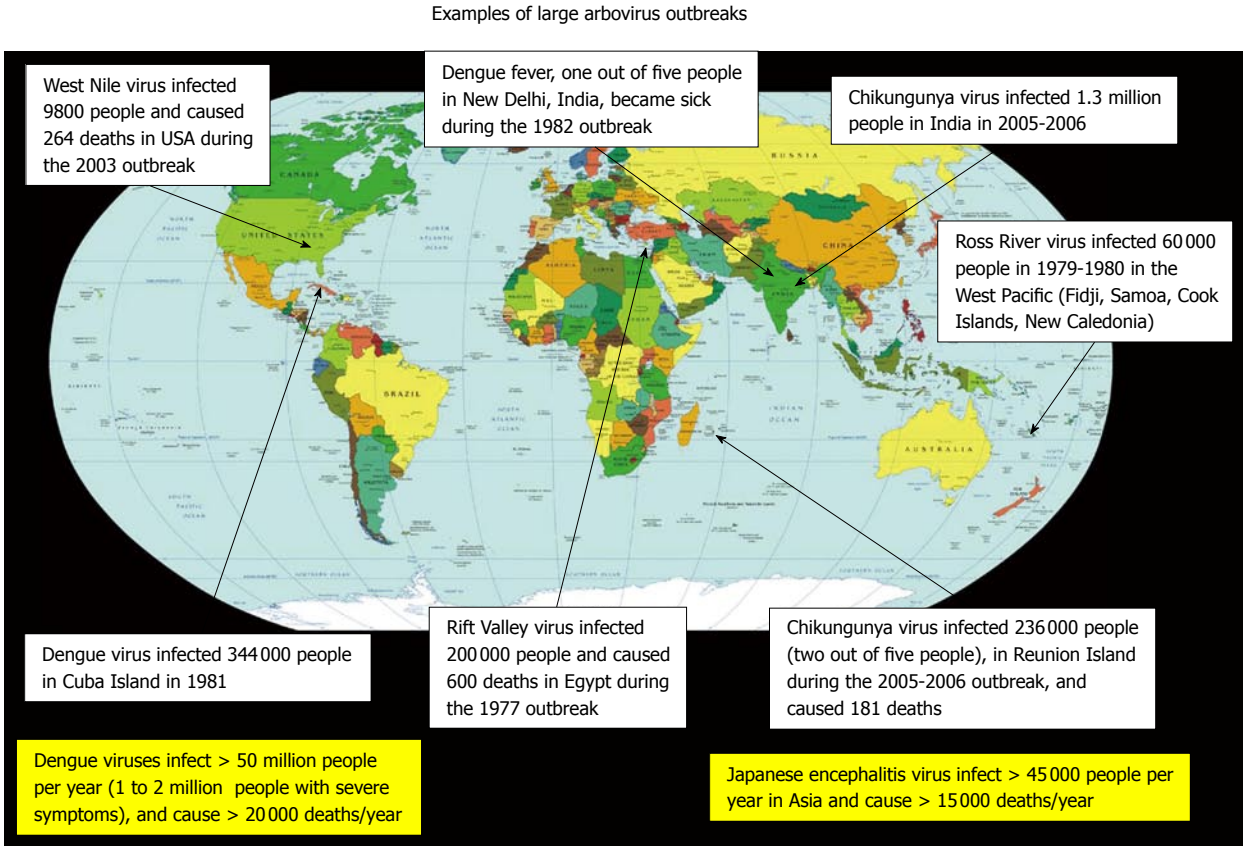

Figure 2 Examples of large arbovirus outbreaks, from the literature. This diagram illustrates several unexpected arbovirus outbreaks that have occurred in regions of the world which are currently the most vulnerable to arboviruses. It is likely that in the coming years other parts of the world will be affected, including regions that are outside of the tropical area.

severe epidemic bursts. To illustrate the resurgence of endemic diseases in more sensitive populations, examples will be given that are related to the situation encountered during the re-emergence of CHIKV. CHIKV belongs to the genus *Alphavirus*, family *Togaviridae*.

Choosing an arbovirus such as CHIKV to discuss the mechanisms of resurgence is not simply the result of chance. Indeed, these viruses illustrate not only the complexity of the interactions between an animal or a human and a virus-infected mosquito, the infection of another animal or a human bitten by an infected mosquito, but also the problems related to migration of human and animal populations, changes in hosts, transports, climate and global warming, water, *etc.*, in other words, to an entire ecosystem apprehended in its entirety. We should also understand the dynamics of interaction at the molecular level as it is the case for the interaction between a CHIKV and membranes of an insect cell or a human cell, and subsequently, between viral proteins and cellular proteins.

As we will see later in this review, resurgence is exactly what happened in the Reunion Island during the CHIKV outbreak in 2006.

PATHOPHYSIOLOGY OF CHIKV-ASSOCIATED DISEASE

The CHIKV-induced DEN-like epidemic in the Reunion Island in 2005-2006 clearly corresponds to a disease previously identified but whose manifestations were associated with a sudden increase in the incidence, severity and change in geographical distribution. The symptoms[19] were most often clinically indistinguishable from those observed in Dengue, and evidence for dual infections has been reported[20]. CHIKV-induced disease appears after an average incubation period of 2-7 d. CHIKV fever is characterized by sudden onset, chills and fever between 39℃ and 40℃, headache (70%), nausea/vomiting (60%), persistent myalgia/arthralgia (40%), and maculopapular rash (60%). Poly-arthralgia, the typical clinical sign of the disease, is very painful. The virus-associated symptoms, were also very similar to those previously observed in Australia during other unusual epidemic episodes caused by a related Ross River virus (RRV) in New South Wales in 1928[21], in Queensland in 1943[22], in the Schouten Islands near Papua New Guinea in 1946[23], and in the Muray Valley in 1956[24]. Until recently, CHIKV-associated syndrome differed from DENV-syndrome by the absence of fatalities and the clinician's attention mainly focused on the rheumatologic consequences of CHIKV infection[25,26], and eventually on neurological and cardiac disorders, or hemorrhagic manifestations. CHIKV associated with a fatal hemorrhagic form was described once in India in 1964 and one case of a child who died from CHIKV infection in Ceylon was also documented in

1967. Yet, these cases were considered as exceptions and did not draw attention of the clinicians and biologists on the potential risks of CHIKV infection. Unfortunately, this lack of fatality can no longer be claimed, since 181 patients had died from CHIKV infection in the Reunion Island, indicating that CHIKV, like other *Alphaviruses*, is fatal for a low percent (around 0.5%-1%) of infected patients.

TEMPORAL AND SPATIAL DISTRIBUTION OF CHIKV OUTBREAKS

In the history of the disease linked to CHIKV, the first known viral isolate was from the serum of a febrile patient during a Dengue-like epidemic burst that occurred on the Makonde Plateau in Tanzania along the border between Tanzania and Mozambique in 1952[27,28]. Although the CHIKV epidemiological data are difficult to collect, it was reported that seroconversion was 31% in Bangkok in 1962[29], 15%-25% in Vellore, India in 1964[30], and 70% in Ibadan (Nigeria) in 1969. CHIKV has also been isolated from patients in Australia[31]. Since the first identification of CHIKV-infected humans, outbreaks of CHIKV have occurred throughout African and Asian countries where it is responsible for illnesses in hundreds to thousands of individuals. Comparative observations of Asian and African strains have been reported[32]. In both Africa and Asia, the re-emergence was unpredictable, with intervals of 7 to 20 years between consecutive epidemics. A representative example refers to the Indonesian outbreaks. The first cases of CHIKV were reported in Samarinda and Balikpapan, East Kalimantan, Indonesia in 1973. The disease then spread to Kuala Tungkai (Jambi Province) in 1980, and Yogyarkarta, Martapura and Ternate in 1983. After a hiatus of 20 years, 24 distinct outbreaks of probable CHIKV were reported between 2001 and 2003. Up to 8068 cases were reported in 29 cities of nine provinces in 2003, including Central Java, Yogyakarta, Banten, West Java, East Java, Bali, West Nusas Tenggara, East Nusa Tenggara and Lampung. In 2004, outbreaks were reported in Keagungan, West Jakarta (50 cases), Magetan, East Java (at least 168 cases), and Central Java (820 cases)[33].

In June 2004, a CHIKV outbreak was reported in Kenya[34]. The next outbreak emerged in the Indian Ocean Islands, during the first months of 2005[35]. More than 5000 cases were reported in Comoros from January to March 2005. A few months later, between March and June, the virus circulated in other islands, i.e. the Reunion, Mayotte, Seychelles, and Mauritius Islands. An outbreak of CHIKV was identified in the Reunion Island between April and June with a peak incidence in May which next decreased in winter season. The local sentinel network in the Reunion Island and the French health service administration (DRASS) reported 12 400 cases infected by CHIKV in the Reunion Island. Surprisingly, a second outbreak occurred at the end of December 2005 and the first couple of months of 2006, and a very fast increase in the number of infected persons of more than ten times (about 186 000 cases) was observed and DRASS announced 93 patients who died from CHIKV. In January 2006, it was also reported in Madagascar. By mid-March, about 214 000 persons had been infected with CHIKV in the Reunion Island, 148 of them died after infection. One month later, about 236 000 persons (up to one-third of the 770 000 people living on the Reunion Island), were infected and 181 of them died. Next, the Mauritius Island (more than 200 cases) and Seychelles (more than 1000 cases), and the French Island Mayotte (4308 cases, probably underestimated, of a population of about 180 000) which are also located in the Indian Ocean have also reported cases of CHIKV-infection in humans. The outbreak of CHIV which occurred in India between 2005 and 2007 has infected 1.5-6.5 million people[36]. Other outbreaks of CHIV took place in Cameroon in 2006 and in Gabon in 2007[37], where 11 500 cases were recorded between April and June.

Although the number of cases and the impact of the disease are not comparable to what has been described in Africa, India and the Indian Ocean area, Europe has not been spared by the CHIKV. In 2007, an infected traveller from India arrived in Italy and, within a few weeks, a small outbreak of more than 200 people was reported in this country where the local transmission was made possible by the enormous population of *Aedes albopictus* and the presence of a CHIKV-positive patient[38]. This European episode is very interesting because it helps understand the dynamics of virus outbreak, based on genetics characteristics of viruses, conditions encountered in interactions of virus with the vector, the geographical distribution of the vector and the climatic conditions favouring the development of an urban cycle of transmission. This will be commented in the subsequent section of this review.

SOUTH OF FRANCE: AN OBSERVATORY FOR INSECT-BORNE PATHOGEN DYNAMICS

In the coming decades, arboviruses will more frequently affect regions of the world that are outside of the tropical areas and it is very likely, for example, that Europe will face important arbovirus epidemics in the future[39]. This could occur through a combination of at least two major phenomena: First, the rapid global movement of people including those recently infected with arboviruses, as was observed in southern France in 2006, in Marseille, with people traveling from the Comorian Islands to France and carrying CHIKV; Secondly, because of changes in the implementation of vectors into new territories as in the cases of *Aedes albopictus* in southern France, perhaps linked to climate changes which likely determine the geographical and temporal distribution of arthropods as well as their life cycle, but most probably by accidental motorway transport. This adaptation of a mosquito is interesting and troubling because it opened the door to

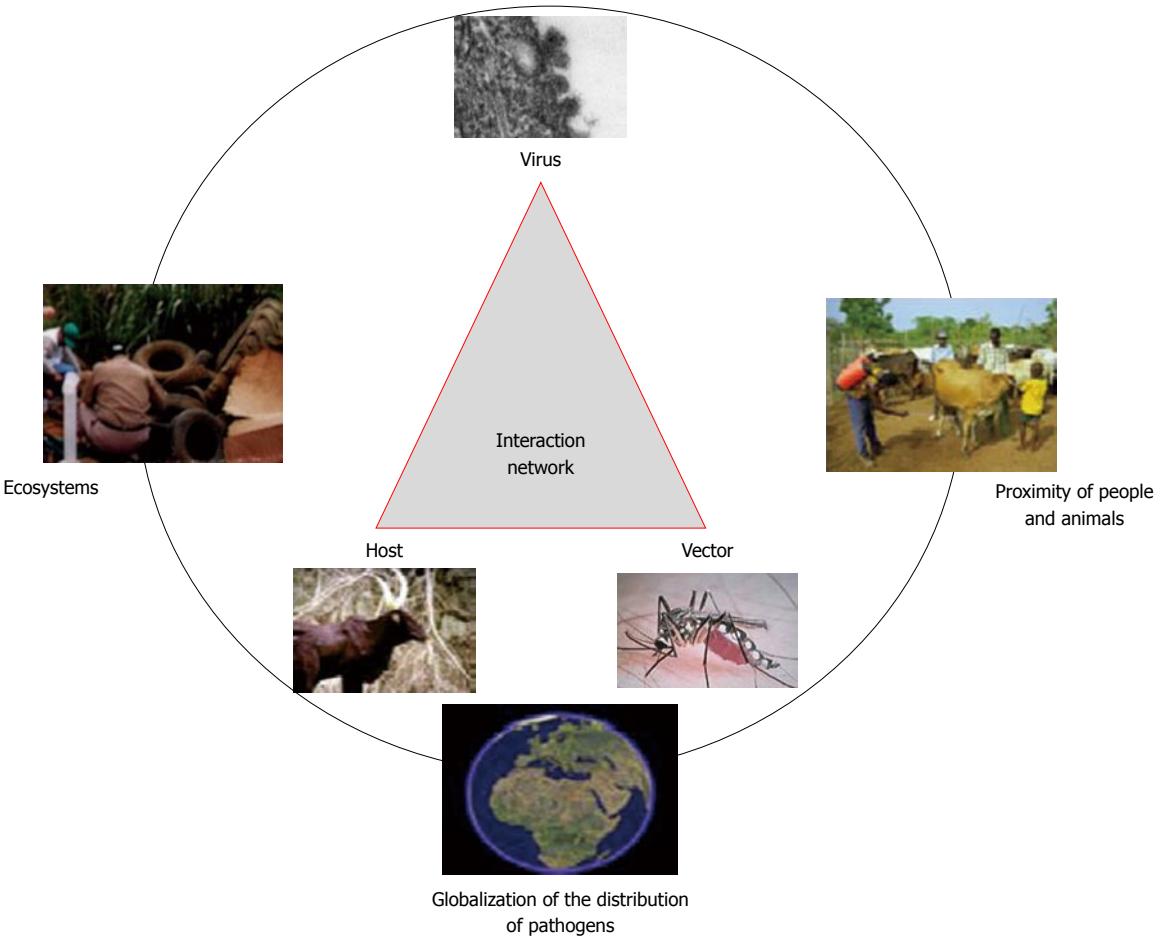

Figure 3 Globalization of the distribution of pathogens. Climate change, ecosystems evolution, anarchic urbanization, human behaviors, migration of humans and animals, development of air transport, extensive agriculture and water control projects, contribute to rapid spread of vectors and arbovirus-induced diseases in the world.

new epidemics. Interestingly, the most frequent vector of CHIKV, *Aedes aegypti* is not present in the South of France. In contrast, *Aedes albopictus* which can carry and transmit the CHIKV to humans, is now well established in the South East of France, in cities such as Nice and Marseille, and it has been reported further West towards Montpellier and further North to Valence and Grenoble[40]. In the event of contamination of mosquitoes by CHIKV, the spread of the disease will depend on many parameters including the abundance of mosquitoes in the area, the number of infected people moving around, the density of the population in the concerned region, the lifespan of infected mosquitoes, the replication time of the pathogen, the ability of mosquito to bite people, *etc.* The ecological conditions make an outbreak very likely, since some port cities of southern France are regularly visited by the people from infected areas and since *Aedes albopictus* tends to colonize urban areas (it is present in wet gardens and also in the water of flower pots at home), and preferably it bites man when it has the choice between man and animal.

Interestingly, two domestic cases of CHIKV and DENV transmission were reported in south of France in September 2010[41]. The two autochthonous cases of Dengue were diagnosed on September 10 and September 17, 2010 in people living in the city of Nice near an imported founder case (note that there were 173 cases of Dengue imported into France in 2010 with two-thirds coming from the Caribbean). The two autochthonous cases of CHIKV infections occurred in the town of Fréjus at monthly intervals, with the first case diagnosed on September 24, 2010 in a small area where resided an imported founder case of CHIKV. These CHIV isolates have been sequenced[42]. These infections have been made possible by the presence of mosquitoes *Aedes albopictus* (Figure 3), which are thought to have arrived in France with the motorway transport between Italy and France. Once installed in the city, mosquitoes tend to move slightly and bite people in the neighborhood.

Moreover, this region of southern France is known for being a place of emergence of other arboviruses including outbreaks of West Nile virus. Initially reported in France in the 1960s, West Nile fever re-emerged in 2000 in the wetland of Camargue where it is episodically reintroduced by migratory birds and caused 76 equine clinical cases, 21 of which died[43]. West Nile virus is frequently associated with ornithophilic *Culex spp.* mosquitoes, which amplify the virus and transmit it to domestic and

migratory birds, facilitating a wide geographical spread. In 2003, 7 human cases were reported in the Var district in Southeastern France. One may question what will be the impact of the introduction of *Aedes albopictus* in Southern France on the potential spread of West Nile virus. Indeed, the diet patterns of haematophagous arthropods are of major importance in the spread of arthropod-borne infectious pathogens. As recently studied[44], *Aedes Albopictus* acquired blood exclusively from human host. Accordingly, invasion of a geographic area by *Aedes Albopictus* could likely support local outbreak of Chikungunya and Dengue viruses induced diseases; in contrast, this mosquito could be expected to have negligible impact on the transmission of zoonotic agents, such as West Nile, since it avoids avian blood feeding in its diet.

It is also worth noting that, each year, there are a few thousand cases of imported malaria in France. The last known case of autochthonous malaria (*Plasmodium vivax*) was reported in Corsica, an Island at the South of France, in 2006[45]. The French Health Authorities have also identified other risks of arboviruses, in particular the Usutu virus, a *Flaviviridae* and the Toscana virus, a *Bunyaviridae*. Two autochtonus cases of Toscana virus-fever, a virus transmitted by *Phlebotomus spp*, were recently reported by the French Institute of Public Health, InVS, in 2011[46].

Vector control will be a requirement to interrupt the transmission of emergent/re-emergent vector-borne diseases.

FIGHT AGAINST HEALTH THREATS: ONE CANNOT BE EFFECTIVE AGAINST WHAT WE DO NOT KNOW WELL

It is of utmost importance to support and conduct research on basic and applied aspects of host, pathogen, and environmental factors that influence disease emergence and re-emergence as well as transmission. For example, CHIKV may survive in wildlife species through constant transmission cycles moving in epizootic waves (sylvatic cycle)[39]. Meteorologic factors such as temperature, rainfall, and humidity influence the transmission dynamic of vector-borne diseases, with increased viral spread following periods of heavy rainfall. Humans entering the areas where infected mosquitoes circulate may serve as incidental hosts for the mosquitoes and thus become infected. These people may then provide a source of virus to infect peridomestic mosquitoes, which next become involved in urban cycle of CHIKV transmission and epidemic episodes in the urban community.

Until recently, CHIKV episodes were restricted to diffuse epidemics in Africa where it was propagated by a variety of mosquitoes from the Aedes family, including *Aedes furcifer*, *Aedes luteocephalus*, *Aedes taylori*, *Aedes africanus*[47]. Then, the virus was imported to Thailand and India where it became an urban disease, transmitted largely by *Aedes aegypti* mosquitoes[39]. It is of particular interest that the CHIKV strain that has caused most of the epidemics in urban areas of the Indian Ocean is believed to have originated in Central/East Africa and transmitted by *Aedes aegypti*. Phylogenetic studies have shown that the earliest CHIKV isolated from the Reunion Island closely resembled those from East Africa, but as the epidemic accelerated, the CHIKV sequence of subsequent isolates changed, resulting in an amino acid substitution in the viral E1 envelope glycoprotein as a consequence of viral genome microevolution[48]. Simultaneously, this mutation appeared only in the CHIKV isolated from *Aedes albopictus*. Until very recently, CHIKV outbreaks in India were primarily associated with *Aedes aegypti* as it is in Africa. The Indian CHIKV-induced disease is recognized as an urban disease whereas the CHIKV African strains are expected to spread under sylvatic nature. Surprisingly, it was evidenced that CHIKV that circulated in India after the Reunion Island outbreak was genetically related to that circulating in the Indian Ocean Islands. This Indian CHIKV carries the mutation that allows a more efficient dissemination in *Aedes albopictus*[49]. CHIKV that were then circulated in Cameroon (2006), Gabon (2007) and Italy (2007), all bear the mutation which favors a transmission of the pathogen by *Aedes albopictus*. In Indian Ocean, Cameroon and Gabon, *Aedes albopictus* has effectively displaced *Aedes aegypti* through interspecific competition. *Aedes albopictus* appears to have adapted to activities of humans such as transportation and water programs, and it has colonized peridomestic storage of used car tires and transportation of plants. *Aedes albopictus* is now present in southern Europe. Although climate change has not yet been scientifically proven to have caused the emergence or re-emergence of any of the vector-borne diseases, a warming climate that would facilitate the introduction of mosquitoes in areas not yet infected, represents a serious hypothesis for the development of arboviruses in new regions of the world. Such considerations must be taken into account to anticipate changes that will undoubtedly lead to future epidemics. They have also important implications for the design of vector control strategies to fight against the virus in the regions at risk of Chikungunya fever.

African strains of CHIKV differ from the Reunion Island isolates of the virus regarding the sensitivity of *Aedes albopictus* for infection and replication of the viruses[50]. Acquisition of a single V226A mutation by the Reunion Island isolates coincides with the acquisition of a dependence on cholesterol in the target cell membrane[51] and their sensitivity to lysomotropic agents during infection of mammalian cells[52].

Research on vector control using environmentally safe insecticides will be required to interrupt the transmission of arboviruses. Other approaches could be to block the virus replication cycle. A better understanding of the replication cycle of CHIKV in both insect cells and mammalian cells should help move forward in this fight[53]. Insects cannot synthesize cholesterol *de novo* and depend on dietary cholesterol for their physiological requirements[54]. Under culture conditions that allows to question the requirement for membrane cholesterol in C6/36 *Aedes*

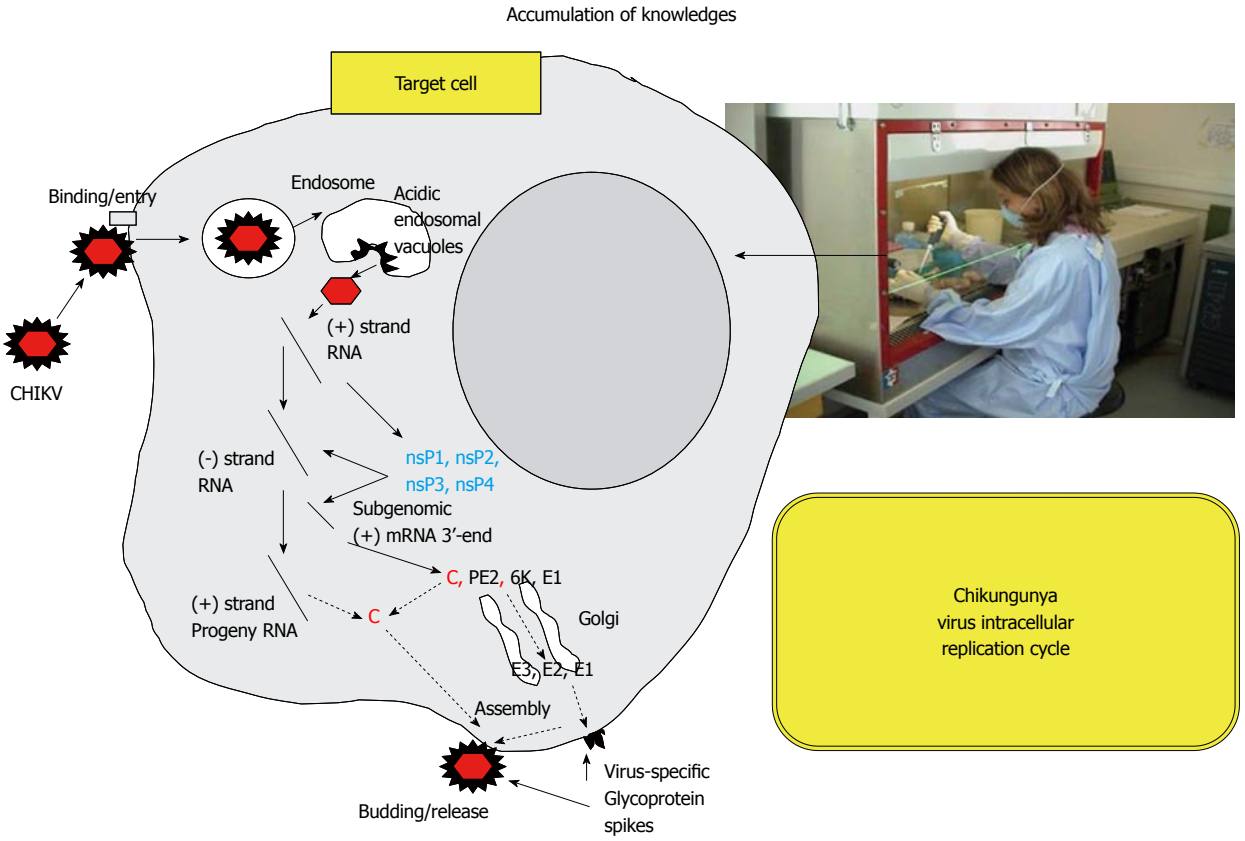

Figure 4 Accumulation of knowledge. We know relatively little about the cycle of CHIKV replication in mammalian cells and insect cells. It remains necessary to conduct experimental work to better understand the virus replication cycle, particularly in order to develop therapeutic agents. Understanding of each stage of the cycle, the molecular crosstalk between the pathogen and the host, and each molecular interaction, may offer a new avenue to fight the pathogen.

albopictus cells infected by CHIKV, we found that cholesterol depletion reduced the infectivity of strains bearing an alanine residue at position 226 of E1 (African CHIKV strain), whereas strains bearing a valine at position 226 of E1 (the Reunion Island CHIKV strain) are markedly less sensitive, indicating that the presence of valine represents a selective advantage for the virus subjected to this selection pressure[55]. Yet, there are still many things to understand in the replication cycle of CHIKV (Figure 4). For example, we have recently demonstrated that entry of CHIKV into mammalian cells occurs by a pH-dependent pathway[52], and that entry of the virus into mosquito cells occurs by a pH-independent pathway[55]. In mammalian cells, CHIKV uses clathrin-independent, Eps15-dependent endocytosis as an entry pathway and requires functional early endosomes and low pH conditions in this compartment for productive infection[52]. The more we move forward in understanding intracellular cycle of the virus, the more chances we will have to design molecules capable of inhibiting the replication of the virus, thus developing them into efficient therapeutics.

WORLD IS CHANGING, ECOSYSTEMS ARE CHANGING, BIOMONITORING MUST ADAPT

The few examples mentioned above illustrate the major development in the relations between pathogen, vector, animal and human target within ecosystems that have never changed so quickly. In this context, efforts should be made on the development of biosurveillance (Figure 5). This can be achieved, for example, by monitoring climate change, exposure to environmental hazards, vulnerability of populations, social behaviors, economic level of populations, hygiene of housing, demographic factors, and status of population health. It is also desirable to better inform people through communication among scholars, politicians and media, and probably consider warning signal coming from new sources, such as encouraging people to self-screen clinical signs of infection to accelerate the specialized care. This also involves a better knowledge of ecology of mosquitoes, the genetics of viruses, virus-target cells interaction in mosquitoes, animal reservoirs and humans, and replication cycle of the viruses[13,39,56]. Improvement of diagnostics and therapeutics is needed for early detection and control of infectious diseases (including preventive measures regarding blood transfusion in areas with a recent outbreak). This seems obvious, but one must know with certainty which pathogenic agent is causing the disease. In the past, similarities between symptoms of CHIKV-disease and DENV-disease, probably accounted for the frequent misclassification and some under-reports of CHIKV fever in areas with endemic DENV and, possibly, the incidence of CHIKV infection was much higher than reported[11]. On the basis of clini-

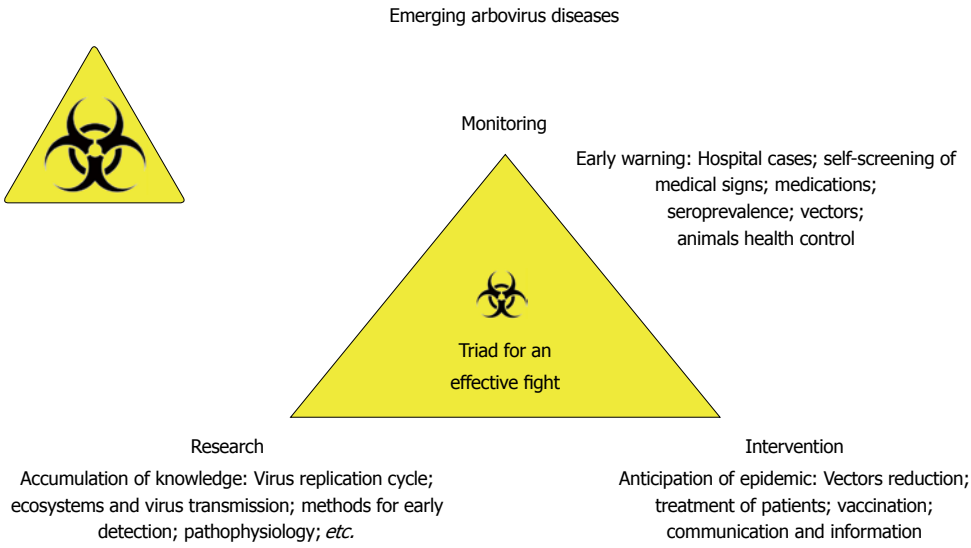

Figure 5 Emerging arbovirus diseases and the biosurveillance. Biosurveillance is based on three main principles: research (accumulation of knowledge in all fields of science), large scale monitoring for early warning and intervention to prevent or reduce the epidemic risks.

cal symptoms, the infections by CHIKV may have been mistaken for infections caused by related viruses, such as RRV[57], or O'Nyong-nyong virus[58].

Emerging infectious pathogens have significantly increased over the past few decades, prompting the need for more rapid outbreak detection and report. Of course, to prevent these health threats, many structures have been created that address this risk, such as the WHO, the Center for Disease Control (CDC, Atlanta), the European CDC, and others. An integrated global alert and response system for EID and other public health emergencies based on strong national public health systems and capacity, and an effective international system for coordinated response has been set up by the WHO[59]. The rapid expansion in Internet access and utilization has also provided a more open route for reporting that could push local governments toward greater transparency[10]. Additionally, NIAID and others (i.e. Welcome Trust, Gates Foundation), support training programs to maintain and develop the national and international scientific expertise required to respond to future health threats.

France obviously contributes to international efforts in the fight against emerging diseases. The country has specific structures such as the Institute for Public Health (InVS) with its division called the National Reference Center for arboviruses, and the International Network of Pasteur Institutes with many locations around the world. Facing the constant threat of emerging diseases, it is necessary to continue the development of a surveillance system of infectious diseases, to develop ways for quick diagnosis and prevention, and to expand international collaborations in case of emergence. But is this enough? Many French experts in the field of infectious diseases now consider the desirability of establishing Centers for Research and Biosurveillance, "Centre de Recherche et de Veille" (CRV), in five major regions of the world, to put themselves in situations dealing with the emergence of new pathogenic agents nearest to the place of emergence.

The need arose to establish a CRV when the crisis of CHIKV outbreak occurred in the Reunion Island. The main idea is simple: it is to set up transdisciplinary approaches to diseases of wildlife and domestic animals which have been highlighted as part of growing disciplines of conservation medicine. The purpose is to focus on more proactive approaches to surveillance, health assessment, and monitoring of wildlife populations as well as health and disease interactions with anthropogenic change and the ecological footprint. Indeed, the French Ministry of Health and the Ministry of Higher Education and Research quickly realized that despite the presence on this island of many specialists in biology, medicine, veterinary medicine and social sciences, the coordination between different institutions and different specialists of EID was poor. Decisions were made at the highest level of government to overcome these difficulties. As a consequence, the Indian Ocean CRV was created in 2007. CRVs should be valuable platforms to conduct biosurveillance under a close working relationship with neighboring countries' governments and academic institutions. The synergy between this biosurveillance system near emergence sites and organizations of global health, is likely a formula for success. The CRV should also be centers where research teams can perform experiments under optimum quality and safety conditions. In addition, it will be necessary to establish quality controls to ensure that data produced by different research groups of the network of teams working in conjunction with the CRV, can be comparable and reproducible. The CRV may also help provide adequate scientific training to health professionals. Finally, the CRV could also be considered as a node of networks for international cohorts of patients or healthy individuals. The operation of these facilities should be provided through the contributions of individ-

ual institution partners, responding to international tenders to programs and the fund raising from international agencies and charitable organizations.

Ideally, in addition to the Indian Ocean CRV, it would be desirable to set up an African CRV, a Southeast Asia CRV, a Caribbean CRV, and a Mediterranean CRV. Of course, it depends largely on the geopolitical situation in these regions of the world, the state of diplomatic relations with the countries of the region, and the wishes expressed by our government in terms of scientific and health priorities.

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GUIDELINES FOR CLINICAL PRACTICE

Porcine reproductive and respiratory syndrome virus vaccines: Immunogenicity, efficacy and safety aspects

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) infection is the leading cause of economic casualty in swine industry worldwide. The virus can cause reproductive failure, respiratory disease, and growth retardation in the pigs. This review deals with current status of commercial PRRS vaccines presently used to control PRRS. The review focuses on the immunogenicity, protective efficacy and safety aspects of the vaccines. Commercial PRRS modified-live virus (MLV) vaccine elicits delayed humoral and cell-mediated immune responses following vaccination. The vaccine confers late but effective protection against genetically homologous PRRSV, and partial protection against genetically heterologous virus. The MLV vaccine is of concern for its safety as the vaccine virus can revert to virulence and cause diseases. PRRS killed virus (KV) vaccine, on the other hand, is safe but confers limited protection against either homologous or heterologous virus. The KV vaccine yet helps reduce disease severity when administered to the PRRSV-infected pigs. Although efforts have been made to improve the immunogenicity, efficacy and safety of PRRS vaccines, a better vaccine is still needed in order to protect against PRRSV.

Key words: Porcine reproductive and respiratory syndrome virus; Vaccine

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INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) causes severe economic loss in swine production industry worldwide[1]. The virus has brought about severe PRRS outbreaks in many countries in Southeast Asia including Thailand, leading to an unusually high mortality of pigs of all ages[2]. The virus also has recently devastated pig industry in China, causing losses of more than 30% of pig populations[3].

PRRSV belongs to the *Arteriviridae* family. The virus possesses enveloped positive-sense, single-stranded RNA genome of approximately 15 kb in size and with nine open-reading frames (ORF)[4]. The up-to-date information of PRRSV ORF is summarized in Table 1. PRRSV can be classified into two genotypes, the North American (NA) and the European (EU). Both genotypes of PRRSV share an approximately 60% nucleotide sequence

homology to each other[4]. Within each genotype, the virus isolates can exhibit up to 20% variability of nucleotide sequences, making them a variety of heterogeneous clusters or subpopulations[5].

PRRSV of either genotype causes reproductive failures in breeding age swine, which are characterized by mummification, stillbirth, late-term abortion and delayed return to estrus[4]. The virus also causes respiratory disorders in growing pigs, which can be subclinical or fatal depending on the virulence of the virus[4]. PRRSV-infected pigs usually suffer from poor growth performance and are highly susceptible to co- or secondary bacterial and other viral infections[4].

The measures used currently to control PRRS include management (e.g. whole herd depopulation/repopulation and herd closure), bio-security, test and removal, and vaccination[6]. Vaccination is used generally for the purpose of reduction of clinical losses, but not of prevention of virus infection. The vaccination strategy costs lowest to the pig producers and is feasible to all sizes of pig producers (i.e. small, medium and large), compared with other PRRS control strategies. There are two types of PRRS vaccines that are commercially available. One is a modified-live virus (MLV) vaccine and the other is a killed virus (KV) vaccine. PRRS MLV vaccine is well recognized for its protective efficacy against PRRSV that are genetically homologous to the vaccine virus. It is of concern, however, for its immunogenicity, cross protective efficacy and safety. PRRS KV vaccine, on the other hand, is well known for its safety, but it only confers limited protection.

This article aims to summarize the current status of commercial PRRS vaccines with respect to their immunogenicity, efficacy and safety. The article also discusses current efforts to develop an ideal PRRS vaccine.

MLV VACCINE

General information

PRRS MLV vaccine is licensed for use in several countries worldwide (http://www.cfsph.iastate.edu/Vaccines/disease_list.php?disease=porcine-reproductive-respiratory-syndrome&lang=en). The MLV vaccines licensed for use in the US are derived from the NA PRRSV, which include Ingelvac® PRRS MLV and ReproCyc® PRRS-PLE (both from VR-2332; Boehringer Ingelheim), and Ingelvac® PRRS ATP (from JA-142; Boehringer Ingelheim). The MLV vaccines licensed for use in the EU countries are, likewise, derived only from the EU PRRSV, which comprise Porcilis PRRS® (from DV; Merck), Amervac-PRRS® (from VP046; Hipra), and Pyrsvac-183® (from All-183; Syva). The MLV vaccines licensed for use in other countries may not be restricted to either virus genotype and may be available for both PRRSV genotypes. Details of the commercial PRRS MLV vaccines are summarized in Table 2.

Immunogenicity

Commercial PRRS MLV vaccine of either NA or EU genotype elicits relatively weak humoral and cell-mediated immune (CMI) responses. PRRSV-specific antibodies appear approximately 2 wk, and peak around 4 wk after vaccination[7]. Majority of the antibodies are against viral nucleocapsid (N) proteins which have no neutralizing activity[7]. These antibodies do confer some clinical protection, but their protective mechanism is yet unknown[7].

PRRSV-specific neutralizing antibodies appear approximately 4 wk after vaccination, and have relatively low titers (approximately 2^3-2^5) throughout the course of immunization[7]. The reason for poor neutralizing titers is not exactly known but is proposedly attributed to the presence of decoy neutralizing epitopes and the heavy glycosylation of the major and minor neutralizing epitopes[8-10].

PRRSV-specific CMI response appears approximately 2-4 wk after vaccination as determined by lymphocyte blastogenesis and interferon γ (IFNγ) production in recall reaction[11,12]. Majority of T cell subsets responsive to PRRSV are $CD4^+CD8^{lo}$ and $CD4^-CD8^+$[11-13], which are identified as porcine memory T helper cells and cytotoxic T cells, respectively[14,15]. The frequency of PRRSV-specific T cells producing IFNγ increases gradually with age, reaching a peak at approximately 32 wk of vaccination[11]. This is extremely delayed compared with T cell response to pseudorabies virus (PRV) MLV vaccine, which appears within 1 wk of vaccination and peaks approximately at 4 wk after vaccination[11]. The reason for delayed and weak CMI response to PRRSV is not thoroughly known, but is reported to be attributed, at least in part, to virus-mediated suppression of type I IFN and other pro-inflammatory cytokines, e.g. interleukin-1 (IL-1), IL-12, and tumor-necrosis factor α (TNFα)[16]. The poor CMI response might be also attributed to the virus capacity to up-regulate anti-inflammatory cytokine production, i.e. IL-10 and transforming-growth factor β, in infected cells, and to induce regulatory T cell response[17-19].

Following a challenge exposure to virulent PRRSV, MLV-vaccinated pigs do not develop systemic anamnestic antibody and CMI responses to the challenge viruses that are genetically homologous to the vaccine virus, but do develop anamnestic immune responses to the genetically heterologous viruses[12,20,21]. This absence of anamnestic antibody and CMI responses is observed also following repeated immunizations with PRRS MLV vaccine[12]. The reason for the absence of anamnestic immune responses to homologous virus, and the presence of anamnestic responses to heterologous virus is yet unknown. These phenomena, however, seem not to affect the protective efficacy of the MLV vaccine[12,20,21].

Protective efficacy

PRRS MLV vaccine effectively protects pigs from PRRSV-mediated reproductive and respiratory diseases. The vaccine helps protect gilts from viremia and helps reduce numbers of pre- and post-natal death and congenitally infected piglets[22]. Piglets born to vaccinated gilts had higher body weight and survival rate at weaning than those born to non-vaccinated control gilts[23]. The MLV vaccine, when

Table 1 Porcine reproductive and respiratory syndrome virus genome and relevant information

ORF	Product	Function	Role in immunity/protection	Ref.
1a	Nsp1α	Papain-like cysteine protease	Potential IFN and TNFα antagonist	[66-68]
	Nsp1β	Papain-like cysteine protease	Potential IFN and TNFα antagonist	[66,68,69]
	Nsp2	Cysteine protease	Potential IFN antagonist	[70]
	Nsp3	Transmembrane protein	NA	[70]
	Nsp4	Serine protease	NA	[70]
	Nsp5	Transmembrane protein	NA	[70]
	Nsp6	NA	NA	[70]
	Nsp7α	NA	Potential antigen for serological determination of persistence infection	[70]
	Nsp7β	NA	Potential antigen for serological determination of persistence infection	[70]
	Nsp8	NA	NA	[70]
1b	Nsp9	RNA-dependent RNA polymerase	NA	[70]
	Nsp10	Helicase	NA	[70]
	Nsp11	Endoribonuclease	Potential IFN antagonist	[70,71]
	Nsp12	NA	NA	[70]
2a	GP2	Minor envelope protein; interacts with CD163	Minor neutralizing epitope	[72]
2b	E protein	Minor envelope protein; possibly form oligomeric ion channel	NA	[72]
3	GP3	Minor envelope protein	Minor neutralizing epitope	[72]
4	GP4	Minor envelope protein; interacts with CD163	Minor neutralizing epitope	[72]
5	GP5	Major envelope protein; interacts with sialoadhesin	Major neutralizing epitope	[72]
6	M protein	Major envelope protein; interacts with heparan sulfate	T cell epitope; minor neutralizing epitope	[72]
7	N protein	Nucleocapsid	Non-neutralizing epitope	[72]

Nsp: Non-structural protein; GP: Glycoprotein; NA: No data available; ORF: Open-reading frames.

Table 2 Recommendation and vaccination schedule of commercial PRRS modified-live virus vaccines

Vaccine[1]	Pigs[2]	Route	Dose (mL)	Program
Ingelvac® PRRS MLV	Gilt/Sow	im	2	At any stage of production[3]
	Piglet/Nursery/Growing	im	2	At any stage of production[3]
ReproCyc® PRRS-PLE	Gilt/Sow	im	5	Primary: 4-6 wk prior to breeding Booster: prior to subsequent breeding
Ingelvac® PRRS ATP	Nursery/Growing	im	2	At 3-18 wk of age
Porcilis PRRS®	Gilt/Sow	im/id	2/0.2	Primary: 2-4 wk prior to breeding Booster: 2-4 wk prior to subsequent breeding/or every 4 mo
	Piglet/Nursery/Growing	im/id	2/0.2	At 2 wk of age or older
Amervac-PRRS®	Nursery/Growing	im	2	At 4 wk of age or older
Pyrsvac-183®	Gilt/Sow	im	2	Primary: 2-4 wk prior to breeding Booster: 3-4 wk prior to subsequent breeding
	Piglet/Nursery/Growing	im	2	At 2-3 wk of age or older

[1]Not recommended for use in porcine reproductive and respiratory syndrome virus-negative farms; [2]Not recommended for use in boars due to negative impact on semen quality[73]; [3]Recommended to revaccinate every 3-4 mo for whole herd vaccination program. im: Intramuscularly; id: Intradermally.

used in PRRSV-infected sows, effectively helps reduce abortion and return to estrus rate, and increase farrowing rate and number of weaning pigs[24,25].

In growing pigs, immunization with PRRS MLV vaccine associates with reduced viremia, respiratory signs, and improved growth performance[13,26,27]. The MLV vaccine, when vaccinated during acute PRRS outbreak or in endemically PRRSV-infected pigs, helps reduce virus shedding and respiratory disease, and improve growth performance[26-28].

Despite good protection, several concerns have been raised with respect to the MLV vaccine efficacy. First, PRRS MLV vaccine confers relatively delayed protection, which is usually detectable around 3-4 wk after vaccination[29]. Second, vaccine protection is rather virus genotype-specific and, to the most extent, strain-specific. Protection conferred by EU PRRS MLV vaccine is seen only after EU, but not NA PRRSV challenge[20,22]. Likewise, protection by NA PRRS MLV vaccine is seen after NA, and to some extent, EU PRRSV challenge[13,29]. And third, immunization with PRRS MLV vaccine might interfere with the protective efficacy of other swine vaccines, e.g. *Mycoplasma hyopneumoniae* bacterin. The MLV vaccine, when administered with certain schedule of the bacterin, might lower the bacterin efficacy[30,31].

Safety

The major concern of PRRS MLV vaccine is reversion to

Table 3 Recommendation and vaccination schedule of commercial PRRS killed virus vaccines

Vaccine	Pigs	Route	Dose (mL)	Program
Progressis®/ Ingelvac® PRRS KV	Gilt	im	2	Primary: twice, 3-4 wk interval, at least 3 wk prior to breeding Booster: 60-70 d of each gestation
	Sow			Primary: twice, 3-4 wk interval, at any stage of production
		im	2	Booster: 60-70 d of each gestation
Suipravac-PRRS	Gilt	im	2	Primary: twice, 3-4 wk interval, when entering the farm Booster: Follow sows' vaccination program
	Sow	im	2	Primary: twice, 3-4 wk interval, during pregnancy or lactation Booster: every 4 mo
Suivac PRRS-INe/ Suivac PRRS-IN	Gilt/Sow	im	2	Primary: three times; 1st at 5-6 mo of age, 2nd at 3-4 wk after 1st, and 3rd at 6-4 wk prior to expected farrowing Booster: twice; 1st at 3-4 wk after the farrowing, and 2nd at 6-4 wk prior to the further expected farrowing
	Boar	im	2	Primary: twice, 4 wk interval, starting at 6 mo of age Booster: every 4-6 mo
	Nursery/Growing	im	2	Three times: 3-4 wk interval, starting at 6-10 wk of age

im: Intramuscularly; KV: Killed virus.

virulence. This is predominantly through genetic mutations of the vaccine virus and/or recombination with field virulent PRRSV[32]. The revert-to-virulent vaccine virus can cause clinical diseases, both reproductive and respiratory, and affect growth performance[23]. The vaccine-like virus can potentially cross placenta during late gestation, and cause mummification and stillbirth[23]. Piglets born to these infected sows can be carriers of PRRSV and can shed the virus to other naive pigs[23]. In addition, the MLV-vaccinated pigs can develop viremia of the vaccine virus at least 4 wk after vaccination, and during this period, the animals can spread the virus to other naïve animals[33].

KV VACCINE

General information

PRRS KV vaccine is licensed for use in EU countries and other parts of the world, but not in the US. In the US, the vaccine appeared once in the market (under the trade name PRRomiSe™; Intervet), but the manufacturer discontinued it in 2005. The PRRS KV vaccines licensed for use in the EU can be derived from both EU and NA PRRSV. These vaccines include Ingelvac® PRRS KV (derived from P120; Boehringer Ingelheim), Suipravac-PRRS (from 5710; Hipra), Progressis® (from proprietary strain; Merial), Suivac PRRS-INe (from VD-E1 and VD-E2; Dyntec), and Suivac PRRS-IN (from VD-E1, VD-E2, and VD-A1; Dyntec). Details of the commercial PRRS KV vaccines are summarized in Table 3.

Immunogenicity

In contrast to PRRS MLV vaccine, vaccination with PRRS KV vaccine does not elicit detectable antibodies as determined by IDEXX ELISA and serum virus neutralization assay[34]. The vaccine also barely elicits CMI response as determined by lymphocyte proliferation and IFNγ production in recall response[12,35].

When PRRS KV vaccine is used in PRRSV-positive pigs, the vaccine helps increase antibody and CMI responses to the infecting virus[12,34]. The enhanced immune responses are detected approximately 2 wk after the second shot of vaccination, and correlate with protection[12,34]. These findings lead to the potential application of PRRS KV vaccine as a therapeutic vaccine in PRRSV-positive farms.

Protective efficacy

PRRS KV vaccine is considered less efficacious than PRRS MLV vaccine. In naïve animals, the vaccine fails to prevent reproductive losses and congenital infection in fetuses[36]. When used off-label in growing pigs and boars, the vaccine fails to reduce viremia, duration and titers of virus shedding in semen, and respiratory signs after virulent PRRSV challenge[29].

The benefit of PRRS KV vaccine is seen more obviously in virus-infected animals. In these cases, the vaccine helps improve reproductive performance, e.g. increased farrowing rate, number of weaned pigs, and health status of piglets born to vaccinated sows[37].

Safety

The PRRS KV vaccine is considered safe. Up to date, there has been no report on the negative impact of PRRS KV vaccine on pig health.

CURRENT EFFORTS ON PRRS VACCINE DEVELOPMENT

Numerous efforts have been made to develop an ideal PRRS vaccine, i.e. vaccine that possesses high immunogenicity, confers broad protection, and is safe[38,39]. These efforts reportedly included use of several adjuvants[40-42], use of mixed strains of PRRSV[43,44], and generation of alternative vaccines, i.e. DNA vaccine[45,46], subunit vaccine[47,48], synthetic peptide vaccine[13], viral vector vaccines using adenovirus[49-51], PRV[52,53], poxvirus[54,55], and

Table 4 Alternative PRRS vaccines

	Encoded ORF/GP	Immunogenicity		Protection		Ref.
		Antibody	CMI	Homologous	Heterologous	
DNA vaccine	ORF1-7	+	+	+	ND	[45,46]
Subunit vaccine	GP5	Poor	Poor	-	ND	[47,48]
Synthetic peptide vaccine	GP5	-	-	ND	-	[13]
Adenovirus vector vaccine	GP3, 4, 5	+	+	ND	ND	[49-51]
PRV vector vaccine	GP5, M	+	+	+	ND	[52,53]
Poxvirus vector vaccine	GP3, 5, M	+	+	+	ND	[54,55]
TGEV vector vaccine	GP5, M	+	ND	+	ND	[56]
Alphavirus-derived replicon	GP5, M	+	+	+	+	[57]
Bacterial vector vaccine	GP5, M	+	-	+	ND	[58]
Insect cell-derived vaccine	ORF3, 5, 7	+	ND	+	ND	[59]
Plant-derived vaccine	GP5	+	+	ND	ND	[60,61]
Gene-deleted MLV (deleted15-mer nsp2 epitope)		+	ND	ND	ND	[65]

+: Success; -: Failure; ND: Not determined.

transmissible gastroenteritis virus[56] as vectors, alphavirus-derived replicon[57], bacterial vector vaccine[58], insect cell-derived vaccine[59], and plant-derived vaccine[60,61] (Table 4). These efforts, however, can achieve at best some, but not all, properties of an ideal PRRS vaccine. In fact, none of these efforts can confer significantly better protection than PRRS MLV vaccine.

Development of mucosal vaccine also has been attempted in order to induce protective mucosal immunity, primarily at the site of PRRSV entry, i.e. respiratory and vaginal[62]. The success of mucosal vaccination concept has been reported in many other virus models, e.g. poliovirus, influenza virus and human immunodeficiency virus[63]. PRRSV glycoprotein 5 and N proteins conjugated with cholera toxin, a potent inducer of mucosal immunity[64], were shown to enhance the antibody response in mucosal surfaces, i.e. intestinal and genital, when the vaccine was administered orally, but the protective efficacy of the vaccine was not evaluated[62]. The vaccine, when administered intramuscularly, however, failed to confer respiratory and viremia protection[13].

There is also an effort to produce a PRRS vaccine that can differentiate infected from vaccinated animals for PRRS eradication[65]. This is accomplished by a deletion of 15-mer of non-structural protein 2 (nsp2) epitope of PRRSV. This gene-deleted vaccine is waiting for evaluation of its protective efficacy in the pigs (Table 4).

FUTURE PROSPECTS

Current major obstacle for development of an ideal PRRS vaccine is the lack of complete knowledge on several aspects of PRRSV, including (1) the virus strategies to suppress and evade host innate and adaptive immune responses; (2) the virus epitope(s) responsible for such immune suppression and evasion; (3) the virus epitope(s) common to both NA and EU PRRSV and can confer broad protection; and (4) the roles of PRRSV non-structural proteins and structural proteins on virus replication, virulence, immunity and protection. Efforts are needed to elucidate all these gap of knowledge. Addressing these questions will be essential to advance our understanding on PRRSV immunology and to provide valuable information for vaccine development.

CONCLUSION

There are two types of commercial PRRS vaccines currently used to control PRRS. PRRS MLV vaccine confers effective genotype/strain-specific protection, but provides only partial protection against genetically heterologous PRRSV. The MLV vaccine elicits relatively late humoral and CMI responses which lead to delayed protection. The vaccine virus has a potential to revert to virulence and cause diseases. PRRS KV vaccine, on the other hand, has poor immunogenicity and poor protective efficacy against either homologous or heterologous PRRSV. The vaccine, however, confers some protection when administered to the PRRSV-infected pigs.

The development of PRRS vaccine is and will be the topic of interest among PRRS researchers for years to come. With efforts from laboratories worldwide, it is possible that we will come up with a better PRRS vaccine.

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REVIEW

Serological diagnosis of Epstein-Barr virus infection: Problems and solutions

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Abstract

Serological tests for antibodies specific for Epstein-Barr virus (EBV) antigens are frequently used to define infection status and for the differential diagnosis of other pathogens responsible for mononucleosis syndrome. Using only three parameters [viral capsid antigen (VCA) IgG, VCA IgM and EBV nuclear antigen (EBNA)-1 IgG],it is normally possible to distinguish acute from past infection: the presence of VCA IgM and VCA IgG without EBNA-1 IgG indicates acute infection, whereas the presence of VCA IgG and EBNA-1 IgG without VCA IgM is typical of past infection. However, serological findings may sometimes be difficult to interpret as VCA IgG can be present without VCA IgM or EBNA-1 IgG in cases of acute or past infection, or all the three parameters may be detected simultaneously in the case of recent infection or during the course of reactivation. A profile of isolated EBNA-1 IgG may also create some doubts. In order to interpret these patterns correctly, it is necessary to determine IgG avidity, identify anti-EBV IgG and IgM antibodies by immunoblotting, and look for heterophile antibodies, anti-EA (D) antibodies or viral genome using molecular biology methods. These tests make it possible to define the status of the infection and solve any problems that may arise in routine laboratory practice.

INTRODUCTION

Epstein-Barr virus (EBV) or human herpesvirus 4 is ubiquitous, and about 90% of adults throughout the world have antibodies against it[1]. Acute infection is usually asymptomatic in immunocompetent children, and manifests itself as mononucleosis in 30%-50% of immunocompetent adolescents and adults[2,3]. Especially in immunocompromised patients, EBV is associated with various lymphoproliferative disorders and some neoplastic diseases, including Burkitt's lymphoma and nasopharyngeal carcinoma.

Like other herpesviruses, EBV has a productive lytic cycle and a latent phase. B lymphocytes are infected after the viral envelope glycoprotein gp350/220 has bound to the CD21 cell receptor, which is also the receptor for the C3d component of complement[4]. During the lytic cycle, regulatory proteins belonging to the immediately early antigen (IEA) and early antigen (EA) groups are synthesized to allow the production of viral DNA (EBV-DNA), the virion structural proteins (viral capsid antigen, VCA) and membrane proteins (MA). The lytic cycle leads to the destruction of infected cells and the production of viral particles, but EBV can also persist in host cells without complete virus production by replicating ex-

trachromosomal nucleic acids (episomes) following the expression of a few selected viral genes[1]. The expression of these genes leads to the immortalization of B cells and their transformation into proliferating blasts[5,6]. In immunocompetent patients, cytotoxic T lymphocytes and NK cells control the growth of transformed cells during primary infection, particularly the CD8+ T cells directed against antigens of the lytic cycle[7]. These cells are also directed against antigens of the latent phase[8], but the response is insufficient to ensure their complete eradication, and the virus can persist throughout life with low or intermittent levels of virion production[9]. After recovery, it has been estimated that about 1 in 10 000-100 000 memory cells contain EBV DNA in episomal form[10]. During the latent phase, EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) are expressed in infected cells. EBNAs represent a complex of at least six proteins (EBNA 1-6). EBNA-1 is responsible for maintaining the episomal state of EBV DNA in infected cells, and EBNA-2 seems to be involved in the immortalization of B lymphocytes[11]. The LMPs (LMP1, LMP2A and LMP2B) may also play a role in the process of immortalization, and oncoprotein LMP-1 appears to be responsible for most of the effects altering the growth of infected B cells[12]. Depending on the expressed antigens, it is possible to distinguish four types of latency, each of which is typical of the diseases associated with EBV (Table 1). As EBNA-3 is a target of CD8+ lymphocytes[7], latent cells are normally eliminated by cytotoxic T lymphocytes in immunocompetent patients[1], whereas the transformed cells can proliferate and cause various lymphoproliferative disorders in immunosuppressed patients[13].

Reactivation of the lytic cycle may occur during latency, possibly because of the recirculation of infected memory B cells in lymphoid tissues[14] which, stimulated by their natural antigens, differentiate into plasma cells. The beginning of the replication cycle is characterized by the initial expression of the BZLF1 gene initial expression and the production of Z Epstein-Barr replication activator protein (ZEBRA), an IEA that starts a cascade process which, by transactivating the expression of itself and other IEAs, regulates the production of the viral DNA polymerase and thymidine kinase required for viral replication[15], and leads to the production of VCA and MA, and the release of infectious virions[16]. Various studies have associated this reactivation with episodes of transplant rejection[17,18], post-transplant lymphoproliferative diseases (PTLDs)[19-21], and clinical diseases in patients with multiple sclerosis[22].

The humoral response includes antibodies against antigens of both the lytic cycle and the latent phase. However, only a few are widely studied and used for diagnostic purposes. Anti-EA antibodies (EA IgG) reflect two patterns, a diffuse (D) and a restricted pattern (R), that were originally observed by means of immunofluorescence on the basis of their distribution inside cells and their differential denaturation by means of fixation and proteolytic enzymes. Although not always present, EA (D) IgG increases during the first 3-4 wk and is no longer detectable after 3-4 mo (approximately 85% of the patients with acute infection are positive for up to 3 mo after symptom onset)[23,24], even though in some cases they can still be detected years after a primary infection[23]. Approximately, 20%-30% of healthy subjects who have previously been infected by EBV have EA (D) IgG[2,23,25]. High titers have also been seen during reactivation[26] and in patients with nasopharyngeal carcinoma[27], who also have high titers of VCA and EA IgA[28]. EA (R) IgG levels may remain high for up to 2 years and, in cases of protracted disease, can be detected after the disappearance of EA (D) IgG[29]. EA (R) IgG has been found in children aged less than 2 years with silent infection, in patients with Burkitt's lymphoma, and also in the previously infected subjects at low levels[28]. High levels of EA (D) and/or EA (R) IgG can also be seen in cases of reactivation and in immunocompromised patients[29].

Anti-MA antibodies seem to be particularly important in limiting the spread of infection and preventing reinfections[30,31]. The membrane antigen consists of three glycoproteins (gp 350, gp 250 and gp 85), which are present on the viral envelope and the membranes of infected cells, and mediate the cell binding of the virus.

Antibodies against the capsid antigen IgG (VCA IgG) typically appear at the time of the onset of the clinical symptoms of acute infection, and remain positive for life[32], whereas IgM antibodies (VCA IgM) usually appear at the same time as VCA IgG and disappear within a few weeks[32-37], although they may persist for several months[35]. Children and adults with primary infection are not always positive for VCA IgM[32,38].

Anti-EBNA-2 IgG (EBNA-2 IgG) appear early, and may be present in up to 30% of the patients in the course of disease[2,23], whereas anti-EBNA-1 IgG (EBNA-1 IgG) is usually undetectable during the first 3-4 wk after the onset of clinical symptoms[32,39] and is therefore indicative of past infection. Furthermore, most patients with chronic infection and immunosuppressed patients are negative for EBNA-1 IgG or have only low levels[40].

Searching for all of these antibodies is a means of defining infection status and can also help in the differential diagnosis as mononucleosis syndrome may be caused by other pathogens such as rubella, mumps, HHV 6, HCMV, HIV, *Toxoplasma gondii*, *etc*.[2,41].

Using only three parameters (VCA IgG, VCA IgM and EBNA-1 IgG), it is generally easy to distinguish acute and past infections in immunocompetent patients[42,43]. The presence of VCA IgG and VCA IgM in the absence of EBNA-1 IgG indicates acute infection, and the presence of VCA IgG and EBNA-1 IgG in the absence of VCA IgM is typical of past infection (Table 2). However, some cases may have different profiles that can create diagnostic doubts, such as the presence of VCA IgG in the absence of VCA IgM and EBNA-1 IgG, the simultaneous presence of VCA IgG, VCA IgM and EBNA-1 IgG, and the presence of EBNA-1 IgG in the absence of VCA IgG

Table 1 Types of latency in Epstein-Barr virus-associated diseases

Type 0 latency (EBERs, BARTs)	AIDS-related plasmablastic lymphoma
Type Ⅰ latency (EBNA1, LMP2, EBERs, BARTs) (BamHI A rightward fragments)	Burkitt's lymphoma
Type Ⅱ latency (EBNA1, LMP1, LMP2, *EBERs*, *BARTs*)	Hodgkin's lymphoma, AIDS-related Burkitt's lymphoma or primary effusion lymphoma Peripheral T cell lymphoma NK/T cell lymphoma, nasal type Nasopharyngeal carcinoma (plus BARF1) Gastric adenocarcinoma (plus BARF1)
Type Ⅲ latency (EBNA1, -2, -3A, -3B, -3C; LMP1, LMP2, *EBERs*, *BARTs*)	Post-transplant lymphoproliferative disorder AIDS-related immunoblastic or brain lymphoma Infectious mononucleosis Chronic active EBV infection Lymphoblastoid cell lines *in vitro* X-linked lymphoproliferative disease

Table 2 Interpretation of Epstein-Barr virus serological profiles in immunocompetent patients

Anti-EBV antibodies			Interpretation
VCA IgM	VCA IgG	EBNA-1 IgG	
Negative	Negative	Negative	No immunity
Positive	Negative	Negative	Acute infection or non-specificity[1]
Positive	Positive	Negative	Acute infection
Negative	Positive	Positive	Past infection
Negative	Positive	Negative	Acute or past infection[1]
Positive	Positive	Positive	Late primary infection or reactivation[1]
Negative	Negative	Positive	Past infection or non-specificity[1]

[1]Further testing required.

and IgM (Table 2). In such circumstances, in addition to following up the patients to assess any changes in the antibody profile, it is also useful to perform other laboratory tests[43].

The detection of antibodies is less useful in immunocompromised patients because of their immune system dysfunctions, and the fact that the type of antibody and its maintenance may vary over time depending on the dynamics of the disease, thus leading to atypical profiles[44]. There is generally an increase in the titers of VCA IgG and EA (D) or EA (R) IgG, with a decrease in the titer or loss of EBNA-1 IgG; there may also be increases in other antibody classes such as EA IgA and, although less frequently, VCA IgM. The variability observed in different patients (this may be complicated by the therapeutic use of immunoglobulins) indicated that a search for EBV DNA by molecular biology methods is useful for the diagnosis and follow-up of patients at risk of developing EBV-related lymphoproliferative disorders[45].

Serology is generally not decisive also in patients with EBV-associated tumors as high values of VCA IgG and EA IgG may persist with low titers of EBNA-1 IgG (Table 3)[15,16] and, once again, a search for EBV DNA is essential. There is one type of EBV-related cancer whose serology is characteristic: nasopharyngeal carcinoma typically leads to high VCA and EA IgA levels as a result of its origin on the nasopharyngeal mucous membrane[46].

LABORATORY METHODS

Various laboratory tests have been used to diagnose EBV infection. In addition to tests for other diagnostically useful parameters (leukocytosis, lymphocytosis with atypical lymphocytes, abnormal liver function test, *etc.*), there are tests for detecting non-specific heterophile antibodies and specific anti-EBV antibodies, as well as molecular biology methods used to detect EBV DNA.

Heterophile antibodies

Heterophile antibodies are antibodies that agglutinate cells of other animal species that are mainly associated with mononucleosis due to EBV but may be, albeit rarely, also detected in other diseases[47]. They peak 2-5 wk after symptom onset and then decline rapidly, although they may rarely persist for 6-12 mo[35,48]. Between 85% and 90% of adolescents and adults are positive during the course of EBV infection: about 50% in the first week, and 60%-90% in the second and third[29]. However, only 50% of children aged 2-5 years are positive at any time during the course of infection, and only 10%-30% of those aged less than 2 years[2]. The rate of false negative results can therefore be high in young children, whereas false positive results are observed in only 2%-3% of the patients with autoimmune diseases[2].

Despite these limitations, detecting heterophile antibodies can be helpful in the case of primary infection mainly because of the simplicity of the tests. In routine clinical practice, these include agglutination tests using sheep, goat or horse red blood cells as antigens after absorption with guinea pig kidney extracts in order to remove natural antibodies against Forssman antigen (the Paul Bunnel reaction), and newer tests based on latex particles with adherent specific bovine antigen for heterophile antibodies (monospot assays). Recently, multiplex flow immunoassays (MFI) have been proposed because

Table 3 Serological profiles in Epstein-Barr virus reactivation and some Epstein-Barr virus-associated diseases

Diseases	VCA IgM	VCA IgG	VCA IgA	EA (D) IgG	EA (R) IgG	EA IgA	EBNA1 IgG
Choronic active infection	+/-	++	+/-	+	++	-	+/-
Burkitt's lymphoma	-	++	-	+/-	++	-	+
Nasopharingeal carcinoma	-	++	+	++	+/-	+	+
Hodgkin's lymphoma	-	++	-	+	-	-	+
Reactivation	+/-	++	+/-	+	+/-	+/-	+/-

they are more sensitive in the case of acute infections[49]. However, given the level of false negative results, negative findings need to be followed by a search for specific antibodies[2,48].

Specific EBV antibodies

The specific tests for anti-EBV antibodies use different substrates or antigens and various technologies, of which those commonly used for the routine screening of EA IgG, EBNA-1 IgG, VCA IgG and IgM are immunofluorescence assays (IFAs) or enzyme immunoassay (EIAs) with enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immunoassay (CLIA) versions or newer multiplex flow immunoassay (MFI). IFAs usually use EBV-transformed B cell lines from patients with Burkitt's lymphoma (e.g. the P3HR-1 or Raj cell lines)[2,23,45,50-52], whereas EIAs use purified native or recombinant proteins, synthetic peptides or fusion proteins (complete proteins or fragments of the proteins encoded by the EBV genes)[42,48]. The type and preparation of the antigens used are probably responsible for the differences in the sensitivity and specificity of the various assays[42,53,54]. IFA has been used as the reference method, although its sensitivity is the same as or less than that of EIAs[43], automated versions of which allow a large number of samples to be tested and are commonly used in laboratories with a large routine workload. The latest CLIAs using synthetic peptides with different cut-off values for VCA IgM and EBNA-1 IgG can better distinguish the stage of infection[55] and, in the case of samples that are simultaneously EBNA-1 IgG, VCA IgG and IgM positive, may help distinguish recent (transient phase) and past infection or reactivation[55,56].

Immunoblotting

In order to confirm the screening assays, various immunoblotting tests have been developed using viral lysates of EBV-transformed cells and recombinant antigens (line blots)[57]. It is considered that the latter is unaffected by antibodies against the cell material that can be found in patients with mononucleosis[23,58]. Some line blots use recombinant antigens coated on the solid phase, such as EBNA-1 (p72), VCA (p18 and p23), EA (p54 and p138) and MA (gp 350/250); the most recent also use IEA (ZEBRA). It has been reported that the sera of patients with acute infection show anti-p23 IgG, anti-p55 IgG and anti-p138 IgG, but not anti-p72 IgG, whereas the sera from patients with past infection show anti-p23 IgG, anti-p72 IgG and anti-p18 IgG[23]. Kinetic studies have found that a strong IgG response to p72 is not observed until 20 d after disease onset. As anti-p72 IgG and anti-p18 IgG are present in patients with past infection, but not in those with acute infection[23], anti-p18 IgG can be considered equivalent to EBNA-1 IgG in terms of significance. In addition, as anti-p18 IgG is not lost in the case of immunosuppression[23], immunoblotting is especially useful in distinguishing acute and past infections in cases that are VCA IgG positive, but EBNA-1 IgG and VCA IgM negative.

Immunoblots for IgM have also been developed to detect VCA IgM in patients with acute infection, but not anti-p72 IgM[23], which may be useful for confirming the specificity of the VCA IgM detected by screening assays.

IgG avidity

The IgG avidity test can assess the degree of IgG maturation. Avidity is low at the beginning of an acute infection, but increases when the immune response matures[59-61]. For example, the maturation kinetics of VCA IgG lasts several weeks and in some cases up to 3 mo after symptom onset[62,63]. Avidity can be measured using an EIA, IFA or immunoblotting[23,57,63]. Two aliquots of the same sample are tested in parallel for the presence of IgG antibodies: one is not treated, and the other is treated with substances that dissociate the antibodies from the antigens (usually 8 M urea). Since the dissociation depends on antibody avidity, the ratio between the treated and untreated sample defines the degree of avidity. A search for avidity can therefore be used to estimate the duration of a primary infection, and differentiate acute and past infection[23,57,63-65].

The published data depend on the examined antibody and therefore the type of antigen used for the test. In addition to a mix of antigens, it is possible to use the whole or specific VCA (p23 or p18), IEA (ZEBRA), EA (p138 or p54) or EBNA (p72). Avidity varies depending on the kinetics of the various antibodies[63,66], e.g. in VCA IgG, it has been reported that avidity is low in the samples collected during the first 12 wk after symptom onset, thus indicating recent infection[67]. With the passing of time, the avidity of VCA IgG may become borderline or high when the avidity of EA IgG is still low[63]. Immunoblotting, which has various antigens coated on the solid phase, can be used to visualize the avidity of various antibodies simultaneously (Table 4), although the maturation of anti-IEA and EA antibodies (BZLF1, p138 and p54) does not seem to indicate immune status as antibodies with a low degree of avidity can be found even during reactivation. Consequently, the manufacturer points

Table 4 Interpretation of IgG avidity test with immunoblotting

IgG	IgG avidity	Interpretation
Negative	Not observed	No infection
Positive BZLF1, p138, p54	Low-high avidity for BZLF1 and/or p138 and/or p54	Acute infection
Positive p23, BZLF1, p138, p54	Low avidity for p23	Acute infection
Positive p23, BZLF1, p138, p54	High avidity for p23	Recent infection
Positive p18, p23, BZLF1, p138, p54	Low-intermediate avidity for p18 and p23	Recent infection
Positive EBNA-1, p18, p23, BZLF1, p138, p54	Low-high avidity for EBNA-1 and/or p18, and possible high avidity for p23	Past infection

out that a reduction in the intensity of the EA and IEA bands alone is not an index of recent infection[68].

The limitations of avidity testing are the individual maturation rates of antibodies and the fact that the tests cannot be used in newborns because of the presence of maternal antibodies.

Molecular biology

A number of different methods, techniques and protocols have been used to determine the presence of EBV DNA and measure viral load[69-72]. Dot blotting, Southern blotting, PCR and *in situ* hybridization have all been applied to various materials, but their differences in sensitivity and specificity have led to the results that need to be considered cautiously[28] as they vary from laboratory to laboratory[73,74]. More recent studies indicate that real-time PCR is particularly sensitive[28], and very useful for defining infection status, especially in immunocompromised patients[45,75,76] and those at risk of developing EBV-related disorders[45]. However, there is still no consensus concerning the best material to use, units of measurement, or the quantitative levels requiring intervention or predicting prognosis[16,74,77-79]. This means that particular care is necessary when comparing the data of different studies[73]: for example, the units of measurement include copies per milliliter, copies per microgram of DNA, copies per 100 000 leukocytes, and copies per positive cell[77]. The targets used may also vary from one method to another: LMP2, BKRF1 or BamHI-W (EBNA-1), BNRF1 (membrane protein), BXLF1 (thymidine kinase), BZLF1 (ZEBRA), BALF5 (viral DNA polymerase) or BHRF-1 (transmembrane protein).

Furthermore, there is much debate concerning the material that should be used to search for EBV DNA, such as whole blood, peripheral blood mononuclear cells (PBMCs), plasma or serum[13,80]. There is also the problem that incorrectly stored whole blood can cause EBV DNA to leave the intracellular compartment and give rise to false positive results in plasma or serum, and false negative results may be due to nucleases that are capable of partially degrading plasma EBV DNA[81].

In general, the best material used to search for EBV DNA depends on where it is, and varies during the course of the disease[13]. The virions produced during primary infection spread in peripheral blood[82,83], and it is also possible to determine the EBV-free or fragmented DNA coming from apoptotic cells[83], and the B cells transformed during the latent phase also pass into the bloodstream. EBV DNA can therefore be determined in serum or plasma as well as in PBMCs[84]. In patients with primary infection, it is frequently detected in whole blood (PBMCs and plasma/serum) within 14 d of symptom onset[85-89]. After the initiation of an immune response, viral load decreases slowly in PBMCs, but rapidly in plasma/serum, and it becomes undetectable after 3-4 wk[90-92], whereas memory cells with EBV may remain latent for a long time in blood. However, it must be kept in mind that there may be individual variations due to individual differences in kinetics, and viral load may increase after an initial decline, and in some cases, it may take as long as a year or more before it reaches stably low levels. Finally, even when this level is reached, the blood of a healthy carrier contains 1-50 copies of EBV DNA per million white blood cells, whereas EBV-DNA is almost always undetectable in plasma or serum[82,85,93-96]. The presence of plasma/serum EBV-DNA is therefore considered a sign of primary infection[13] or reactivation, and the viral load correlates with disease severity[85,88,92]. A search for EBV DNA may be more sensitive than serology in the early stages of the disease[89], and some studies have found that it correlates better with clinical acute infection than the avidity of VCA IgG[86]. However, in immunocompetent patients with acute infection, it is not usually necessary to look for EBV DNA as serology is sufficient except in cases with negative or doubtful serological findings in which there is a strong clinical suspicion of infection[89,97,98].

A search for EBV DNA is particularly important in immunocompromised patients with an incomplete humoral response and patients who have received transfusions or immunoglobulins that confound serological test results[28]. It has been reported that immunocompromised patients have higher baseline viral levels than healthy carriers[99,100], which decline after treatment. A search for EBV DNA is also useful in patients with EBV-related tumors, except for those with AIDS-related brain tumors in whom the blood levels are low because of the blood-brain barrier[99,101]. In EBV-related cancers, episomal or naked EBV DNA from apoptotic tumor cells is found

Table 5 Additional tests in the case of an isolated viral capsid antigen IgG pattern

Tests	Advantages	Disadvantages
EBV IgG immunoblotting	Useful in distinguishing acute from past infection	Individual antibody production; expensive
IgG avidity	Useful in distinguishing acute from past infection	Individual maturation. Not useful in newborns
Molecular biology	Useful in distinguishing acute from past infection	Uncorrected conservation of blood sample, presence of nucleasis; expensive; organisational problems
Heterophile antibodies	Useful in distinguishing acute from past infection if positive; inexpensive and simple	Not very sensitive (especially in children)
Anti-EA(D) IgG	Of some use in distinguishing acute from past infection; costs the same as a screening test	Not useful in at least 10% of cases

EBV: Epstein-Barr virus.

in serum and plasma[84,102], which may also contain tumor cells with latent EBV infection[13] and virions from a small number of tumor cells undergoing lytic infection. The most suitable material varies with the tumor[13] and depends on where EBV DNA is mainly found during the course of the disease (PBMCs or serum/plasma).

ATYPICAL PROFILES IN IMMUNOCOMPETENT PATIENTS

The presence of VCA IgG and IgM in the absence of EBNA-1 IgG indicates acute infection, whereas the presence of VCA IgG and EBNA-1 IgG in the absence of VCA IgM is typical of past infection. These profiles cover the vast majority of situations found in routine laboratory practice. However, the profile of a minority of cases may give rise to doubts or require confirmation.

Isolated VCA IgM

VCA IgM usually appears at the same time as VCA IgG, but because they can be detected earlier, a profile of isolated VCA IgM is usually thought to indicate an early stage of acute infection. Nevertheless, it is recommended to assess the specificity of the result because it may be made aspecific by interfering rheumatoid factor and autoantibodies, or cross-reacting factors such as HCMV or parvovirus B19[67]. A search for these factors and immunoblotting for IgM may be as helpful as the determination of other parameters of acute infection such as heterophilic antibodies or HBV DNA[98]. A search for anti-EA (D) IgG can also be useful as about 85% of the patients with acute infection are positive for these antibodies for up to 3 mo after the onset of symptoms[23,24].

Isolated VCA IgG

In some cases, VCA IgM may not be produced or appear 1-2 wk after VCA IgG, or present for a very short time or at low concentrations and it may not be detected by conventional tests[2,32,103,104]. In addition, about 5% of the patients do not produce EBNA-1 IgG after EBV infection[103,105] or their levels remain below the limit of detection[37,105]. Furthermore, even when they are produced, they may be lost over time particularly, but not exclusively, in immunocompromised patients[23,103,106]. Consequently, isolated VCA IgG may be found in cases of past infection with the loss or disappearance of EBNA-1 IgG, as well as in cases of acute infection with the delayed or early disappearance of VCA IgM. This pattern can be found in approximately 7% of cases in routine laboratory practice and in about 8% of all subjects with at least one marker of EBV infection; it also tends to become more frequent with advancing age[107].

Such cases require further diagnostic investigations (Table 5), including immunoblotting for IgG, avidity tests for VCA IgG, searches for viral genome, or heterophile antibodies or EA (D) IgG[43], or a repetition of the test after about 30 d in order to identify any change in the antibody profile. However, this last option inevitably delays the diagnosis until the second sample is collected, and physicians tend to avoid it if the symptoms improve over time, especially in the case of children, they may find it traumatic, which means the second sampling usually involves only a small number of patients[107].

Immunoblottting for IgG allows the interpretation of cases with isolated VCA IgG[23]. Some tests use recombinant p72 antigen (EBNA-1), p18 and p23 (VCA), p54 and p138 (EA) and gp 350/250 (MA). As anti-p18 IgG can be considered equivalent in meaning to EBNA-1 IgG (because they are both produced late during EBV infection), their presence indicates past infection, and their absence indicates an acute infection. Immunoblotting can therefore distinguish cases with the loss or non-appearance of EBNA-1 IgG, which however develop an anti-p18 IgG response, from those with primary EBNA-1 IgG negativity (acute infection)[23,108]. Using this method to examine cases of isolated VCA IgG, about 20% was identified as acute infections and 80% as past infections[107].

A VCA IgG avidity test can also be used to distinguish acute and past infection[64,67], which are respectively characterized by low and high levels of avidity; studies of patients with isolated VCA IgG have found low levels in about half of the cases, and high levels in the remaining half[75,86]. When using avidity test, it is important to have all the patients' temporal clinical data in order to interpret the results more appropriately.

The detection of EBV DNA is particularly useful in the case of serologically indeterminate EBV infec-

Table 6 Additional tests in the case of a simultaneous Epstein-Barr virus nuclear antigen 1 IgG, viral capsid antigen IgG and IgM positive pattern

Tests	Advantages	Disadvantages
EBV IgM immunoblotting	Useful only in verifying the specificity of EBV IgM	Not useful in distinguishing late primary infection (transient) from reactivation; expensive
HCMV IgM Parvovirus IgM	Useful in verifying the specificity of EBV IgM	Not useful in distinguishing late primary infection (transient) from reactivation
EBV IgG immunoblotting	Only useful in verifying the specificity of EBNA-1 IgG	Not useful in distinguishing late primary infection (transient) from reactivation; expensive
IgG avidity	Useful in distinguishing primary infection (transient) from reactivation	Individual maturation
Molecular biology	Useful for EBV reactivation follow-up	Difficult to distinguish late primary infection (transient) from reactivation in a single sample; expensive; organisational problems
Heterophile antibodies	Useful in distinguishing late primary infection (transient) reactivation when positive; inexpensive and simple	Not very sensitive (especially in children)
Anti-EA(D) IgG	Useful for EBV reactivation follow-up	Not useful in distinguishing late primary infection (transient) from reactivation in a single sample
CLIA for EBV antibodies with differential cut-off values	Useful in distinguishing primary infection (transient) from past infection; can be used for screening	Requires further study

EBV: Epstein-Barr virus; EBNA: EBV nuclear antigen; CLIA: Chemiluminescence immunoassay.

tion[75,86,98] because its presence in serum or plasma indicates primary infection or reactivation[13]. Some studies have found that the presence of EBV DNA in patients with isolated VCA IgG is usually associated with a low degree VCA IgG avidity[75,86] (especially when the sample is taken early in the course of suspected infectious mononucleosis), and in some cases it has been found to correlate better than VCA IgG avidity[86]. Furthermore, in cases of past infection diagnosed on the basis of a high level of VCA IgG avidity, none of the serum samples were positive for EBV DNA[86].

A search for heterophile antibodies may be useful in patients with isolated VCA IgG if the result is positive, although there have been reports of their presence for 6-12 mo[28,35,48]. About 60% of patients with acute infection and isolated VCA IgG present heterophile antibodies, as against none of those with past infections[107]. However, nothing can be said in the absence of heterophile antibodies, especially in children aged less than 5 years, and other tests must be used.

One possible alternative is to search for EA (D) IgG because about 85% of patients with acute infection are positive for EA (D) IgG for up to 3 mo after symptom onset[23,24]. However, as high titers can be found during EBV reactivation[26], as well as in 20%-30% of healthy subjects with past infection[2,25] and in patients with nasopharyngeal carcinoma[27], the diagnostic use of these antibodies is still being debated. In general, a search for EA (D) IgG alone does not identify the stage of disease[43,109], but combining these antibodies with other parameters can be useful in laboratory diagnoses[110] because it has been found that only 12% of the patients with isolated VCA IgG and past infection are EA (D) IgG positive, as against 90% of subjects with isolated VCA IgG and acute infection[111].

Simultaneous presence of EBNA-1 IgG, VCA IgG and VCA IgM

VCA IgM may persist for several months after an acute infection[35], and may also reappear during EBV reactivation[33,112]. Consequently, EBNA-1 IgG, and VCA IgG and IgM may be simultaneously present in patients with primary EBV infection if VCA IgM persist and EBNA-1 IgG have already been produced (a phase that has been variously defined as "recent infection", "primary infection, transient phase or convalescence", "past infection, IgM persisting"), or in those with reactivation and the simultaneous presence of VCA IgM and EBNA-1 IgG[42]. Reactivation is still relatively rare and often short in immunocompetent subjects, and is generally considered of no clinical relevance[113,114]; however, it can cause serious complications in immunocompromised patients.

This serological pattern is uncommon (approximately 5% in normal routine laboratory practice)[107], and further diagnostic tests are needed to distinguish transient infection and reactivation (Table 6). First of all, it is important to verify the specificity of VCA IgM because there may be false positive results[115-117] during the course of infection with other pathogens, such as HCMV, parvovirus B19, *Toxoplasma gondii*, hepatitis A or HIV[118-124]. It has been shown that primary HCMV infection often causes a further antibody response in anti-EBV IgM[125], and cross-reactive antibody responses against conserved epitopes are well known among herpes viruses, such as the glycine-alanine epitope shared by EBV and HCMV[119]. In some studies, up to about 50% of VCA IgM in patients with past infection may be due to non-specific reactivity[67] and a positive result should be confirmed (e.g. by immunoblotting for EBV IgM)[124]. It is also possible to test for cross-reactivity or polyclonal IgM, especially in the case of HCMV and parvovirus B19[98], and a search for HCMV or parvovirus B19 DNA can also help in

some cases[98]. Moreover, as false positive reactions may also result from the presence of autoantibodies or rheumatoid factor, it can be useful to look for these interfering factors. However, it needs to be pointed out that cold agglutinins, rheumatoid factor and autoantibodies can be found for a short period during the course of EBV mononucleosis because of the polyclonal activation of B-cells[126], therefore, the presence of rheumatoid factor does not automatically mean a falsely positive VCA IgM result. Finally, EBNA-1 IgG positivity in patients with primary infection may also result from aspecific reactivity, which can be detected by immunoblotting for IgG antibodies[124]. Furthermore, the recently developed EIA for antibodies to EBNA-1 IgG based on recombinant or synthetic peptides may be more sensitive than its predecessors, and allow their identification early in the course of primary EBV infection[124].

Once the specificity of the results obtained has been established, additional diagnostic approaches are necessary (Table 6). In addition to repeating the test after a reasonable period of time in order to detect any changes in the antibody profile, VCA IgG avidity has proved to be particularly useful because low levels of avidity have been found in the course of recent infection, and high levels in the course of past infection or reactivation[62-64]. Some studies have found that fewer than half of immunocompetent patients with this profile and a low degree of avidity have primary infection, and about 20% or less a reactivation that is probably clinically insignificant[112,124]. In order to fully understand the IgG avidity test and evaluate the results in relation to antibody maturation time, it is important to know how long after the onset of symptoms the blood sample was taken.

The use of heterophile antibodies is controversial because some studies have shown that they are very sensitive (94% of infected patients with transient infection)[124], whereas others have found very few cases of simultaneous positivity for EBNA-1 IgG, VCA IgG, VCA IgM and heterophile antibodies[109].

The use of molecular biology seems to be a rather delicate question. Patients with latent infection have an almost constant number of circulating infected B cells in peripheral blood and, in the case of reactivation, these differentiate into plasma cells, leading to the start of the replicative cycle and increased EBV DNA levels in PBMCs and serum/plasma. It has been reported that a search for EBV DNA in PBMCs and serum/plasma is important for an immediate diagnosis of reactivation but, although this is true in the patients who are followed up over time in order to detect any changes in viral load, the finding of EBV DNA in a single sample should not necessarily be seen as a sign of reactivation[95]. Consequently, as EBV DNA is present in cases of reactivation or primary infection, it is unlikely that testing one sample will be able to distinguish the two situations[75,95].

Persistent or reactivated EBV infection is characterized by high titers of EA (D) IgG, especially in immunocompromised patients[26,29]. After reactivation, the levels of these antibodies increase with VCA IgG, whereas the levels of EBNA-1 IgG decrease. An increase in the titer of EA (D) IgG can therefore be considered a useful marker of reactivation. However, as EA (D) IgG can also be found in 85% of primary infections and 20%-30% of past infections, and simultaneous VCA IgM, VCA IgG, EBNA-1 IgG and EA (D) IgG positivity has been seen in both the transient phase and reactivation[76], the detection of anti-EA (D) IgG seems to be useful only if serial sampling is possible. Other antibodies have been found in cases of reactivation. It has been suggested that EBNA IgM may be useful in identifying reactivation[114]. The combination of negative EBNA IgG and positive EBNA IgM, in addition to a high degree of avidity for VCA IgG within 3 mo of an acute infection, should reliably indicate reactivation rather than primary EBV infection[67].

It has also been suggested that VCA IgA may be a marker of reactivation. These antibodies reach a peak level 3-4 wk after primary infection and decline slowly, but may last indefinitely. Different studies have found them in 35%-74% of acute cases[34,127,128], but they were also seen in 10% of healthy subjects. High levels were also found in patients with immunodeficiencies, recurrent parotitis, multiple sclerosis or nasopharyngeal cancer, as well as in pregnant women and elderly subjects[26,129-132]. Consequently, a search for VCA IgA is considered useful only in the diagnosis and management of patients with nasopharyngeal carcinoma[133-136].

EA (R) IgG, EA IgM, EA IgA, VCA IgG-3 and the EBNA-1/EBNA-2 ratio have also been used as markers of reactivation in some studies[18,95,114,137-139] but, as many of these antibodies are also present in primary infections, and some even in past infections, the usefulness of a single sample in patients with the simultaneous presence of EBNA-1 IgG, VCA IgG and IgM has yet to be evaluated.

Finally, the use of different cut-off values proposed by the most recent CLIAs for determining VCA IgG, VCA IgM and IgG EBNA-1 in parallel seems to be a promising approach as it should make it possible to distinguish the different stages of EBV infection, especially the difference between transient infection and past infection or reactivation[55,56].

Isolated EBNA-1 IgG

VCA IgM usually appears at the same time as VCA IgG antibodies, but they disappear completely within a few weeks[32-37], whereas patients are VCA IgG positive for the rest of their lives[32]. EBNA-1 IgG cannot usually be detected during the first 3-4 wk after the onset of clinical symptoms[32,140] and as VCA IgG antibodies persist but EBNA-1 IgG antibodies may disappear, especially in the case of immunosuppression[23,103,106], a pattern of isolated VCA IgG without VCA IgM or EBNA-1 IgG has been widely documented as mentioned above, whereas the presence of EBNA-1 IgG without VCA IgG is generally considered implausible[43]. However, some immunoblotting studies have found that 2% of subjects who have

experienced previous infections may be negative for VCA IgG (anti-p23 or anti-p18)[23] and, although rare, this pattern can be found in routine laboratory tests and may represent about 1.7% of all EBNA-1 IgG-positive samples[39]. An isolated EBNA-1 IgG pattern therefore gives rise to some interpretative doubts: it is possible to envisage aspecific EBNA-1 IgG, a lack of VCA IgG production or their loss over time. At clinical level, the problem is to discover whether the patient has experienced a past infection or is still susceptible. As the doubt is to distinguish past infection and non-specificity, it is useless to search for heterophile antibodies or EBV DNA. The presence of EA (D) IgG may help indicate a past infection as approximately 20%-30% of healthy subjects with a history of EBV infection have EA (D) IgG[2,23,25], but nothing can be said in the case of negativity. However, immunoblotting for IgG can solve the problem as it can confirm the real presence of EBNA-1 IgG. One immunoblotting study found that such patients were not only all anti-p72 (EBNA-1) positive, but also anti-p23 (VCA) positive, which was not detected by the screening tests using p18 antigen alone to detect VCA IgG[140]. It therefore seems that EBNA-1 IgG does not exist without the presence of at least one VCA IgG, such as anti-p23.

CONCLUSION

A number of tests have recently been developed that may help clarify doubtful results in the serological diagnosis of EBV infection, and it is now possible to reach a conclusion without having to wait for a second sample taken after a certain lapse of time. What tests should be used after screening depends on various factors in addition to their scientific and technical suitability; these include organizational and economic questions (such as differences in costs and the reimbursements foreseen by the National Health Service or insurance companies), as well as the availability of space and adequately trained personnel. Furthermore, the number of routine tests (the number of samples with inconclusive results) affects the decision to undertake further more or less expensive laboratory tests or to send the sample (or the patient) to a reference laboratory. Finally, the type of patient may also be decisive. If the laboratory has to deal not only with samples from immunocompetent patients, but also with samples from immunosuppressed patients and/or patients with other EBV-related diseases, the choice of methods should privilege those suitable for all patients.

In conclusion, considerable progress has been made in the serological diagnosis of EBV infection and, using appropriate algorithms and methodologies, it is possible to solve all of the problems that may arise during the course of routine laboratory practice.

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Innovation in Severe Acute Respiratory Infections
Sitges, Spain

January 20, 2012
Exploiting Bacteriophages for Bioscience, Biotechnology and Medicine
Welwyn Garden City,
United Kingdom

January 20-22, 2012
International Science Symposium on HIV and Infectious Diseases
Chennai, India

February 26-29, 2012
10th American Society for Microbiology Biodefense and Emerging Diseases Research Meeting
Washington, DC, United States

March 11-15, 2012
8th International Symposium on Pneumococci and Pneumococcal Diseases
Foz de Iguaçu City, Brazil

March 12-16, 2012
Vaccine World Summit India
Hyderabad, India

March 14-17, 2012
22nd Annual Meeting of the German Society for Virology
Essen, Germany

March 21-26, 2012
2012 HIV Vaccines
Keystone, CO, United States

March 21-26, 2012
Viral Immunity and Host Gene Influence
Keystone, CO, United States

March 26-31, 2012
Cell Biology of Virus Entry, Replication and Pathogenesis
Whistler, BC, Canada

March 26-31, 2012
Frontiers in HIV Pathogenesis, Therapy and Eradication
Whistler, BC, Canada

April 20-21, 2012
2012 Molecular Virology Workshop
Daytona Beach, FL, United States

April 22-25, 2012
2012 28th Clinical Virology Symposium
Daytona Beach, FL, United States

April 24-27, 2012
European Molecular Biology Organization Workshop - Antigen presentation and processing
Amsterdam, Netherlands

May 19-22, 2012
European Molecular Biology Organization and European Molecular Biology Laboratory Symposium - New perspectives on immunity to infection
Heidelberg, Germany

June 11-16, 2012
Antiviral RNAi: From Molecular Biology Towards Applications
Pultusk, Poland

June 28-29, 2012
7th International Workshop on Hepatitis C - Resistance and New Compounds
Cambridge, MA, United States

July 16-20, 2012
European Molecular Biology Organization Conference Series - Viruses of microbes: From exploration to applications in the -omics era
Brussels, Belgium

July 19–20, 2012
7th International Workshop on HIV Transmission - Principles of Intervention
Washington, DC, United States

July 21-25, 2012
31st Annual Meeting of American Society for Virology
Madison, WI, United States

July 30 - August 1, 2012
3rd Annual Symposia of Hepatitis Virus
Guangzhou, China

August 20-22, 2012
2nd World Congress on Virology
Las Vegas, NV, United States

September 7-9, 2012
Viral Hepatitis Congress 2012
The Johann Wolfgang Goethe University, Frankfurt, Germany

October 18-20, 2012
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Berlin, Germany

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GENERAL INFORMATION

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The columns in the issues of *WJV* will include: (1) Editorial: To introduce and comment on the substantial advance and its importance in the fast-developing areas; (2) Frontier: To review the most representative achievements and comment on the current research status in the important fields, and propose directions for the future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (6) Review: To systemically review the most representative progress and unsolved problems in the major scientific disciplines, comment on the current research status, and make suggestions on the future work; (7) Original Articles: To originally report the innovative and valuable findings in virology; (8) Brief Articles: To briefly report the novel and innovative findings in virology; (9) Case Report: To report a rare or typical case; (10) Letters to the Editor: To discuss and make reply to the contributions published in *WJV*, or to introduce and comment on a controversial issue of general interest; (11) Book Reviews: To introduce and comment on quality monographs of virology; and (12) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on the research in virology.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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